

# Benfotiamine Counteracts Glucose Toxicity Effects on Endothelial Progenitor Cell Differentiation via Akt/FoxO Signaling

Valentina Marchetti,<sup>1</sup> Rossella Menghini,<sup>1</sup> Stefano Rizza,<sup>1</sup> Alessia Vivanti,<sup>1</sup> Tiziana Feccia,<sup>1</sup> Davide Lauro,<sup>1</sup> Akiyoshi Fukamizu,<sup>2</sup> Renato Lauro,<sup>1</sup> and Massimo Federici<sup>1</sup>

Dysfunction of mature endothelial cells is thought to play a major role in both micro- and macrovascular complications of diabetes. However, recent advances in biology of endothelial progenitor cells (EPCs) have highlighted their involvement in diabetes complications. To determine the effect of glucotoxicity on EPCs, human EPCs have been isolated from peripheral blood mononuclear cells of healthy donors and cultured in the presence or absence of high glucose (33 mmol/l) or high glucose plus benfotiamine to scavenge glucotoxicity. Morphological analysis revealed that high glucose significantly affected the number of endothelial cell colony forming units, uptake and binding of acLDL and Lectin-1, and the ability to differentiate into CD31- and vascular endothelial growth factor receptor 2-positive cells. Functional analysis outlined a reduced EPC involvement in de novo tube formation, when cocultured with mature endothelial cells (human umbilical vein endothelial cells) on matrigel. To explain the observed phenotypes, we have investigated the signal transduction pathways known to be involved in EPC growth and differentiation. Our results indicate that hyperglycemia impairs EPC differentiation and that the process can be restored by benfotiamine administration, via the modulation of Akt/FoxO1 activity. *Diabetes* 55:2231–2237, 2006

Postnatal blood vessel formation was for a long time attributed to the migration and proliferation of preexisting fully differentiated endothelial cells, a mechanism known as angiogenesis (1–3). However, recent studies have demonstrated that circulating bone marrow-derived endothelial progenitor cells (EPCs) tightly contribute to adult blood vessel formation

(4,5). The EPCs promote in vivo re-endothelization and are able to be incorporated into new vessels in animal models of hind limb ischemia (6,7). EPCs are involved in processes like myocardial ischemia and infarction, wound healing, and endogenous endothelial repair (8–11). Furthermore, in vivo studies in animal models and in vitro studies using EPCs from type 1 diabetic patients revealed a potential role for glucotoxicity in impairing EPC function (7,12–14).

High glucose induces pathological alterations through increased formation of advanced glycosylation end product, activation of aldose reductase and protein kinase C, and increased flux through the hexosamine pathway. All of these mechanisms seem to reflect a single hyperglycemia-induced process of overproduction of superoxide anion by the mitochondrial electron transport chain (15). Superoxide inhibits the glycolytic enzyme glyceraldehyde phosphate dehydrogenase, diverting upstream metabolites from glycolysis toward the glucose-driven signaling pathways that cause hyperglycemic damage (16). These processes may be in part reduced by transketolase activation through its cofactor thiamine. In fact, both thiamine and benfotiamine have been shown to correct microvascular and macrovascular complications of diabetes, although at a different extent, blocking three major pathways of hyperglycemic damage (16–19).

Interestingly, the phosphatidylinositol 3-kinase (PI 3-kinase)/Akt pathway is crucial for both endothelial cell function and EPC differentiation (20–22). The PI 3-kinase/Akt pathway is known to direct cellular processes like differentiation and stress resistance through a tight regulation of the forkhead family of transcription factors (FoxO1/3a/4). FoxO1 and FoxO3a were recently found to play a role in angiogenesis and vasculogenesis (23–26). We and others have recently shown that both genetic and metabolic factors impair activation of the PI 3-kinase/Akt/FoxO pathway in mature endothelial cells (27–29). In this study, we investigated the impact of glucose toxicity on the ability of EPCs to differentiate into mature endothelial cells, and we also tested benfotiamine capacity to bypass the negative effects of high glucose concentrations.

## RESEARCH DESIGN AND METHODS

**EPC isolation and culture.** EPCs were obtained by isolating peripheral mononuclear cells from human blood buffy coats using Ficoll density centrifugation. Recovered cells were washed twice with PBS. Unselected mononuclear cells were plated on fibronectin-coated culture dishes (Biocoat; Becton Dickinson Labware) at a density of  $10^6$  cells/ml in Medium 199 (Invitrogen), supplemented with 20% fetal bovine serum, 100 units/ml penicillin/streptomycin (Invitrogen), and 0.05 mg/ml bovine pituitary extract (Invitrogen) and in

From the <sup>1</sup>Department of Internal Medicine, University of Rome Tor Vergata, Rome, Italy; and the <sup>2</sup>Center for Tsukuba Advanced Research Alliance, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan.

Address correspondence and reprint requests to Dr. Massimo Federici, Department of Internal Medicine, University of Rome Tor Vergata, Via Montpellier 1, 00133 Rome, Italy. E-mail: federicm@uniroma2.it.

