Regulation of Angiogenesis by Glycogen Synthase Kinase-3β*

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REGULATION OF ANGIOGENESIS BY GLYCOCEN SYNTHASE KINASE-3β

Glycogen synthase kinase-3β (GSK3β) plays important roles in metabolism, embryonic development, and tumorigenesis. Here we investigated the role of GSK3β signaling in vascular biology by examining its function in endothelial cells (ECs). In EC, the regulatory phosphorylation of GSK3β was found to be under the control of phosphoinositide 3-kinase-, MAPK-, and protein kinase A-dependent signaling pathways. The transduction of a nonphosphorylatable constitutively active mutant of GSKβ promoted apoptosis under the conditions of prolonged serum deprivation or the disruption of cell-matrix attachments. Conversely, the transduction of catalytically inactive GSK3β promoted EC survival under the conditions of cellular stress. Under normal cell culture conditions, the activation of GSK3β signaling inhibited the migration of EC to vascular endothelial growth factor or basic fibroblast growth factor. Angiogenesis was inhibited by GSK3β activation in an in vitro Matrigel plug assay, whereas the inhibition of GSK3β signaling enhanced capillary formation. These data suggest that GSK3β functions at the nodal point of converging signaling pathways in EC to regulate vessel growth through its control of vascular cell migration and survival.

Glycogen synthase kinase-3 (GSK3) is a highly conserved and ubiquitously expressed serine/threonine kinase that phosphorylates proteins containing clustered serine or threonine residues that are separated by four amino acids (1). GSK3α and GSK3β are encoded by different genes, and they are 85% homologous in their amino acid sequence. Both isoforms have similar substrate specificity and are regulated in parallel in response to growth factor stimulation (2). However, the disruption of the GSK3β gene in mice results in embryonic lethality, indicating that GSK3α cannot completely substitute for a loss of GSK3β (3). Although GSK3 was originally identified as a kinase that phosphorylates glycogen synthase, subsequent studies have demonstrated that it has a broader range of substrates including β-catenin, tau, myelin basic protein, cyclin D1, GATA4, c-Jun, c-Myc, CREB, initiation factor eIF2B, heat shock factor-1, and p53 (2). Through the phosphorylation of this diverse set of substrates, GSK3 regulates embryonic development and proliferative responses in adult tissues and is implicated in several human disease states including tumorigenesis, Alzheimer’s disease, and diabetes.

GSK3 signaling is inactivated in cells that are stimulated by mitogens. Growth factor-induced inactivation involves phosphorylation of the N-terminal serine residue (Ser-21 for GSK3α and Ser-9 for GSK3β). This phosphorylation can be mediated by several upstream protein kinases including p90RSK, p70S6K, integrin-linked kinase, Akt, and protein kinase A (PKA) (4). GSK3 is also regulated by Wnt signaling during embryonic development, leading to the specification of cell fate (5). Mitogenic and Wnt signaling differentially regulates GSK, and this can elicit distinct downstream responses (2).

Relatively little is known about the role of GSK3 signaling in the cardiovascular system. Recently, two studies have shown that GSK3β signaling inhibits cardiac myocyte hypertrophy, an effect that may be mediated through its regulation of the nuclear factor of activated T cells or GATA4 transcription factors (6, 7). It has also been shown that GSK3β promotes apoptosis in cultured vascular smooth muscle cells (8). However, its function in endothelial cells (ECs) and its role in blood vessel formation have not been examined. In this study, we examined the upstream signaling pathways that control GSK3β phosphorylation and inactivation in ECs. To determine the functional significance of this signaling pathway, GSK3 activity was modulated using adenoviral vectors expressing mutant GSK3 proteins, and the effects on survival, migration, NO production, and angiogenesis were assessed.

