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Visfatin exerts angiogenic effects on human umbilical vein endothelial cells through the mTOR signaling pathway

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ABSTRACT

The biologically active factors known as adipocytokines are secreted primarily by adipose tissues and can act as modulators of angiogenesis. Visfatin, an adipocytokine that has recently been reported to have angiogenic properties, is upregulated in diabetes, cancer, and inflammatory diseases. Because maintenance of an angiogenic balance is critically important in the management of these diseases, understanding the molecular mechanism by which visfatin promotes angiogenesis is very important. In this report, we describe our findings demonstrating that visfatin stimulates the mammalian target of the rapamycin (mTOR) pathway, which plays important roles in angiogenesis. Visfatin induced the expression of hypoxia-inducible factor 1α (HIF1α) and vascular endothelial growth factor (VEGF) in human endothelial cells. Inhibition of the mTOR pathway by rapamycin eliminated the angiogenic and proliferative effects of visfatin. The visfatin-induced increase in VEGF expression was also eliminated by RNA interference-mediated knockdown of the 70-kDa ribosomal protein S6 kinase (p70S6K), a downstream target of mTOR. Visfatin inactivated glycogen synthase kinase 3β (GSK3β) by phosphorylating it at Ser-9, leading to the nuclear translocation of β-catenin. Both rapamycin co-treatment and p70S6K knockdown inhibited visfatin-induced GSK3β phosphorylation at Ser-9 and nuclear translocation of β-catenin. Taken together, these results indicate that mTOR signaling is involved in visfatin-induced angiogenesis, and that this signaling leads to visfatin-induced VEGF expression and nuclear translocation of β-catenin.

1. Introduction

Obesity is strongly linked to diabetes mellitus and several other metabolic and cardiovascular disorders [1]. Recently, adipose tissue has emerged as an endocrine organ that is the primary producer of adipocytokines, a group of secreted, biologically active growth factors and cytokines that include adiponectin, interleukin-6, leptin, and visfatin (pre-B-cell colony-enhancing factor; PBEF) [2]. These adipocytokines have various biological functions and can have cardiovascular effects [3].

Visfatin was originally cloned from human peripheral blood lymphocytes and characterized as nicotinamide phosphoribosyltransferase (Nampt), an enzyme that synthesizes nicotinamide mononucleotide from nicotinamide [4,5]. Subsequently, it was recognized as an adipocytokine preferentially produced by visceral fat and renamed visfatin. Interestingly, visfatin has been reported to have insulin-mimetic effects in various cultured cell lines and in insulin-sensitive tissues, although these effects remain controversial [6,7].

Several recent studies have suggested that visfatin has angiogenic properties [8–11]. Angiogenesis, the process of new blood vessel formation from existent vasculature, is important not only in tissue homeostasis, embryonic development, and wound healing but also in some pathologic conditions, including tumor growth, diabetes, and inflammation [12]. Angiogenesis is also important as a therapeutic target for myocardial infarction, ischemic stroke, and other ischemic conditions.

Several studies have shown that visfatin levels are elevated in diabetes, cancer, and inflammatory disease [13–16]. Visfatin activates Akt (the serine/threonine protein kinase B) and induces the expression of vascular endothelial growth factor (VEGF) in human endothelial cells [9]. However, the precise downstream signaling mechanism underlying these effects and its ability to induce angiogenesis are not yet fully understood. Since maintaining angiogenic homeostasis is very important in preventing the progression of these diseases, understanding the molecular mechanisms by which visfatin promotes angiogenesis is an important research goal.

The serine/threonine kinase known as mTOR (mammalian target of rapamycin) is a critical regulator of cellular proliferation and metabolism. The mTOR pathway integrates the signals of various factors, including Ras, Akt, and NF-κB, that respond to nutrients,

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energy status, and growth factors [15,16]. Dysregulation of mTOR signaling is frequently associated with tumor growth, metastasis, and angiogenesis [17]. Rapamycin, an inhibitor of the mTOR pathway, is used clinically for anticancer therapy and to prevent stent restenosis [18].

In the present study, we investigated the possible involvement of the mTOR signaling pathway in visfatin-induced angiogenesis and characterized the downstream molecular effectors of visfatin. Our data show for the first time that visfatin induces angiogenesis in human endothelial cells *via* activation of the mTOR pathway. They also demonstrate that activation of the mTOR pathway increases VEGF protein expression and β-catenin nuclear translocation by inactivating glycogen synthase kinase 3β (GSK3β).

2. Materials and methods

2.1. Materials

Recombinant visfatin and a phosphoinositide 3 (PI3)-kinase inhibitor, LY294002 were purchased from Peprotech (Rocky Hill, NJ) and Calbiochem (San Diego, CA), respectively. Visfatin protein used in this study was >98% pure (SDS-PAGE analysis) and contained 0.01 ng/μg LPS as determined by the *Limulus* amebocyte lysate similar to the previous study [19]. Rapamycin, differentiation inducing factor-3 (DIF-3), lithium chloride (LiCl), and a specific competitive inhibitor of visfatin, FK866 were purchased from Sigma (St Louis, MO). Recombinant human VEGF was from R&D Systems (Minneapolis, MN). Growth factor-reduced Matrigel was purchased from BD Biosciences (San Jose, CA). The primary antibodies used in this study were specific for Ser-473-phosphorylated Akt, Ser-2448-phosphorylated mTOR, Ser-9-phosphorylated GSK3β, Thr-389-phosphorylated 70-kDa ribosomal protein S6 kinase (p70S6K), Ser-241-phosphorylated phosphoinositide-dependent kinase-1 (PDK1), HIF1α (Cell Signaling Technology, Beverly, MA), VEGF (R&D Systems), β-catenin (BD Biosciences), cyclin D1 (BD Biosciences), visfatin (Phoenix Pharmaceuticals, Belmont, CA), and β-actin (Sigma).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs; Clonetics, Walkersville, MD) at passages 3–6 were grown in SingleQuots endothelial cell growth medium-2 (EGM-2; Clonetics) at 37 °C in a humidified 5%-CO_2 atmosphere. The medium was replaced with endothelial cell basal medium-2 (EBM-2; Clonetics) supplemented with 0.1% fetal bovine serum (FBS) 5 h before all experimental treatments, with the exception of tube formation and aortic ring-sprouting assays.