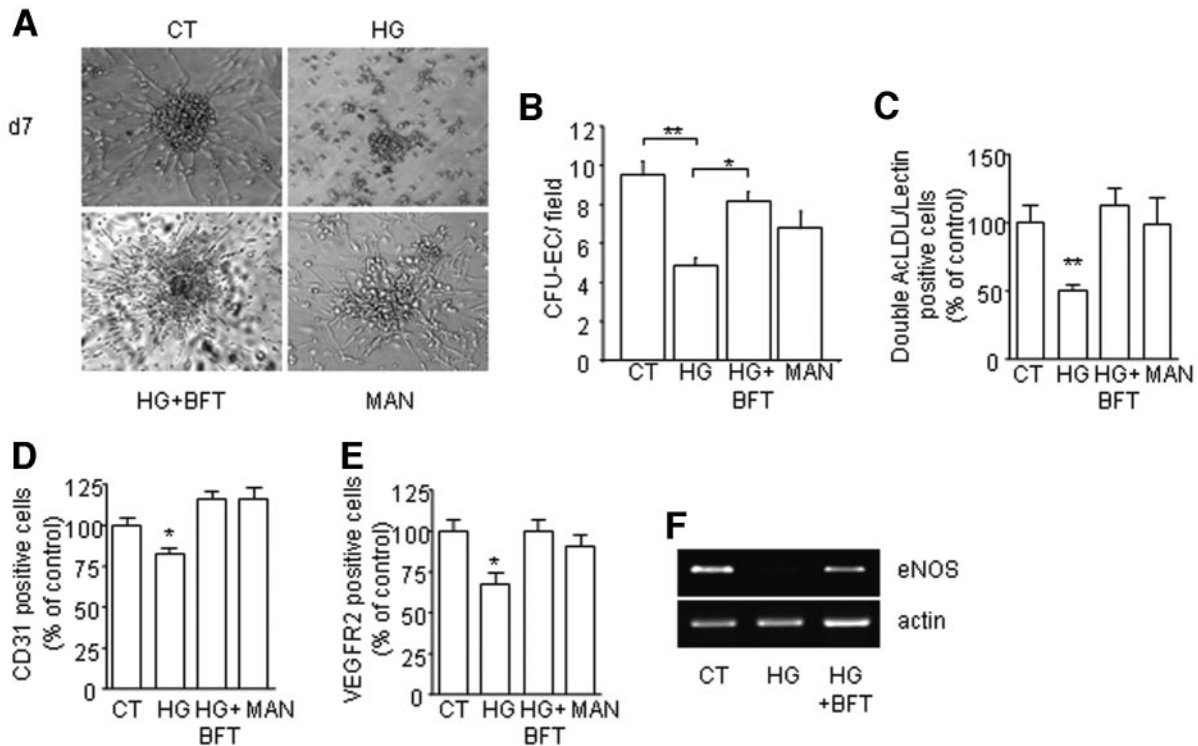
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Dil, dioctadecyl-tetramethylindo-carbocyanine perchlorate; Dil-acLDL, Dil-labeled acetylated LDL; eNOS, endothelial nitric oxide synthase; EC-CFU, endothelial cell colony forming unit; EPC, endothelial progenitor cell; FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; JNK, Jun NH<sub>2</sub>-terminal kinase; PI 3-kinase, phosphatidylinositol 3-kinase; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

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**FIG. 1. High glucose (HG) impairment of CFU-EC morphology and number is counteracted by benfotiamine (BFT).** *A:* CFU-EC at 7 days after the preplating phase in the various conditions; EPCs appeared as spindle cells separating from the central cluster of the CFU-EC. *B:* The number of CFU-EC was counted in 10 fields for each type of treatment in cultures from at least 10 blood donors. The number of CFU-EC was decreased by high glucose and increased in benfotiamine (\*\* $P < 0.001$  CT vs. high glucose, \* $P < 0.01$  high glucose vs. benfotiamine, ANOVA-1,  $n = 10$ ). *C:* EPCs, identified through double positivity to Lectin-1/Dil-acLDL, were reduced by high glucose and recovered by benfotiamine (\* $P < 0.05$  CT vs. high glucose, \*\* $P < 0.01$  high glucose vs. high glucose plus benfotiamine, ANOVA-1,  $n = 4$ ). *D:* Expression of VEGFR2 (*D*) and CD31 (*E*) in EPCs (\* $P < 0.05$  with respect to CT, ANOVA-1,  $n = 4$ ). *F:* eNOS mRNA by RT-PCR at day 15 in EPCs.

the presence or absence of high glucose (33 mmol/l) and high glucose and benfotiamine (50  $\mu$ mol/l). An initial preplating step on fibronectin-coated dishes was performed, to exclude contamination from mature endothelial cells (11,30). After 48 h, the nonadherent cells were collected, counted, and replated on fibronectin-coated culture dishes or 4-well glass slides for the final assay. Culture medium was changed every 3 days.

**EPC proliferation and differentiation.** Adherent cells were stained for the uptake of dioctadecyl-tetramethylindocarbocyanine perchlorate (Dil)-labeled acetylated LDL (Dil-acLDL; Molecular Probes) and the binding of fluorescein isothiocyanate (FITC)-labeled Ulex europaeus agglutinin I (Sigma). Cells were first incubated at 37°C in the presence of 10  $\mu$ g/ml Dil-acLDL for 2 h. Cells were then washed three times and fixed for 15 min in PBS containing 4% paraformaldehyde. Finally, cells were incubated for 1 h at room temperature with 10  $\mu$ g/ml FITC-labeled Ulex europaeus agglutinin I. The number of total attached cells was evaluated by 4'-6-diamidino-2-phenylindole-2HCl (Sigma) staining. Cells were visualized using a laser confocal microscope (Nikon): double-positive cells were judged EPCs and quantified by examining 15 or more random microscopic fields. To confirm the endothelial profile, indirect immunostaining was performed using antibodies directed against vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2; Santa Cruz) and CD31 (Santa Cruz) for 1 h and then for 1 h further with a fluorescein-conjugated anti-rabbit IgG or phycoerythrin anti-mouse IgG (Molecular Probes); nuclei were stained 4'-6-diamidino-2-phenylindole-2HCl. Only spindle-shaped cells were included in the count. Cells were visualized by an inverted fluorescent microscope (Nikon) and quantified by examining 15 or more random microscopic fields.

**Western blotting and nuclear extracts.** Adherent cells were serum deprived for 16 h and then stimulated with insulin ( $10^{-7}$  mol/l; Sigma) for 15 min at 37°C. Protein extracts were prepared as previously described (27). The nuclear and cytosolic fractions were separated by use of a commercially available kit according to the manufacturer's protocol (Pierce). The purity of the nuclear fractions was ensured by immunoblotting with topoisomerase-1 or histone H1 (Santa Cruz). Western blotted proteins (50  $\mu$ g/lane) were immunodetected with anti-phospho-(Ser473) Akt antibody, anti-Akt, anti-phospho-p44/p42 map kinase (Thr202/Tyr204), anti-p44/p42, anti-phospho-FKHR (Thr24)/FKHRL1 (Thr32) antibody, and anti-FoxO1 antibody (all from Cell Signaling). The anti-acetylated (K242/K245) FoxO1 antibody was previously

described (31). The phosphorylated and acetylated specific levels of the proteins studied were normalized for the amount of the respective total protein on the same filter. Actin (total cell homogenate) and topoisomerase-1 or histone 2b (nuclear) were used to normalize protein loading.

**Matrigel assay.** Matrigel (Becton Dickinson) was thawed and placed in 24-well plates for 30 min at 37°C to allow solidification. For fluorescent labeling of EPCs, cells were incubated with Dil (Molecular Probes) at a concentration of 5.2  $\mu$ g/ml in serum-free culture medium for 10 min at 37°C. Dil-labeled EPCs ( $2 \times 10^4$ ) were coplated with  $4 \times 10^4$  human umbilical vein endothelial cells (HUVECs) and incubated overnight at 37°. The tubule was defined as a structure exhibiting a length four times its width. The number of EPCs in tubules was determined counting at least 10 random fields.