**EXPERIMENTAL PROCEDURES**

**Materials**—LY294002 was purchased from Cell Signaling Technology (St. Louis, MO). PD98059, bisindolylmaleimide 1, H-89, 8-bromo-3,5-cyclic AMP, forskolin, and 3-isobutyl-1-methoxyxanthine were purchased from Calbiochem. Recombinant human VEGF-165, basic FGF-157, and PDGF-BB were purchased from R & D Systems (Minneapolis, MN).

**Cell Culture and Adenoviral Vectors**—HUVECs were cultured in endothelial growth medium (Clonetics). 4–6 passage cells were used in this study. To examine the regulation of GSK3β phosphorylation, HU-
VECs were serum-starved for 15 h, treated with the indicated agents for 1 h, and stimulated with 10% fetal bovine serum. To examine the role of Akt in the regulation of GSK3β phosphorylation, HUVECs were transfected with a replication-defective adenovirus expressing myristoylated Akt1, dominant-negative Akt1, or β-galactosidase (9, 10). To examine serum deprivation-induced apoptosis, HUVECs were transduced with the indicated adenoviral construct and cultured in serum-free media for 1–4 days. Some assays employed a replication-defective adenoviral vector expressing catalytically inactive GSK3β (GSK3β-KM) where lysine residues at positions 85 and 86 were mutated to methionine and alanine, respectively. Another vector expressed the nonphosphorylatable constitutively active mutant of GSK3β (GSK3β-S9A) where the serine residue at position 9 was mutated to alanine (11, 12). To examine the role of GSK3β in anchorage-dependent growth, HUVECs were exposed to 70% ethanol for 15 min, stained with Giemsa solution, and counted in three random fields per well. Each experiment was performed in duplicate, and four separate experiments were performed in each group.

Cell Viability Assay—Cell viability was assessed by WST-1 assay, flow cytometry analysis of hypodiploid DNA, observations of pyknotic nuclei, and annexin V/propidium iodide double staining by modification of procedures described previously (9, 11). HUVECs in 96-well plates were infected with adenovirus and analyzed using tetrazolium salt WST-1 as instructed by the manufacturer (Roche Molecular Biochemicals). DNA fragmentation was assessed by flow cytometry. For these assays, HUVECs were infected with adenoviruses at serum-starved for 2–4 days. At several time points after serum starvation, the attached and floating HUVECs were harvested and fixed in cold 90% ethanol for 20 min and then resuspended in staining buffer consisting of 1 mg/ml RNase A, 20 μg/ml propidium iodide, and 0.01% Nonidet P-40. DNA content was analyzed by flow cytometry on the FL-2 channel, and gating was set to exclude debris and cellular aggregates. For each analysis, 10,000 events were counted. Alternatively, pyknotic nuclei were assessed by Hoechst staining. For these assays, HUVECs were fixed with 4% paraformaldehyde for 30 min at room temperature, carefully washed with PBS twice, and stained with 10 mg/ml solution of Hoechst 33342 (Sigma) at room temperature with light shielded. Cell nuclei were visualized using Vpseudocolored with Hoechst for double staining. These experiments, serum-deprived HUVECs were cultured in 4-well slide chambers (Nunc). The medium was removed, and cells were covered with staining solution that contained annexin V fluorescein, propidium iodide, and binding buffer (Roche Molecular Biochemicals) for 15 min at room temperature. To examine the anchorage-deprivation-induced apoptosis, HUVECs in suspension culture were centrifuged at 200 × g for 5 min. The cell pellet was resuspended in staining solution, incubated for 15 min at room temperature, and spread on glass slides.