2.3. Western blotting

Cells were lysed in a lysis buffer (Cell Signaling Technology) containing 50 mM NaF, 2 mM Na_3VO_4, 100 μg/ml phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma). Protein concentrations in the lysate samples were determined using Bradford Assay Reagent (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin (BSA) as a standard. Equal amounts of cell lysates were subjected to SDS-PAGE, and the separated proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The membrane was incubated with primary antibody overnight at 4 °C and then with the appropriate secondary antibody for 1 h at room temperature. Bands were detected using an ECL system (Santa Cruz Biotechnology, Santa Cruz, CA). All experiments were performed at least three times.

2.4. Tube formation assay

Tube formation assays were carried out using Matrigel according to the manufacturer's protocol (BD Biosciences). Briefly, HUVECs were cultured on the surface of the Matrigel, treated with visfatin (1 μg/ml) in the presence or absence of rapamycin (25 nM), and the extent of capillary tube formation 15 h after treatment was observed in three random microscopic fields. As a positive control, the cells were treated with VEGF (20 ng/ml) rather than visfatin.

2.5. Aortic ring-sprouting assay

Aortic ring-sprouting assays were performed using a protocol modified from a previous study [20]. Each well of a 24-well plate was coated with Matrigel, and the Matrigel was allowed to polymerize for 30 min at 37 °C. Aortas were extracted from 6-week-old male C57BL/6 mice, and the adipose tissue was carefully removed under a microscope. The aortas were cut into 1-mm-thick slices, rinsed four times with EGM-2 medium, placed on the Matrigel-coated plates, and covered with additional Matrigel. The Matrigel was allowed to solidify at 37 °C, and the EGM-2 medium was added. After 24 h, the medium was replaced with fresh EBM-2 media supplemented with 2% FBS, ascorbic acid, hydrocortisone, heparin, and amphotericin, and visfatin, rapamycin, and/or VEGF were added. After 4 days of incubation, vascular sprouts from the aortic tissues were photographed. Mice were housed and treated in accordance with the Animal Care Guidelines of the Korean National Institutes of Health Animal Facility.

2.6. 5-bromodeoxyuridine (BrdU) incorporation assay

BrdU incorporation was assayed using a Cell Proliferation ELISA BrdU (colorimetric) kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, HUVECs treated with visfatin in the presence or absence of rapamycin were labeled with BrdU for 8 h, fixed, and washed. Anti-BrdU-POD working solution and substrate solution were added, and BrdU incorporation was quantified by measuring the absorbance at 370 nM on an automated ELISA microplate reader (Molecular Devices, Sunnyvale, CA).

2.7. In vitro wound-healing migration assay

HUVECs were seeded into 12-well plates and grown to 90% confluence. Wounds were made by careful scraping of the cell layer with sterile cell scrapers. The medium was replaced with EBM-2 media supplemented with 0.1% FBS and visfatin with or without rapamycin. After 24 h at 37 °C, the cells were photographed, and the cells that had migrated into the wound area were counted manually. At least three independent experiments were performed.

2.8. Transfection of siRNA

HUVECs grown to 30% confluence were transfected with p70S6K or β-catenin siRNA (Invitrogen, Carlsbad, CA) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. After 48 h, the cells were treated with visfatin. The siRNA sequences were as follows: human p70S6K, 5′-UUC UCC UCC ACU GAG AUA CUC AAG G-3′ (sense) and 5′-CCU UGA GUA UCU CAG UGG AGG AGA A-3′ (anti-sense); human β-catenin, 5′-UUA CCA CUC AGA GAA GGA GCU GUG G-3′ (sense) and 5′-CCA CAG CUC CUU CUC UGA GUG GUA A-3′ (anti-sense). At a final siRNA concentration of 50 nM, the transfection efficiency was about 80% efficiency.

2.9. Immunocytochemical analysis

After HUVECs were cultured on four-well Lab-Tek chamber slides (Nalge Nunc, Rochester, NY), the medium was replaced with the EBM-

2 medium supplemented with 0.1% FBS and visfatin with or without rapamycin. After 4 h, the cells were fixed in 4% paraformaldehyde for 20 min and washed three times with phosphate-buffered saline (PBS). The cells were permeabilized by incubation in 0.2% Triton X-100 in PBS for 2 min, blocked with 5% BSA in PBS (5% BSA/PBS) for 1 h, and then incubated with a mouse monoclonal antibody against human β-catenin (1:200 in 2% BSA/PBS) or with a rabbit polyclonal antibody against cleaved caspase-3 (1:100 in 2% BSA/PBS) overnight at 4 °C. After three washes with PBS, the cells were incubated with Alexa 594-conjugated goat anti-mouse secondary antibody (Invitrogen) or with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Invitrogen) in 2% BSA/PBS. The cells were rinsed with PBS, mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA), and imaged under a fluorescence microscope.

3. Results

3.1. Visfatin activates mTOR and inactivates GSK3β

When we examined the phosphorylation of PDK1, Akt, mTOR, and GSK3β in HUVECs grown in the presence of visfatin, Western blot analysis with phospho-specific antibodies confirmed that visfatin increases PDK1 and Akt phosphorylation as described previously (Fig. 1) [8,9,11]. In addition, visfatin time- and dose-dependently increased mTOR phosphorylation at Ser-2448 and GSK3β phosphorylation at Ser-9, thereby activating mTOR and deactivating GSK3β (Fig. 1). It also time-dependently increased the total expression of β-catenin. Because visfatin has been reported to increase VEGF expression, and HIF1α is a key transcription factor for VEGF transcription [21–23], we also examined the effect of visfatin on HIF1α expression. We found that visfatin increased HIF1α expression in a dose-dependent manner (Fig. 1B).

3.2. The mTOR signaling pathway is involved in visfatin-induced angiogenesis in vitro and ex vivo

Our finding that visfatin induces activation of mTOR suggested that the mTOR signaling pathway is involved in visfatin-induced angiogenesis. To investigate this hypothesis, we examined the effects of rapamycin, a specific inhibitor of mTOR, on visfatin-induced angiogenesis. First, we examined the formation of capillary-like tubes by HUVECs plated on Matrigel. As shown in Fig. 2A, visfatin treatment increased tube formation by the HUVECs, but rapamycin significantly inhibited this effect. Next, we examined the effect of rapamycin on visfatin-induced angiogenesis in an *ex vivo* mouse aorta model (Fig. 2B). Incubation of mouse aortic tissue embedded in Matrigel with visfatin for 4 days increased microvessel sprouting from the tissue, but the addition of rapamycin significantly inhibited visfatin induced-microvessel outgrowth (Fig. 2B).