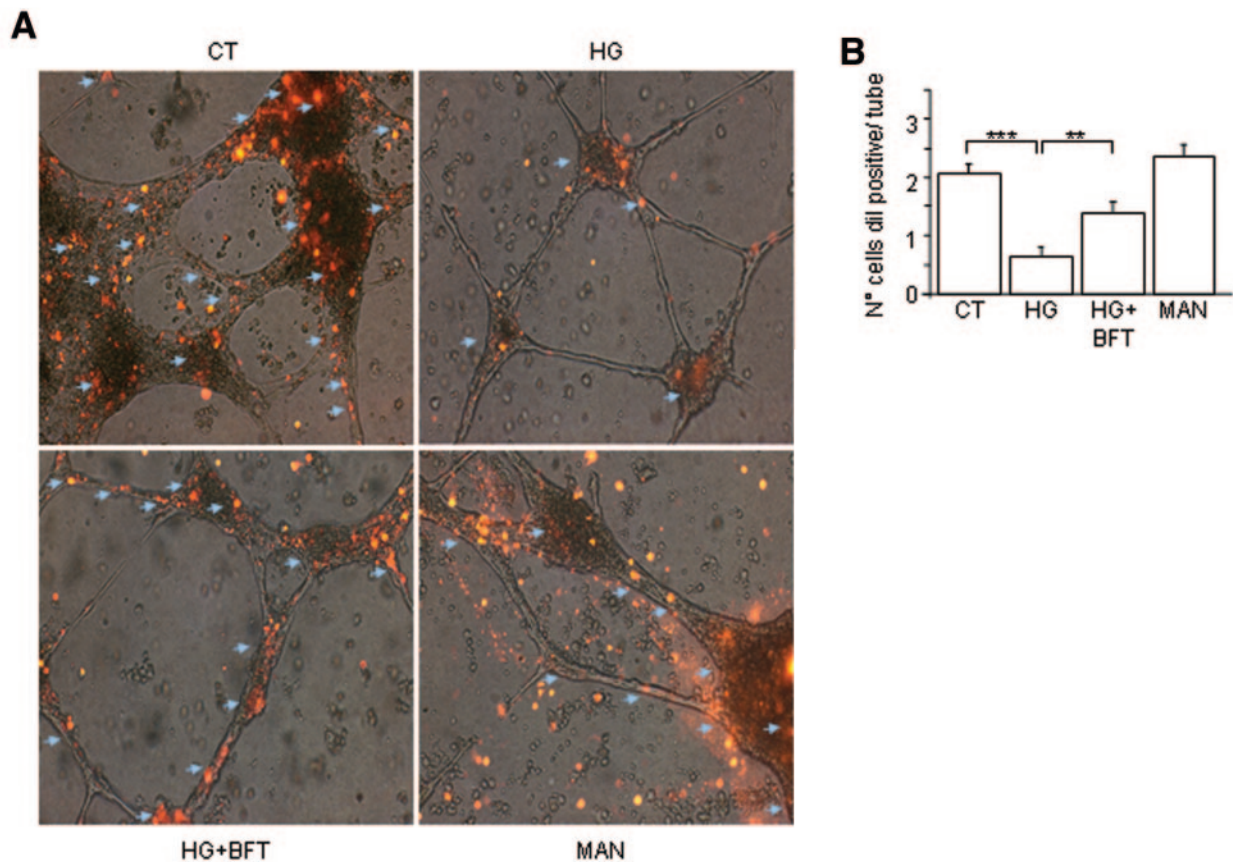
**Evaluation of apoptosis by flow cytometry.** The percentage of cells undergoing apoptosis was quantified as previously described (32).

**RNA isolation and RT-PCR.** Total RNA was isolated using the TRIzol reagent (Invitrogen) as described by the manufacturer. For RT-PCR, 1  $\mu$ g total RNA was reverse-transcribed into cDNA with 1 unit/ml M-MLV reverse transcriptase (Invitrogen) at 42°C for 45'. Primers sequences for human FoxO1, FoxO3, FoxO4, Bim-eL, FasL, catalase, and  $\beta$ -actin are available upon request. The PCRs were carried out at 60°C with Platinum *Taq* Polymerase (Invitrogen).

**Statistical analysis.** Results for continuous variable are expressed as means  $\pm$  SE. Comparisons between groups were analyzed by two-tailed Student's *t* test or ANOVA, as appropriate.  $P < 0.05$  was deemed significant. All analyses were performed with GraphPad Prism 4.

## RESULTS

**EPC differentiation and function are impaired by high glucose and recovered by benfotiamine.** EPCs when plated on fibronectin give rise to endothelial cell colony forming unit (EC-CFU) (11). An EC-CFU at 7 days of differentiation consists of multiple thin, flat cells emanating from a central cluster of spherical cells. To detect effects of high glucose on EC-CFU number and EPC