In Vivo Angiogenesis Assay—The formation of new vessels in vivo was evaluated by Matrigel plug assay (Becton Dickinson) by a modification of the procedures described previously (12). For these experiments, local amounts of heparin (10 units/ml) and basic FGF (1 μg/ml) (R&D) were mixed, and 5 ml of this solution was mixed on ice with 10 ml of Matrigel such that the final concentration of basic FGF was 250 ng/ml. Solutions of adenoviral vectors encoding β-galactosidase, GSK3β-KM, or GSK3β-S9A were mixed in with Matrigel solution on ice (2 × 10⁶ plaque forming units of virus/500 μl). 50 μl of Matrigel containing equal growth factor and adenovirus was injected subcutaneously near the right mid-abdomen of C57BL mice (Jackson Laboratories). Mice were sacrificed 10 days after the injection. The Matrigel plugs with the adjacent subcutaneous tissues were carefully recovered by en bloc resection, embedded in OCT compound, and quick-frozen in liquid nitrogen. Immunohistochemistry for hemagglutinin (HA) or CD31 (platelet endothelial cell adhesion molecule-1) and histochemistry for alkaline phosphatase were performed on adjacent frozen sections. The primary antibodies were anti-HA rabbit polyclonal antibody (1:20 dilution, Santa Cruz Biotechnology) and anti-platelet endothelial cell adhesion molecule-1 goat polyclonal antibody (1:20 dilution, Santa Cruz Biotechnology). The secondary antibodies were biotinylated horse anti-goat IgG antibody (1:1000 dilution, Vector Laboratories) and biotinylated goat anti-rabbit IgG antibody (1:100 dilution, Vector Laboratories). Other components for immunohistochemistry were from the LSAB-2 kit (Dako).

Assay of Endothelial Cell Nitric Oxide Production—NO mediates many of its biological effects by activating soluble guanylyl cyclase, resulting in increased intracellular cGMP concentration. Therefore, eNOS-derived NO production was monitored in intact cells by measuring the accumulation of cGMP sensitive to the eNOS inhibitor LNAME by modification of the procedures previously described (13). HUVEC cultured in 6-well plates were incubated for 60 min in HEPES-buffered physiologic salt solution (22 mM HEPES (pH 7.4), 124 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.5 mM CaCl₂, 0.16 mM HPO₄²⁻, 5 mM NaHCO₃, 5.6 mM D-glucose) supplemented with 200 μM 3-isobutyl-1-methylxanthine and 200 μM t-Arg in the absence or presence of L-NAME (500 μM). HUVECs were lysed by the addition of 6% ice-cold trichloroacetic acid. The level of cGMP present in the cell lysate was determined using an enzyme immunoassay (Cayman Chemical) according to the manufacturer's instructions. The level of cGMP was determined in triplicate, and the experiment was performed in duplicate, and four separate experiments were performed in each group.

Statistical Analysis—Data are shown as the mean ± S.E. All data were evaluated with a two-tailed paired Student’s t test or compared by one-way ANOVA, Student-Newman-Keuls post-hoc test.

RESULTS

Differential Regulation of GSK3β and Akt by MAPK and PKA Signaling—We first assayed for the presence of GSK3β in ECs and examined the signal transduction pathways that are involved in its regulatory phosphorylation at serine 9. Stimulation with fetal bovine serum (PBS) was used as a general EC activator because it contains many growth factors and cytokines and promotes signaling through multiple pathways. The stimulation of serum-deprived HUVECs with 10% PBS for 1 h led to a marked increase in GSK3β phosphorylation (Fig. 1A). The phosphorylation of serine 9 results in autoinhibition of
GSK3β because the phosphorylated N terminus binds to the active site as a competitive pseudosubstrate (1). The phosphorylation of GSK3β was paralleled by an increase in the phosphorylation of Akt, a candidate upstream kinase that is regulated by phosphoinositide 3-kinase (PI3K) (4). The serum-induced phosphorylation of GSK3β was inhibited by pretreatment with the PI3K inhibitor LY294002 or the MAPK inhibitor PD98059. In contrast, Akt phosphorylation was blocked by LY294002, but the administration of PD98059 significantly elevated the level of Akt phosphorylation. These treatments did not detectably alter the total level of Akt (data not shown) or GSK3β (Fig. 1) protein expression.