We next examined endothelial cell mobility by counting the HUVECs that migrated into a wound scratched into a confluent cell monolayer with a cell scraper. Visfatin significantly increased the mobility of the HUVECs, but co-treatment with rapamycin significantly inhibited this effect (Fig. 2C); in fact, it reduced cell mobility to less than that of the control. Similarly, when we explored the HUVEC proliferation using BrdU incorporation and MTT assays, we observed that visfatin significantly increased HUVEC proliferation, but co-treatment with rapamycin significantly inhibited this effect of visfatin (Fig. 2D and Supplementary Fig. 1A). To explore the involvement of apoptosis in this rapamycin-induced growth inhibition, fixed HUVECs were stained with an antibody against cleaved caspase-3 (Supplementary Fig. 1B). The number of cells positive for cleaved caspase-3 did not increase significantly after rapamycin exposure (Supplementary Fig. 1B). A terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay yielded similar results (data not shown). These results suggest that rapamycin does not exert its antiproliferative and antiangiogenic effects *via* apoptosis.

3.3. Visfatin increases VEGF expression by affecting the mTOR signaling pathway

Visfatin has been reported to increase VEGF expression *via* the Akt signaling pathway [9]. To explore whether the visfatin-induced expression of VEGF also involves the mTOR signaling pathway, HUVECs were treated for 24 h with visfatin in the presence or absence of rapamycin. When we measured VEGF expression and normalized it to β-actin expression, we found that visfatin treatment increased VEGF expression by half, and that co-treatment with rapamycin abolished this increase (Fig. 3A). To more specifically explore the downstream signaling mechanism, we analyzed the phosphorylation of p70S6K, an mTOR downstream kinase, in HUVECs treated with visfatin in the presence or absence of rapamycin for 4 h. In the absence of rapamycin, a visfatin-induced increase in VEGF expression was evident within 4 h, as was an increase in phosphorylation of p70S6K at Thr-389 (Fig. 3B). These effects were eliminated by rapamycin co-treatment. Visfatin also increased HIF1α expression in a rapamycin-inhibitable manner, but it increased Akt phosphorylation at Ser-473 in a rapamycin-independent manner (Fig. 3B), suggesting that visfatin increases VEGF expression via the mTOR signaling pathway.

To confirm that mTOR signaling is involved in visfatin-induced VEGF expression, we examined the effect of knockdown of p70S6K, which reportedly mediates mTOR phosphorylation at Ser-2448 [24]. The increase in VEGF expression induced by incubation with visfatin for 24 h was substantially smaller in p70S6K siRNA-transfected HUVECs than in control siRNA-transfected cells (Fig. 3C, D).

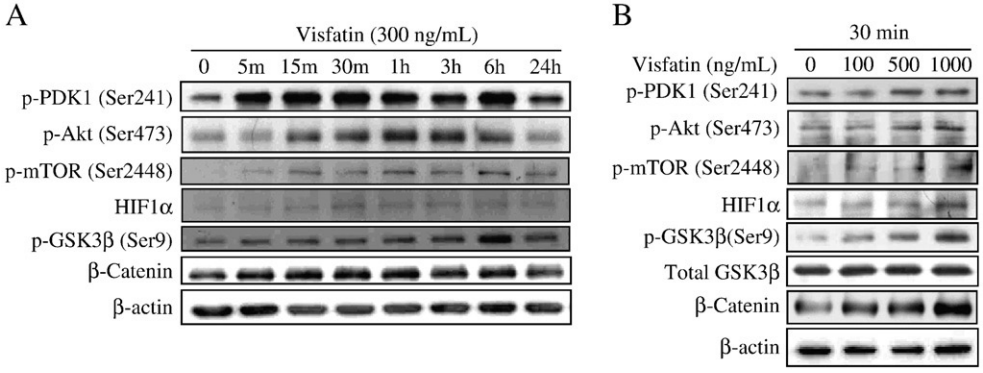

Fig. 1. Visfatin activates mTOR and inactivates GSK3β signaling pathways in HUVECs. Serum-starved HUVECs were treated with visfatin and analyzed for the indicated proteins by Western blotting. Results shown are representative of at least three independent experiments. (A) HUVECs were treated with 300 ng/ml visfatin for the indicated lengths of time. (B) HUVECs were treated with the indicated doses of visfatin for 30 min.