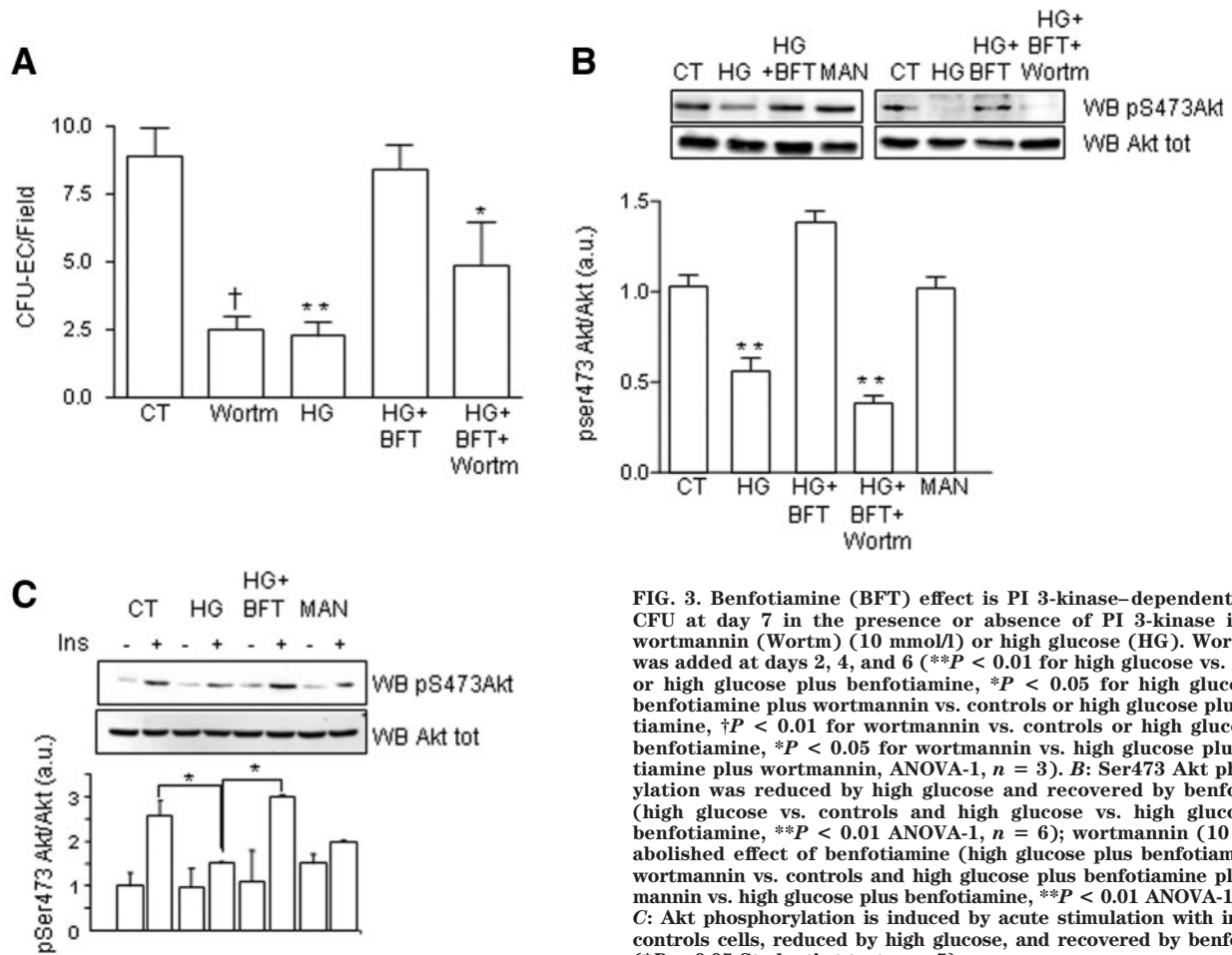
**FIG. 2.** High glucose (HG) impairment of EPC participation in tubulization is counteracted by benfotiamine (BFT). **A:** Fluorescent-labeled Dil EPCs (red) were coplated with HUVECs (transparent) to form tubule structures on Matrigel (controls through mannitol [MAN]). Superimposed light and fluorescent image of identical fields reveal that control EPCs made a substantial contribution to tubule networks with HUVECs (controls). In contrast, EPCs cultured in high glucose (HG) condition showed minimal incorporation into the developing vascular network (high glucose). Light blue arrows indicate tubules with EPC incorporation. **B:** Quantification of EPC tubulization revealed that high glucose significantly reduced EPC incorporation into tubules, whereas benfotiamine partially recovered high glucose effects (\*\* $P < 0.001$  controls vs. high glucose ANOVA-1,  $n = 4$ ). The benfotiamine treatment inhibits the high glucose effects (\* $P < 0.01$  high glucose vs. high glucose plus benfotiamine, ANOVA-1,  $n = 4$ ).

differentiation into endothelial cells, freshly isolated EPCs were cultured in control medium and in the presence of high glucose, at a concentration that was demonstrated to completely impair EPC function in cell culture (high glucose, 33 mmol/l) (33); mannitol (30 mmol/l) was used as a control for osmotic stress. At day 7, EPCs cultured in control medium formed endothelial cell colonies with several thin, flat, spindle-like-shaped cells emanating from a cluster of rounded/polygonal cells (Fig. 1A). In contrast, EPCs under high glucose treatment remained round/polygonal (Fig. 1A). Benfotiamine recovered effects of high glucose (Fig. 1A). The EC-CFU number was significantly decreased in EPCs cultured in the presence of high glucose, but when high glucose treatment was integrated with benfotiamine, the number of EC-CFUs was comparable with the control (Fig. 1B). Mannitol had no effect on EC-CFU number. Next, we visualized EPCs by double labeling with Dil-acLDL and lectin and counted them after 7 days in culture (Fig. 1C). High glucose significantly reduced the number of double-positive cells, whereas benfotiamine treatment recovered glucose toxicity. The endothelial phenotype of EPCs was additionally confirmed by checking the expression of markers like the VEGFR2 (Fig. 1D), CD31 (Fig. 1E), and the endothelial nitric oxide synthase (eNOS) after 15 days in culture (Fig. 1F). Interestingly, high glucose reduced the number of cells expressing VEGFR2 and CD31, whereas benfotiamine

counteracted the observed effects on the expression of the two markers (Fig. 1D and E). Next, we investigated whether the administration of benfotiamine was able to increase EPC integration into vascular structures using a Matrigel assay. Cocultivation of EPCs and HUVECs on Matrigel led to the formation of an extensive tubule network in all of the different growing media that we used. Fluorescent tagging of EPCs with Dil enabled delineation of EPCs from HUVECs. Analysis of superimposed light and fluorescent images of identical fields revealed that in high glucose conditions, a lower amount of EPCs was incorporated into new tubules when compared with controls, whereas benfotiamine partially recovered high glucose effects (Fig. 2A and B).

**Wortmannin reduces positive effects of benfotiamine on EPC-CFUs.** The PI 3-kinase/Akt pathway is known to play a major role in EPC differentiation (20,21), and previous studies suggested that its activation is impaired by high glucose in endothelial cells (28). Therefore, we incubated EPC cultured in the various conditions in the presence or absence of the PI 3-kinase inhibitor wortmannin. Results showed that wortmannin reverted the positive effects of benfotiamine on EC-CFU number in high glucose-treated EPC (Fig. 3A).