The transduction of ECs with a replication-defective adenovirus construct expressing a dominant-negative form of Akt1 inhibited the serum-stimulated phosphorylation of GSK3β (Fig. 1B). Conversely, transduction with a vector expressing a constitutively active form of Akt1 promoted GSK3β phosphorylation in the absence of serum (Fig. 1C). These data are consistent with pharmacological data showing that LY294002 blocks serum-induced GSK3β phosphorylation (Fig. 1A), and collectively, they suggest that the PI3K/Akt signaling axis is a mediator of GSK3β phosphorylation in response to mitogen activation.

PKA-mediated regulation of GSK3β in HUVECs was indicated by the ability of the PKA activators 8-bromo-cyclic AMP or forskolin to induce the phosphorylation of GSK3β in serum-deprived cells (Fig. 1D). This induction was blocked by pretreatment with the PKA inhibitor H89. In contrast to GSK3β, phosphorylation of the PKA substrate GSK3β was significantly increased by pretreatment with 8-Br-cAMP or forskolin.
regulation, the exposure of cells to H89 promoted the phosphorylation of Akt, consistent with previous data (14). Collectively, these data show that although PI3K signaling participates in GSK3β/H9252 regulation, Akt and GSK3β phosphorylations are differentially regulated by MAPK and PKA signaling pathways.

To modulate the intracellular GSK3β activity in ECs, replication-defective adenoviral vectors that express either a non-phosphorylatable constitutively active mutant (GSK3β-S9A) or a catalytically inactive mutant (GSK3β-KM), which functions as a dominant-negative form (15, 16), were employed. As shown in Fig. 1E, HUVECs transduced with GSK3β-S9A showed a decline in phosphorylated GSK3β, indicative of an activation of GSK3β signaling. An activation of GSK3β signaling was also indicated by a marked increase in the phosphorylation of the downstream substrate β-catenin. In contrast, transduction with GSK3β-KM led to an increase in GSK3β protein that was phosphorylated at serine 9.

Role of GSK3β in Vascular Cell Migration—When HUVECs were transduced with GSK3β-S9A (50 m.o.i.), their chemotactic activities toward VEGF or bFGF were significantly decreased (Fig. 2A). Under the conditions of these assays, VEGF was a more potent chemoattractant than bFGF. The transduction of GSK3β-KM slightly enhanced the directional migration of HUVECs toward VEGF or bFGF, but this was not statistically

![Graph A](image1.png)

**Fig. 2.** GSK3β signaling controls vascular cell chemotaxis toward angiogenic growth factors. A, HUVECs were infected with adenovirus at 50 m.o.i. for 1 day in the presence of serum and then serum-starved for 5 h before the chemotaxis assay was performed. Quantitative data of Giemsa-stained ECs that migrated through filters toward VEGF (50 ng/ml) or basic FGF (25 ng/ml) are shown. Cells were counted in three randomly chosen low power fields (LPF) per well. Each experiment was performed in duplicate, and four separate experiments were performed (*, p < 0.01 between β-galactosidase (β-gal) and GSK-S9A, n = 8). B, GSK3β signaling controls HAoSMC chemotaxis toward growth factors. Quantitative data of Giemsa-stained smooth muscle cells that migrated through filters toward PDGF-BB (50 ng/ml) or basic FGF (50 ng/ml) are shown. HAoSMCs were infected with adenovirus at 100 m.o.i. for 1 day before the chemotaxis assay. Migrating cells were counted in three randomly chosen low power fields per well. Each experiment was performed as duplicate, and four separate experiments were performed. (*, p < 0.01 between β-galactosidase and GSK-S9A or GSK-KM, n = 4).
significant. The antimigratory effect of GSK3β-S9A was not due to a cytotoxic effect of this vector, because adenov-GSK3β-S9A did not decrease viability as assessed by a WST-1 assay under these cell culture conditions (relative values: β-galactosidase, 100 ± 6%; GSK3-KM, 104 ± 8%; GSK-S9A, 98 ± 6%). Furthermore, quantitative analysis of pyknotic nuclei did not reveal any increase or decrease in cell death by adenovirus-mediated GSK3β transduction under these conditions (data not shown). However, at later time points of serum deprivation (>2 days), the modulation of GSK3β signaling influenced cellular survival (see below).