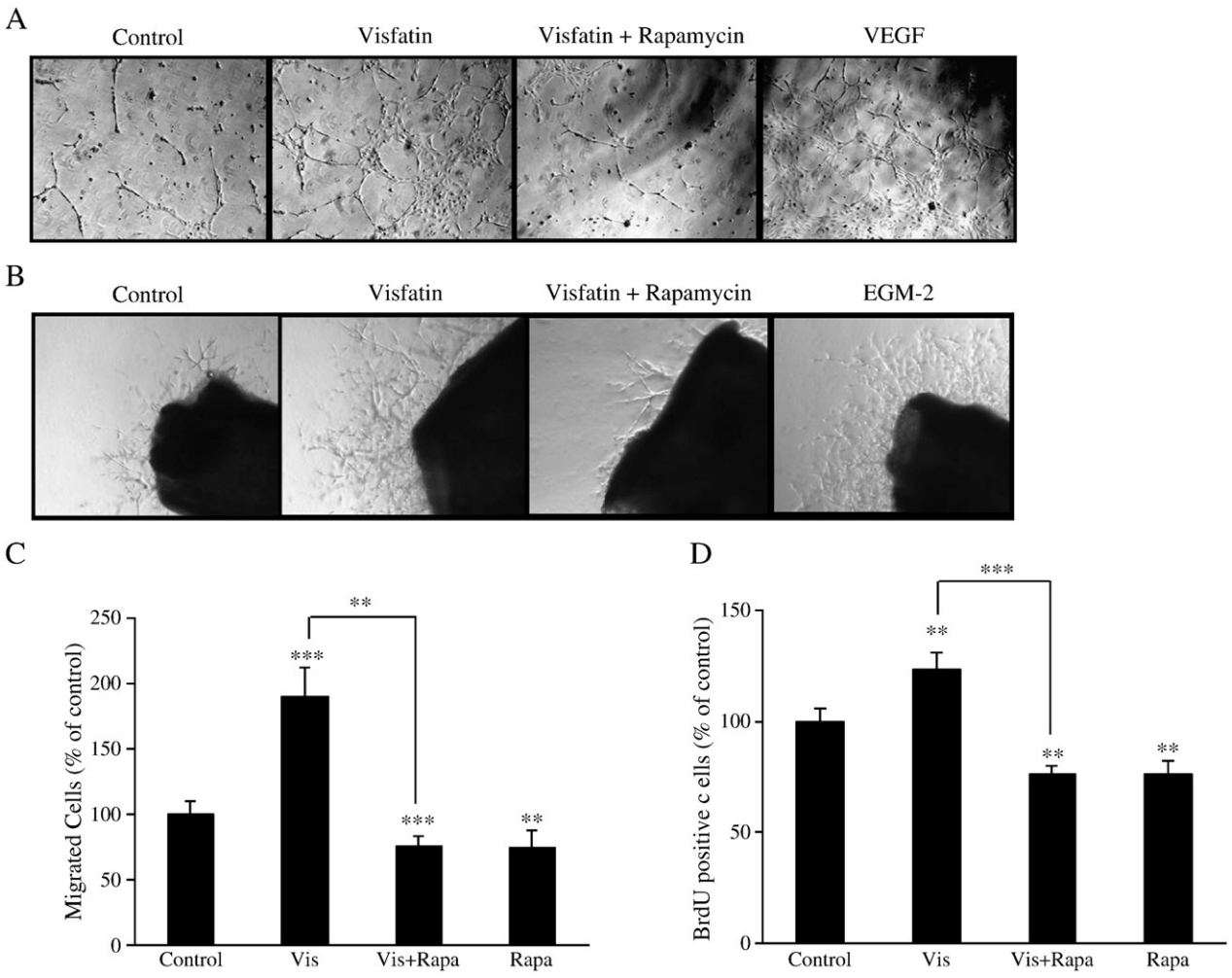

Fig. 2. Rapamycin inhibits visfatin-induced angiogenesis. (A) HUVECs were seeded on Matrigel in the presence of visfatin (1 μg/ml) with or without rapamycin (25 nM) for 15 h and then examined for the formation of capillary-like tubes. VEGF (20 ng/ml) was used as a positive control. (B) Mouse aortic tissues were embedded into Matrigel and incubated for 4 days in the presence of visfatin (1 μg/ml) with or without rapamycin (25 nM) and then examined for visfatin-induced microvessel outgrowth. As a positive control, tissues were incubated in EGM-2 medium for 4 days. (C) Confluent HUVEC monolayers were wounded with a cell scraper, and incubated with visfatin (1 μg/ml) in the presence or absence of rapamycin (25 nM), and assessed for cell mobility by counting the cells that had moved into the wounded area after 24 h. Values are expressed as means ± SD ($n = 6$). (D) Endothelial cell proliferation was examined by measuring BrdU incorporation in HUVECs preincubated with rapamycin (25 nM) prior to stimulation with visfatin (1 μg/ml). Values are expressed as means ± SD ($n = 4$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs control or vs visfatin- and rapamycin-treated samples using Student's *t*-test.

Knockdown of p70S6K also reduced mTOR phosphorylation at Ser-2448 (Fig. 3D), consistent with the results of Chiang et al. [24].

3.4. The mTOR signaling pathway is involved in visfatin-induced inactivation of GSK3β and nuclear translocation of β-catenin

Because we observed that visfatin increases the phosphorylation of GSK3β at Ser-9 residue (Fig. 1), thereby inactivating it, and p70S6K has been reported to regulate GSK3β phosphorylation [25], we investigated whether the mTOR signaling pathway is involved in visfatin-induced phosphorylation of GSK3β. After treating HUVECs with visfatin in the absence or presence of rapamycin for 4 h, we analyzed GSK3β phosphorylation at Ser-9 (Fig. 4A). Rapamycin inhibited the induction of both GSK3β phosphorylation and β-catenin expression by visfatin (Fig. 4A). Knockdown of p70S6K expression produced effects similar to those of rapamycin treatment (Fig. 4B).

To investigate whether visfatin promotes nuclear translocation of β-catenin, we examined the cytoplasmic and nuclear fractions of HUVECs incubated with visfatin in the presence or absence of rapamycin. Visfatin treatment increased β-catenin levels in the nuclear extracts, but co-treatment with rapamycin reduced the magnitude of this increase (Fig. 4C), as did p70S6K knockdown (Fig. 4D). Immunocytochemical analysis confirmed that visfatin promotes β-catenin nuclear translocation and that this effect of visfatin is inhibited by rapamycin (Fig. 4E). These results indicate that the mTOR signaling pathway is involved in visfatin-induced GSK3β inactivation and β-catenin nuclear translocation.

3.5. β-catenin is involved in visfatin-induced angiogenesis

To explore the role of β-catenin in visfatin-induced angiogenesis, we used RNA interference to knock down β-catenin expression in HUVECs. β-catenin knockdown significantly inhibited the visfatin-induced migration of HUVECs (Fig. 5A). Because β-catenin induces the expression of cyclin D1, which acts as a mitogenic signal sensor [26], we next investigated whether knockdown of β-catenin would affect cyclin D1 expression in visfatin-treated HUVECs. Visfatin treatment significantly increased the expression of cyclin D1 in control cells but not in β-catenin siRNA-transfected cells (Fig. 5B). In addition, β-catenin knockdown nearly eliminated the visfatin-induced expression of VEGF (Fig. 5B) and inhibited the visfatin-induced formation of capillary-like tubes by HUVECs seeded on Matrigel beds (Fig. 5C).