To verify that wortmannin was effectively reducing activation of Akt in EPC, we assessed levels of Akt phosphorylation on Ser473. Results showed that high



**FIG. 3.** Benfotiamine (BFT) effect is PI 3-kinase-dependent. **A:** EC-CFU at day 7 in the presence or absence of PI 3-kinase inhibitor wortmannin (Wortm) (10 mmol/l) or high glucose (HG). Wortmannin was added at days 2, 4, and 6 (\*\* $P < 0.01$  for high glucose vs. controls or high glucose plus benfotiamine, \* $P < 0.05$  for high glucose plus benfotiamine plus wortmannin vs. controls or high glucose plus benfotiamine, † $P < 0.01$  for wortmannin vs. controls or high glucose plus benfotiamine, \* $P < 0.05$  for wortmannin vs. high glucose plus benfotiamine plus wortmannin, ANOVA-1,  $n = 3$ ). **B:** Ser473 Akt phosphorylation was reduced by high glucose and recovered by benfotiamine (high glucose vs. controls and high glucose vs. high glucose plus benfotiamine, \*\* $P < 0.01$  ANOVA-1,  $n = 6$ ); wortmannin (10 mmol/l) abolished effect of benfotiamine (high glucose plus benfotiamine plus wortmannin vs. controls and high glucose plus benfotiamine plus wortmannin vs. high glucose plus benfotiamine, \*\* $P < 0.01$  ANOVA-1,  $n = 3$ ). **C:** Akt phosphorylation is induced by acute stimulation with insulin in controls cells, reduced by high glucose, and recovered by benfotiamine (\* $P < 0.05$  Student's  $t$  test,  $n = 5$ ).

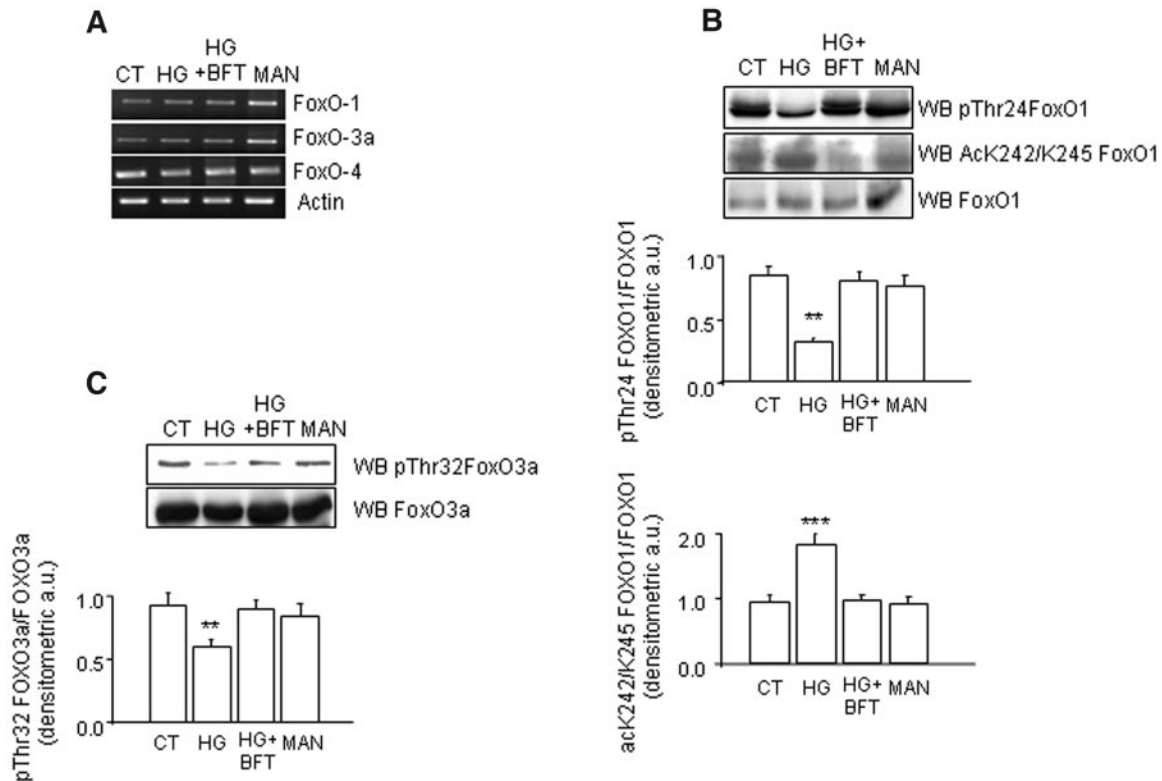
glucose significantly inhibited Ser473 Akt phosphorylation, whereas benfotiamine significantly reverted high glucose effects (Fig. 3A, left panel). Remarkably, wortmannin significantly blocked the positive effects of benfotiamine against glucotoxicity, suggesting that benfotiamine effect is PI 3-kinase dependent (Fig. 3B). To verify that the benfotiamine restoring effect on Akt phosphorylation was observed also after acute growth factor stimulation, EPCs were serum starved for 16 h and then incubated with insulin  $10^{-7}$  mol/l. In high glucose-treated cells, insulin-dependent Akt phosphorylation on Ser473 was significantly reduced compared with controls, high glucose plus benfotiamine, and mannitol (Fig. 3C). By contrast, neither high glucose nor benfotiamine affected phosphorylation of extracellular signal-related kinase1/2, p38, Jun NH<sub>2</sub>-terminal kinase (JNK), and IKK $\beta$  (data not shown).

**Akt/FoxO activation in EPCs is impaired by high glucose and recovered by benfotiamine.** Because the PI 3-kinase/Akt pathway acts to differentiate EPCs into endothelial cells through the modulation of FoxO transcription factors (23–26,35), we investigated their expression under different culture conditions. FoxO1, FoxO3a, and FoxO4 mRNA levels in EPCs were comparable under the various treatments (Fig. 4A). By contrast, FoxO phosphorylation on Akt-dependent consensus sequence (Thr24 for FoxO1 and Thr32 for FoxO3a) was markedly reduced by high glucose compared with controls, high glucose plus benfotiamine, and mannitol (Fig. 4B and C). Another important posttranslational modification controlling

FoxO1 activity is the acetylation on K242 and K245, which are known to be regulated by CBP (acetylase) and SIRT1 (deacetylase) (31). We found that in high glucose-treated EPCs, acetylation of FoxO1 on both the residues is significantly increased compared with controls and restored by treatment with benfotiamine (Fig. 4B).

Because we identified an altered FoxO1 phosphorylation profile in high glucose, we inquired whether this could reflect an anomalous cellular localization. Using confocal microscopy, we observed that high glucose induces a shift versus nuclear localization of FoxO1 when compared with controls, whereas benfotiamine attenuated high glucose effects (Fig. 5A). The number of EPCs showing FoxO nuclear accumulation was markedly increased in high glucose-treated cells compared with control and benfotiamine-attenuated high glucose effects. To verify that FoxO1 was preeminently nuclear only in high glucose, we performed Western blot analysis of EPC nuclear extracts. Results confirmed that high glucose induces a nuclear accumulation of FoxO1 protein with respect to controls, benfotiamine, and mannitol conditions (Fig. 5B).

Having found a reciprocal modulation of phosphorylation/acetylation states in FoxO1, we analyzed FoxO target genes involved in the regulation of cell cycle, apoptosis, and oxidative stress. In high glucose EPCs, we observed an increased expression of Bim-el and FasL (both significant) and p27 (not significant), whereas catalase was significantly reduced ( $P < 0.05$ ; Fig. 5C). Because FoxO targets suggest a tendency to favor apoptosis and reduced resistance to oxidative stress, we estimated the number of



**FIG. 4.** FoxO factors are expressed in EPCs, and their posttranslational regulation is modulated by high glucose (HG). **A:** FoxO mRNA analyzed by RT-PCR in EPCs under different treatments. **B:** FoxO1 phosphorylation/acetylation in EPCs was modified in high glucose-treated EPCs and recuperated by benfotiamine (BFT), similarly to the control (pThr24FoxO1,  $**P < 0.01$  ANOVA-1 for high glucose vs. controls, high glucose plus benfotiamine, and mannitol [MAN],  $n = 5$ ) (acK242/K245FoxO1,  $***P < 0.001$  *t* test for high glucose vs. controls, high glucose plus benfotiamine, and mannitol,  $n = 3$ ). **C:** FoxO3a phosphorylation was reduced by high glucose and improved by benfotiamine ( $***P < 0.001$  ANOVA-1 for high glucose vs. controls, high glucose plus benfotiamine, and mannitol,  $n = 4$ ).

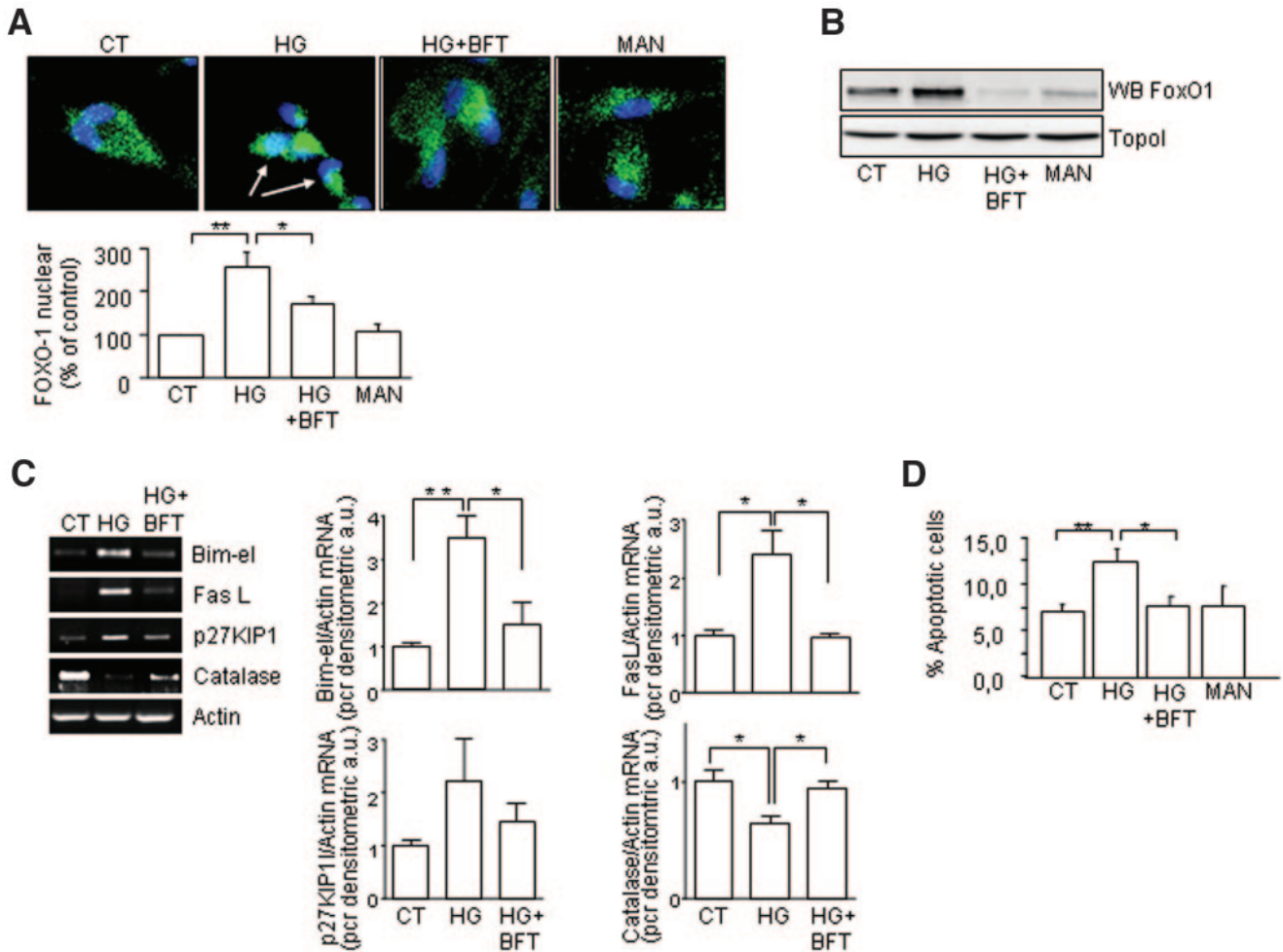
apoptotic cells under these treatments. We found a two-fold increase of apoptosis in high glucose compared with the other treatments (Fig. 5D).

## DISCUSSION

Type 1 and type 2 diabetes are both associated with endothelial cell dysfunction and reduced neovascularization (35,36). It is now accepted that EPCs contribute to maintenance of endothelial layer and take part in neovascularization. Although both in vitro and animal studies suggested that a hyperglycemic state was associated with impaired EPC biology and function, they lack direct evidence that glucose toxicity determined by hyperglycemia may impair EPCs (12–14). To study the effects of high glucose, we isolated EPCs from peripheral blood monocytes and cultured them in a two-step protocol that has been previously demonstrated to reduce artifact results because of the presence in cultures of circulating mature endothelial cells (11). High glucose was confirmed to reduce EPC plasticity toward endothelial cells at different levels.