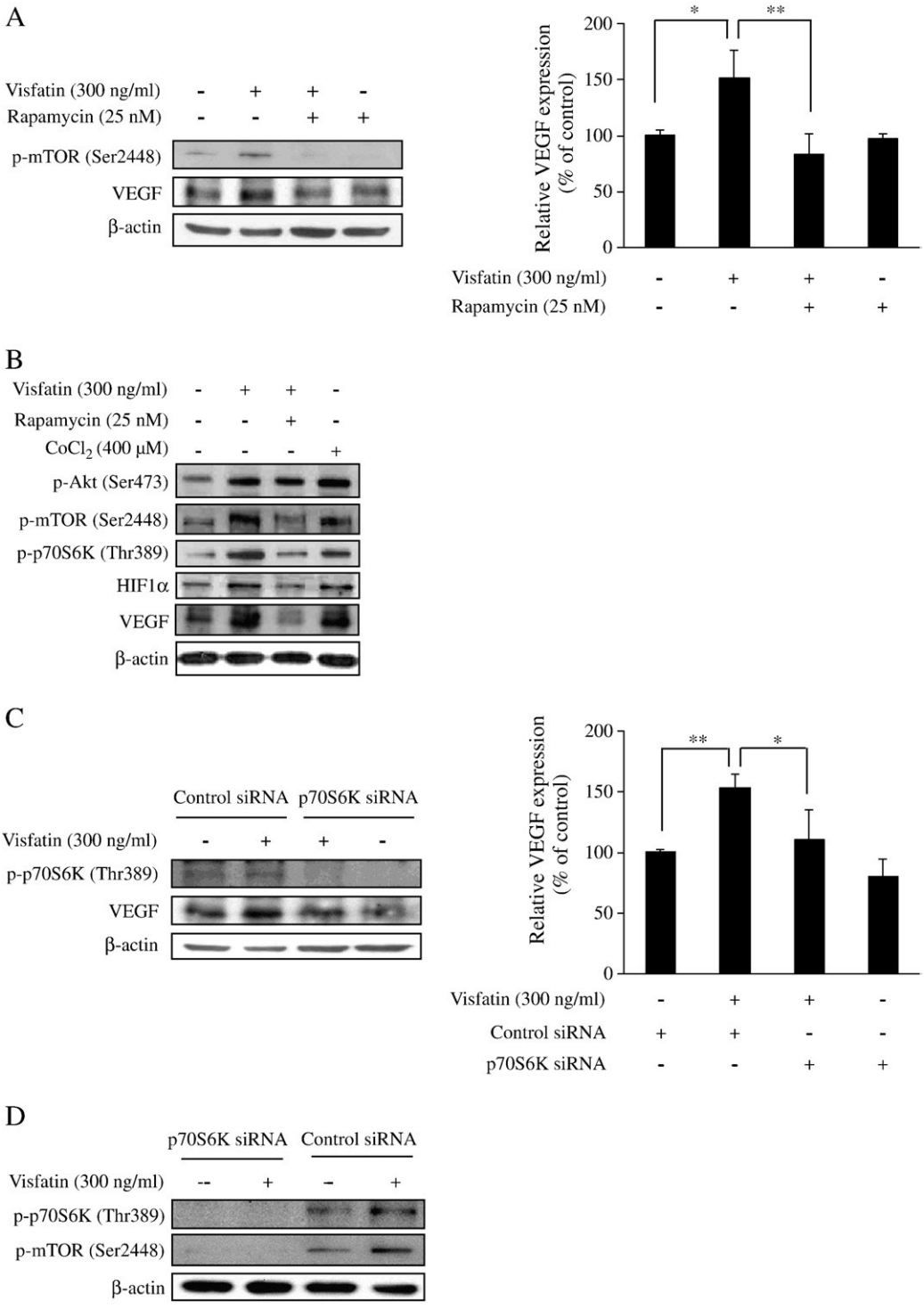

Fig. 3. Western blot analysis of visfatin-treated HUVECs shows that the mTOR signaling pathway is involved in visfatin-induced expression of VEGF. (A) HUVECs were incubated with visfatin (300 ng/ml) in the presence or absence of rapamycin (25 nM) for 24 h and assessed for VEGF expression and mTOR phosphorylation at Ser-2448 by Western blotting. Values are expressed as means ± SD ($n = 4$). *$P<0.05$, **$P<0.01$ using Student's t-test. (B) HUVECs were incubated with visfatin (300 ng/ml) in the presence or absence of rapamycin (25 nM) or with $CoCl_2$ (400 μM) for 4 h and assessed for expression of VEGF and HIF1α and for phosphorylation of mTOR (Ser-2448), Akt (Ser-473), and p70S6K (Thr-389) by Western blotting. Results shown are representative of at least three independent experiments. (C) HUVECs transfected with control siRNA or p70S6K siRNA were treated with or without visfatin (300 ng/ml) for 24 h and assessed for VEGF expression and p70S6K phosphorylation at Thr-389 by Western blotting. Values are expressed as means ± SD ($n = 4$). *$P<0.05$, **$P<0.01$ using Student's t-test. (D) HUVECs transfected with control siRNA or p70S6K siRNA were treated with or without visfatin (300 ng/ml) for 4 h and assessed for p70S6K phosphorylation at Thr-389 and mTOR phosphorylation at Ser-2448 by Western blotting. Results shown are representative of at least three independent experiments.

Visfatin-induced tube formation was completely blocked by the addition of rapamycin to β-catenin siRNA-transfected HUVECs (Fig. 5C). These results suggest that β-catenin and mTOR play key roles in visfatin-induced angiogenesis.

3.6. Akt acts upstream of mTOR and GSK3β signaling

Both PDK1 and Akt bind directly to phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] and phosphatidylinositol (3,4)-