To attenuate the damage determined by glucose toxicity, we elected to use benfotiamine, a thiamine derivative that was shown to dampen effects of the hexosamine, intracellular advanced glycosylation end products, and protein kinase C pathways (19–22,35). Our results indicate that benfotiamine improves the expression of endothelial cell markers in EPCs, restores eNOS levels, and recovers the ability of EPCs to participate in angiogenic processes. To elucidate the molecular mechanisms allowing commitment of EPCs to endothelial cell in the presence of

benfotiamine, we performed analysis of signaling pathways. In particular, glucose toxicity acts to dampen the activity of the PI 3-kinase/Akt pathway, a major signal for EPC differentiation (28,29,36). The PI 3-kinase/Akt pathway is known to direct cellular processes like differentiation and stress resistance through the modulation of FoxO transcription factors activity. In fact, FoxOs activate or repress transcriptional programs involved in metabolic control, cell cycle, oxidative stress sensing, and apoptosis, depending on the integration of external signals received by growth factors like insulin, IGF-I, or VEGF (22–26,37–39). However, the intimal mechanisms regulating FoxO activity are still unclear. A first layer of control is FoxO phosphorylation. Akt phosphorylates and inhibits FoxO transcriptional activity, through cytoplasm restraining, whereas JNK phosphorylation has a permissive role for FoxO activity (40). Our data confirm that hyperglycemia, impairing Akt but not JNK, may affect FoxO restrictive phosphorylation, thus allowing its nuclear localization. Once FoxO is localized within the nucleus, a second layer of control is obtained through acetylation by CBP or deacetylation by SIRT1 (31,39). The complex regulation of FoxO transcriptional activity is still a matter of debate, although it has been suggested that the balance of acetylation and deacetylation of FoxO could regulate its activity from transcription of genes involved in cell cycle and apoptosis (Bim-el and FasL) toward transcription of genes involved in oxidative stress sensing (Catalase) (41). Our data are in keeping with this hypothesis. In fact, we observed that in the presence of low phosphorylation/high



**FIG. 5.** FoxO1 nuclear localization. **A:** FoxO1 localization in EPCs by confocal microscopy. *Top*, the arrows indicate EPC with FoxO1 nuclear localization; *bottom*, the bar graph indicates the number of EPCs showing nuclear FoxO1 is increased in high glucose (HG) compared with controls and benfotiamine (BFT) (\*\* $P < 0.01$ , \* $P < 0.05$ , ANOVA-1,  $n = 4$ ). **B:** Western blot analysis of nuclear extracts. High glucose induces a nuclear accumulation of FoxO1 with respect to controls, benfotiamine, and mannitol (MAN) conditions; topoisomerase-1 was used to confirm quality of nuclear extracts. **C:** Expression of FoxO targets is modulated by high glucose and reverted by benfotiamine (Bim-el, \*\* $P < 0.01$  controls vs. high glucose and \* $P < 0.05$  high glucose vs. high glucose plus benfotiamine; FasL, \*\* $P < 0.01$  for high glucose vs. controls and high glucose plus benfotiamine; p27, not significant; Catalase, \* $P < 0.05$  controls vs. high glucose and high glucose plus benfotiamine; ANOVA-1). **D:** Analysis of apoptosis after propidium iodide staining by flow cytometry. The percentage of apoptotic cells is increased in high glucose compared with controls and benfotiamine (\*\* $P < 0.001$  high glucose vs. controls, \*\* $P < 0.01$  high glucose vs. high glucose plus benfotiamine, ANOVA-1,  $n = 4$ ).

acetylation states, as determined by glucose toxicity, the transcription of apoptotic/cell cycle inhibition genes like FasL and Bim-el is favored. Conversely, in both the control and benfotiamine treatments, the expression of some apoptotic genes was attenuated, whereas expression of an oxidative stress resistance gene, like Catalase, was increased.

Thus, it may be hypothesized that FoxO1 expression is necessary for adequate EPC differentiation, but it should be flanked by a tight posttranslational control through phosphorylation and acetylation to exert the more appropriate effect to physiological request, either regulation of cell cycle or oxidative stress sensing.

Interestingly, because benfotiamine, when incubated with high glucose, appeared to improve over the control, some aspects of EPC differentiation and phenotype, such as double uptake of acetylated LDL/lectin binding, CD31, and VEGFR2 expression, it is conceivable that it may have also some independent effects on EPC differentiation. In particular, from analysis of phosphorylated Akt and phosphorylated/acetylated FoxO status in EPCs, it is intriguing to observe that although benfotiamine was able only to

partially counteract glucotoxicity effects on Akt, it seems to have greater effects on both FoxO1 phosphorylation and acetylation state. Because FoxOs translate some but not all the effects of Akt, further studies beyond the scope of this study will be necessary to investigate whether benfotiamine may promote EPC differentiation through specific regulation of FoxO transcription factors.

In conclusion, we have shown that benfotiamine is able to dampen glucose toxicity effects on endothelial progenitors and have identified in benfotiamine and in the Akt/FoxO1 pathway possible targets in efforts to increase endothelial cell regeneration through EPCs.