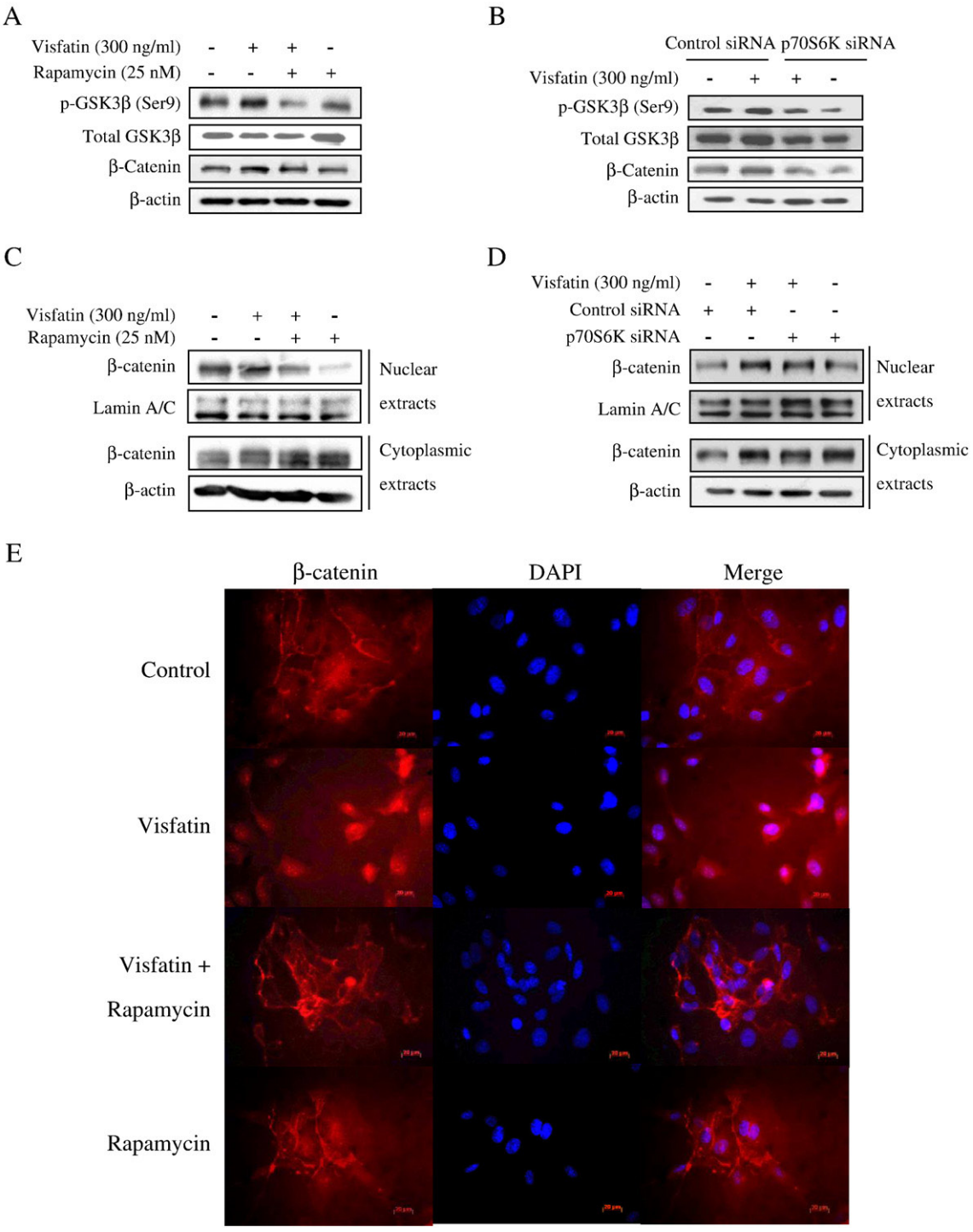

Fig. 4. Western blot (A–D) and immunocytochemical (E) analyses showing that the mTOR signaling pathway is involved in visfatin-induced inactivation of GSK3β and nuclear translocation of β-catenin. (A, C, and E) HUVECs were incubated with visfatin (300 ng/ml) in the presence or absence of rapamycin (25 nM) for 4 h. (B and D) HUVECs transfected with control siRNA or p70S6K siRNA were incubated with visfatin (300 ng/ml) for 4 h. (A and B) GSK3β phosphorylation at Ser-9 was analyzed by Western blotting. (C and D) The cytoplasmic and nuclear fractions were separated, and β-catenin translocation into the nucleus was analyzed by Western blotting. Lamin A/C and β-actin were faintly detected in cytoplasmic and nuclear fractions, respectively (data not shown). (E) After the cells were fixed and permeabilized, they were incubated with mouse anti-β-catenin antibody and then with Alexa 594-conjugated anti-mouse antibody (red). Nuclei were counter-stained with DAPI (blue). The cells were imaged under a fluorescence microscope at 400×. In A–E, the results shown are representative of at least three independent experiments.

bisphosphate [PtdIns(3,4)P_2], which are produced by activated PI3-kinase and are restricted to the plasma membrane. Therefore, activation of PI3-kinase causes PDK1 and Akt to translocate to the plasma membrane, where activated PDK1 phosphorylates and activates Akt [27]. Therefore, when we observed that visfatin induces the phosphorylation of PDK1 and Akt (Fig. 1), we next investigated the effect of visfatin-induced activation of Akt on mTOR and GSK3β using LY294002, an inhibitor of PI3-kinase (Fig. 6). Pretreatment of HUVECs with this inhibitor attenuated visfatin-induced mTOR activation and GSK3β inactivation. In addition, LY294002 pretreatment abolished visfatin-induced β-catenin expression. These results suggest that PI3-kinase and Akt act upstream of mTOR and GSK3β.