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## REFERENCES

- Folkman J: Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1:27–31, 1995
- Risau W: Mechanisms of angiogenesis. *Nature* 386:671–674, 1997
- Carmeliet P, Jain RK: Angiogenesis in cancer and other diseases. *Nature* 407:249–257, 2000
- Asahara T, Kawamoto A: Endothelial progenitor cells for postnatal vasculogenesis. *Am J Physiol Cell Physiol* 287:C572–C579, 2004
- Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM: Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275:964–967, 1997
- Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM: Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* 85:221–228, 1999
- Schatteman GC, Hanlon HD, Jiao C, Dodds SG, Christy BA: Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. *J Clin Invest* 106:571–578, 2000
- Dimmeler S, Zeiher AM, Schneider MD: Unchain my heart: the scientific foundations of cardiac repair. *J Clin Invest* 115:572–583, 2005
- Urbich C, Dimmeler S: Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res* 95:343–353, 2004
- Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, Zeiher AM, Dimmeler S: Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 89:E1–E7, 2001
- Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T: Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 348:593–600, 2003
- Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, Levine JP, Gurtner GC: Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation* 106:2781–2786, 2002
- Fadini GP, Miorin M, Facco M, Bonamico S, Baesso I, Grego F, Menegolo M, de Kreutzenberg SV, Tiengo A, Agostini C, Avogaro A: Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. *J Am Coll Cardiol* 45:1449–1457, 2005
- Loomans CJ, de Koning EJ, Staal FJ, Rookmaaker MB, Verseyden C, de Boer HC, Verhaar MC, Braam B, Rabelink TJ, van Zonneveld AJ: Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. *Diabetes* 53:195–199, 2004
- Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001
- Hammes HP, Du X, Edelstein D, Taguchi T, Matsumura T, Ju Q, Lin J, Bierhaus A, Nawroth P, Hannak D, Neumaier M, Bergfeld R, Giardino I, Brownlee M: Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy. *Nat Med* 9:294–299, 2003
- Babaei-Jadidi R, Karachalias N, Ahmed N, Battah S, Thornalley PJ: Prevention of incipient diabetic nephropathy by high-dose thiamine and benfotiamine. *Diabetes* 52: 2110, 2003
- Ceylan-Isik AF, Wu S, Li Q, Li SY, Ren J: High-dose benfotiamine rescues cardiomyocyte contractile dysfunction in streptozotocin-induced diabetes mellitus. *J Appl Physiol* 100:150–156, 2006
- Stracke H, Hammes HP, Werkmann D, Mavrakis K, Bitsch I, Netzel M, Geyer J, Kopcke W, Sauerland C, Bretzel RG, Federlin KF: Efficacy of benfotiamine versus thiamine on function and glycation products of peripheral nerves in diabetic rats. *Exp Clin Endocrinol Diabetes* 109:330–336, 2001
- Llavadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A, Walsh K, Isner JM, Asahara T: HMG-CoA reductase inhibitor mobilizes bone marrow-derived endothelial progenitor cells. *J Clin Invest* 108:399–405, 2001
- Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, Rutten H, Fichtlscherer S, Martin H, Zeiher AM: HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *J Clin Invest* 108:391–397, 2001
- Ackah E, Yu J, Zoellner S, Iwakiri Y, Skurk C, Shibata R, Ouchi N, Easton RM, Galasso G, Birnbaum MJ, Walsh K, Sessa WC: Akt1/protein kinase Balpha is critical for ischemic and VEGF-mediated angiogenesis. *J Clin Invest* 115:2119–2127, 2005
- Daly C, Wong V, Burova E, Wei Y, Zabski S, Griffiths J, Lai KM, Lin HC, Ioffe E, Yancopoulos GD, Rudge JS: Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FOXO1. *Genes Dev* 18:1060–1071, 2004
- Hosaka T, Biggs WH III, Tieu D, Boyer AD, Varki NM, Cavenee WK, Arden KC: Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. *Proc Natl Acad Sci U S A* 101:2975–2980, 2004
- Furuyama T, Kitayama K, Shimoda Y, Ogawa M, Sone K, Yoshida-Araki K, Hisatsune H, Nishikawa S, Nakayama K, Nakayama K, Ikeda K, Motoyama N, Mori N: Abnormal angiogenesis in Foxo1 (Fkhr)-deficient mice. *J Biol Chem* 279:34741–34749, 2004
- Potente M, Urbich C, Sasaki KI, Hofmann WK, Heeschen C, Aicher A, Kollipara R, Depinho RA, Zeiher AM, Dimmeler S: Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. *J Clin Invest* 119:2382–2392, 2005
- Federici M, Pandolfi A, De Filippis EA, Pellegrini G, Menghini R, Lauro D, Cardellini M, Romano M, Sesti G, Lauro R, Consoli A: G972R IRS-1 variant impairs insulin regulation of endothelial nitric oxide synthase in cultured human endothelial cells. *Circulation* 109:399–405, 2004
- Federici M, Menghini R, Mauriello A, Hribal ML, Ferrelli F, Lauro D, Sbraccia P, Spagnoli LG, Sesti G, Lauro R: Insulin-dependent activation of endothelial nitric oxide synthase is impaired by O-linked glycosylation modification of signaling proteins in human coronary endothelial cells. *Circulation* 106:466–472, 2002
- Du XL, Edelstein D, Dimmeler S, Ju Q, Sui C, Brownlee M: Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. *J Clin Invest* 108:1341–1348, 2001
- Ito H, Rovira II, Bloom ML, Takeda K, Ferrans VJ, Quyyumi AA, Finkel T: Endothelial progenitor cells as putative targets for angiostatin. *Cancer Res* 59:5875–5877, 1999
- Daitoku H, Hatta M, Matsuzaki H, Aratani S, Ohshima T, Miyagishi M, Nakajima T, Fukamizu A: Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. *Proc Natl Acad Sci U S A* 101:10042–10047, 2004
- Federici M, Hribal ML, Ranalli M, Marselli L, Porzio O, Lauro D, Borboni P, Lauro R, Marchetti P, Melino G, Sesti G: The common Arg972 polymorphism in insulin receptor substrate-1 causes apoptosis of human pancreatic islets. *FASEB J* 15:22–24, 2001
- Seeger FH, Haendeler J, Walter DH, Rochwalsky U, Reinhold J, Urbich C, Rossig L, Corbaz A, Chvatchko Y, Zeiher AM, Dimmeler S: p38 mitogen-activated protein kinase downregulates endothelial progenitor cells. *Circulation* 111:1184–1191, 2005
- Urbich C, Knau A, Fichtlscherer S, Walter DH, Bruhl T, Potente M, Hofmann WK, de Vos S, Zeiher AM, Dimmeler S: FOXO-dependent expression of the proapoptotic protein Bim: pivotal role for apoptosis signaling in endothelial progenitor cells. *FASEB J* 19:974–976, 2005
- Brownlee M: The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* 54:1615–1625, 2005
- Miele C, Riboulet A, Maitan MA, Oriente F, Romano C, Formisano P, Giudicelli J, Beguinot F, Van Obberghen E: Human glycosylated albumin affects glucose metabolism in L6 skeletal muscle cells by impairing insulin-induced insulin receptor substrate (IRS) signaling through a protein kinase C alpha-mediated mechanism. *J Biol Chem* 278:47376–47387, 2003
- Accili D: Lilly lecture 2003. The struggle for mastery in insulin action: from triumvirate to republic. *Diabetes* 53:1633–1642, 2004
- Accili D, Arden KC: FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell* 117:421–426, 2004
- Matsumoto M, Accili D: All roads lead to FoxO. *Cell Metab* 1:215–216, 2005
- Wang MC, Bohmann D, Jasper H: JNK extends life span and limits growth by antagonizing cellular and organism-wide responses to insulin signaling. *Cell* 121:115–125, 2005
- Crow MT: Sir-iving cardiac stress: cardioprotection mediated by a longevity gene. *Circ Res* 95:953–956, 2004