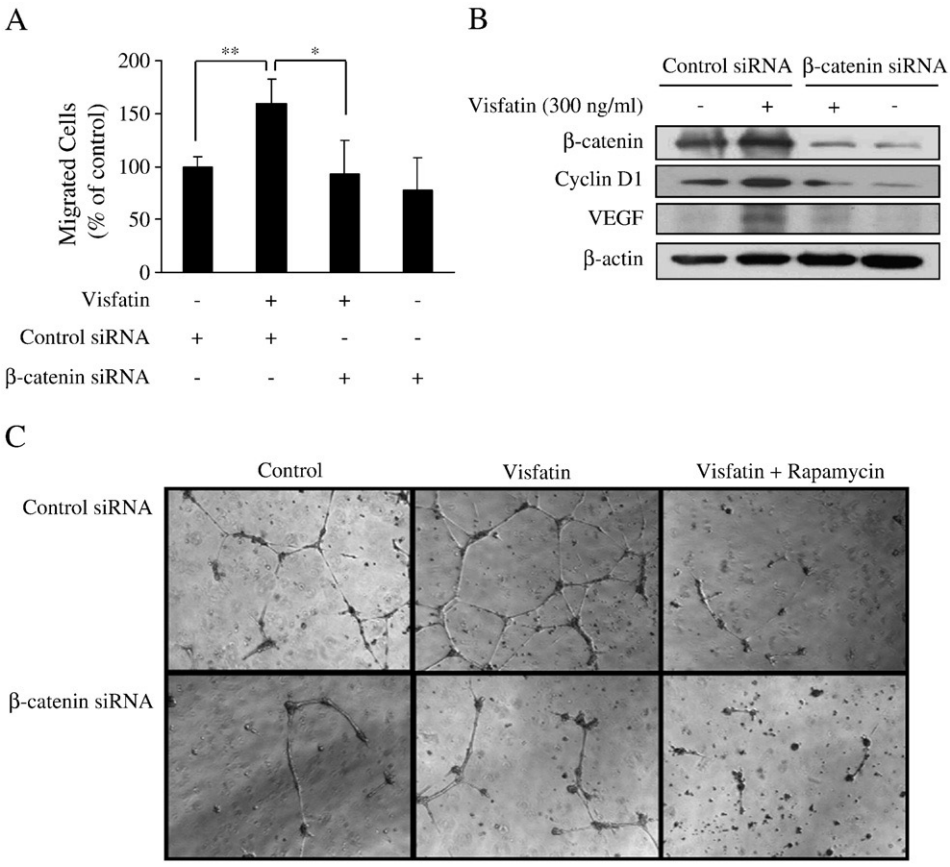

Fig. 5. Knockdown of β-catenin expression by siRNA infection inhibits visfatin-induced angiogenesis. HUVECs were transfected with control or β-catenin siRNA for 48 h and analyzed for mobility (A); expression of β-catenin, cyclin D1, and VEGF (B); and the effect of rapamycin on microvessel outgrowth. (A) After confluent transfected HUVEC monolayers were wounded with a cell scraper, the cells were incubated with or without visfatin (1 μg/ml) for 24 h, and the cells that migrated into the wounded area were counted. Values are expressed as means ± SD ($n = 4$). *$P<0.05$, ** $P<0.01$ using Student's t-test. (B) Transfected HUVECs were treated with visfatin (300 ng/ml) for 24 h and analyzed for expression of the indicated proteins by Western blotting. (C) Transfected HUVECs were detached using trypsin-EDTA, seeded on Matrigel beds, and incubated with visfatin (1 μg/ml) in the presence or absence of rapamycin (25 nM) for 15 h. and assessed for microvessel outgrowth. In A–C, the results shown are representative of at least three independent experiments.

4. Discussion

Visfatin plays important roles in angiogenesis, acting through several different mechanisms [8–11,28]. It up-regulates the expression of VEGF, VEGF receptor 2 (VEGF-R2), and endothelial nitric oxide synthase by activating the mitogen-activated protein kinase and Akt signaling pathways [8,9,11]; it up-regulates fibroblast growth factor-2 in an Erk1/2-dependent manner [10] and it enhances IL-6 expression through STAT3 activation, which promotes angiogenesis [28]. Although the activation of Akt signaling by visfatin is well established, the events occurring downstream of Akt activation remain unexplored [8,9,11]. We have now demonstrated for the first time that visfatin activates mTOR signaling and inactivates GSK3β signaling in human endothelial cells, thereby enhancing VEGF and cyclin D1 expression in these cells.

The mTOR protein is a serine/threonine kinase that plays a key role in cell growth and homeostasis. Hypoxia activates the mTOR pathway to promote angiogenesis and cell proliferation [22,29]. Several studies have found that the activation of PI3-kinase and the subsequent activation of mTOR enhance the expression of HIF1-dependent genes, including VEGF [21–23]. HIF1 is a heterodimeric transcription factor composed of the inducible and constitutively expressed subunits HIF1α and HIF1β, respectively [21]. Under hypoxic conditions, mTOR activation enhances the activity of HIF1α by inhibiting proteolytic degradation, resulting in elevated VEGF expression; this effect is reversible by rapamycin [21,23]. Since mTOR activation is needed to prevent HIF1α degradation, HIF1α seems to be increased after 30 min of visfatin treatment (Fig. 1).

The mTOR protein has three phosphorylation sites, Ser-2446, Ser-2448, and Ser-2481. Ser-2448 phosphorylation, which is usually induced by insulin, correlates with mTOR activation [30–32] and, in our study, was increased by visfatin. Thus, the induction of mTOR Ser-2448 phosphorylation by visfatin indicates that visfatin activates the mTOR pathway, which is also demonstrated by the increased phosphorylation of p70S6K at Thr-389 (Fig. 3B). Although mTOR has long been known to be phosphorylated at Ser-2448 via the PI3-kinase/Akt signaling pathway, p70S6K was recently revealed to be the major protein kinase responsible for this phosphorylation [24]. In accordance with the results of Chiang et al. [24], we found that p70S6K knockdown abolished the visfatin-induced increase in mTOR Ser-2448 phosphorylation and VEGF expression (Fig. 3D). Thus, the mTOR pathway is important in the induction of VEGF expression by visfatin in endothelial cells.

GSK3β is a serine/threonine kinase that plays important roles in metabolism, embryonic development, and tumorigenesis [33]. GSK3β has two sites of reversible phosphorylation: an inhibitory site (Ser-9) and an activating site (Tyr-216). Visfatin-induced phosphorylation of GSK3β at Ser-9 thus leads to the inactivation of GSK3β (Fig. 1). The phosphorylation of β-catenin at Ser-37, Ser-33, and Thr-41 by GSK3β results in the ubiquitination and degradation of β-catenin. The recent finding that β-catenin induces the expression of VEGF-A and VEGF-C implicates β-catenin in angiogenesis [33,34]. In our study, β-catenin knockdown diminished visfatin-induced cell proliferation and tube formation (Fig. 5). β-catenin has been reported to play dual roles in cells; it maintains cell architecture and polarity at adherens junctions and it translocates into the nucleus to increase the expression of

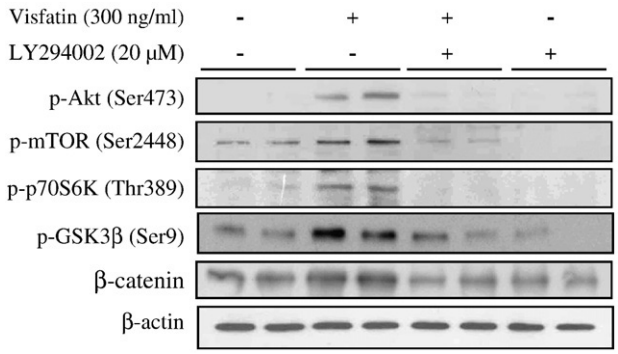

Fig. 6. The PI3-kinase/Akt signaling pathway is involved in visfatin-induced mTOR activation and GSK3β inactivation. HUVECs were pretreated with the PI3-kinase inhibitor LY294002 (20 μM), incubated with visfatin (300 ng/ml) for 4 h, and analyzed for levels of the indicated proteins by Western blotting. Results shown are representative of at least three independent experiments.

various genes, including c-myc and cyclin D1 [26,33]. In unstimulated endothelial cells, β-catenin primarily localizes at the cell membrane, but visfatin induces its nuclear translocation, leading to increased expression of cyclin D1 (Fig. 4C). In addition, β-catenin knockdown diminishes cyclin D1 and VEGF expressions, thereby inhibiting the induction of an angiogenic phenotype by visfatin (Fig. 5).

Interestingly, visfatin-induced GSK3β phosphorylation at Ser-9 depends on the mTOR pathway (Fig. 4A, B). Our results are consistent with those of Zhang et al. [25], who reported that GSK3β can be regulated downstream of mTOR complex 1 and that p70S6K, rather than Akt, is the predominant GSK3β regulatory kinase, particularly under conditions of continuous activation of the mTOR pathway [25]. In other words, activation of mTOR signaling by visfatin contributes to GSK3β inactivation in human endothelial cells.

In contrast, GSK3β inhibition can result in mTOR activation according to Inoki et al. [34]. We therefore investigated the effect of visfatin-induced inactivation of GSK3β on mTOR activation using DIF-3 and LiCl to induce GSK3β activation and inactivation, respectively (Supplementary Fig. 2) [26,35]. LiCl pretreatment did not affect visfatin-induced mTOR activation, but DIF-3 pretreatment diminished this activation (Supplementary Fig. 2A) and inhibited visfatin-induced tube formation (Supplementary Fig. 2B). In immunocytochemical experiments, neither DIF-3 nor LiCl treatment were found to increase caspase-3 cleavage (Supplementary Fig. 2C), suggesting that angiogenic inhibition by DIF-3 is not attributable to apoptosis induction. TUNEL assay yielded similar results (data not shown). This finding is consistent with that reported by Takahashi-Yanaga et al. [35]. Thus, the effects of visfatin on mTOR and GSK3β may result from reciprocal regulation. Further studies are needed to confirm this hypothesis.

It is still unknown whether the exogenous visfatin initiated the angiogenic effects via cellular surface receptor or intracellular target. Thus, we measured both intracellular and extracellular visfatin levels after visfatin treatment in HUVECs (Supplementary Fig. 3). After visfatin treatment for 30 min, visfatin levels in medium were increased in a dose-dependent manner, while visfatin levels were not significantly increased in cell lysates. In contrast, visfatin levels were not significantly changed in medium after visfatin treatment for 24 h, while visfatin levels were significantly increased in cell lysates (Supplementary Fig. 3A, B). These results indicate that exogenous visfatin may penetrate the membrane and directly function inside cells after 24 h. However, the activation of signaling molecules such as Akt, mTOR, and GSK3β already occurred after 30 min of visfatin treatment (Fig. 1A, B). Thus, the activation of signaling molecules by visfatin in the present study may occur via cellular surface receptor.

Visfatin plays a key role in the salvage pathway for NAD+ biosynthesis. It has been demonstrated that a specific competitive inhibitor of visfatin, FK866, causes cellular NAD+ depletion, and has anti-angiogenic activity in murine renal cell carcinoma [36,37]. We examined the effect of FK866 on visfatin-induced angiogenesis *in vitro* and *ex vivo* (Supplementary Fig. 4A, B). Interestingly, FK866 could abolish the angiogenic effects of visfatin *in vitro* and *ex vivo*. Thus, NAD+ levels seem to be important for visfatin-induced angiogenesis. Correlation between signaling pathways and NAD+ levels in visfatin-induced angiogenesis needs to be further elucidated.

In the present study, visfatin induced angiogenesis at concentrations higher than 300 ng/ml similar to the previous studies [9,10,28]. Although plasma visfatin concentrations in humans are usually <100 ng/ml, visfatin concentrations in tissues such as cancer and adipose tissues can be much higher. In addition, exogenous visfatin could enter into cells as shown in Supplementary Fig. 3.

Visfatin also has a reported association with inflammation [38], which is closely linked to angiogenesis through a mechanism that is not well understood [39]. The possibility that visfatin plays a key role in bridging the gap between angiogenesis and inflammation is another reason that understanding the mechanism by which visfatin promotes angiogenesis is critically important.

Previous studies have reported that the PI3-kinase/Akt signaling pathway is an important regulator of visfatin-induced angiogenesis [9,11]. In the present study, we showed that mTOR and GSK3β signaling is involved in the effects of visfatin on angiogenesis. Taken together (see Fig. 7), our results demonstrate that visfatin-induced angiogenesis requires mTOR and GSK3β signaling downstream of the PI3-kinase/Akt signaling pathway. Inhibition of PI3-kinase by the inhibitor LY293002 also inhibits visfatin-induced activation of mTOR and visfatin-induced inactivation of GSK3β. Activation of mTOR inactivates GSK3β, which promotes the nuclear translocation of β-catenin, thereby increasing the expression of cyclin D1 and VEGF. In contrast, GSK3β inactivation may also activate mTOR. Activation of mTOR also increases HIF1α and VEGF expression in endothelial cells. Collectively, these results show that the activation of mTOR and the inactivation of GSK3β play key roles in visfatin-induced angiogenesis. The HIF1α and β-catenin transcription factors are known to regulate VEGF expression, and both were upregulated by visfatin exposure in our experiments using endothelial cells (Fig. 1), suggesting that HIF1α and β-catenin may physically interact with each other to enhance VEGF expression. Further studies are needed to elucidate the precise mechanism underlying this putative interaction and to determine which transcription factor plays an executive role in VEGF expression.

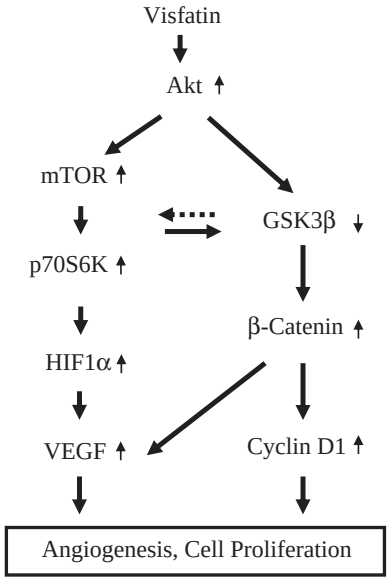

Fig. 7. Proposed scheme for the signaling pathways involved in visfatin-induced angiogenesis. See Discussion for details. The dotted arrow indicates a pathway that remains to be elucidated.

In conclusion, our data provide the first evidence that visfatin-induced mTOR activation and GSK3β inactivation play important roles in visfatin-induced angiogenesis. Furthermore, visfatin increases HIF1α and β-catenin expressions, leading to increased expression of VEGF and cyclin D1. Our findings demonstrate that visfatin and these pro-angiogenic factors are engaged in functional crosstalk *via* the mTOR and GSK3β pathways. This newly identified mechanism of visfatin signaling provides novel insights into the diverse effects of visfatin and will enhance understanding of its functional roles in human diseases.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2011.02.009.

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