Because the role of GSK3β on the migration of vascular cells had not been described previously, the effects of GSK3β-S9A and GSK3β-KM on the migration of HΑoSMC toward bFGF or PDGF were also assayed. The transduction of GSK3β-S9A significantly inhibited the chemotactic activities of HΑoSMC toward PDGF or bFGF in the Boyden chamber analysis (Fig. 2B). Conversely, the transduction of GSK3β-KM significantly enhanced the chemotaxis of HΑoSMC toward either growth factor. Under these experimental conditions, GSK3β-S9A or GSK3β-KM did not show a significant cytotoxic or cytopoietic effect, respectively, as assessed by WST-1 assay (relative values: β-galactosidase, 100 ± 7%; GSK3-KM, 101 ± 7%; GSK-S9A, 96 ± 5%) or by visual examination of cells for apoptotic morphology (data not shown).

GSK3β Signaling Controls Serum Depetration-induced Apoptosis of ECs—As shown above, the transduction of GSK3β-S9A or GSK3β-KM had no effect on cell viability under normal cell culture conditions with mitogens present in the media or after short periods of serum deprivation. Therefore, to examine the role of GSK3β signaling in EC viability, HUVECs were infected with the adenoviral vectors expressing GSK3β mutants and incubated in serum-free media for 2–4 days to promote apoptosis (9). Under these culture conditions, the transduction of GSK3β-KM significantly reduced the subdiploid fraction of DNA detected by fluorescence-activated cell sorter analysis, whereas the constitutively active GSK3β-S9A increased DNA degradation (Fig. 3A). These data were corroborated by assessing the impact of GSK3β signaling modulation on the frequency of pyknotic nuclei in Hoechst 33342-stained HUVEC cultures (Fig. 3B). Consistent with these data, the transduction of GSK3β-KM significantly promoted viability as assessed by the WST-1 assay of mitochondrial function, whereas GSK3β-S9A reduced mitochondrial function at these late time points (Fig. 3C).

GSK3β Regulates EC Anoikis—Adhesion to extracellular matrix is an important determinant of EC survival under conditions of neovascularization (17). Thus, the role of GSK3β signaling in HUVEC anoikis was assessed. As shown in Fig. 4A, there was a time-dependent decrease in phosphorylated GSK3β following the placement of HUVEC in suspension culture. The decrease in GSK3β phosphorylation was paralleled by decreases in the phosphorylation and protein level of Akt, a regulator of EC anoikis (9). To assess the role of GSK3β signaling in EC anoikis, HUVECs were transduced with GSK3β-KM or GSK3β-S9A prior to placement in suspension cultures. Annexin V staining revealed that the expression of GSK3β-KM protected HUVECs from anoikis compared with control HUVECs that were transduced with β-galactosidase (Fig. 4B). Conversely, GSK-S9A promoted cell death under these conditions. These findings were confirmed by a reattachment assay where HUVECs were reseeded on the adhesive plate after 1 day of the suspension culture (Fig. 4, C and D). Cells expressing GSK-KM displayed a higher frequency of successful reattachment than control cells, whereas HUVEC transduced with GSK-S9A displayed a lower frequency of reattachment following incubation in suspension culture.

GSK3β Regulates Angiogenesis—A Matrigel plug assay in mice was employed to test the role of GSK3β signaling in angiogenesis in vivo. Adenoviral vectors (2 × 10⁸ plaque-forming units) were incorporated in the Matrigel plugs along with bFGF (250 ng/ml) prior to subcutaneous implantation in the abdomen of C57BL6 mice for 10 days prior to recovery. In this assay, the Matrigel serves as a reservoir for the viral vector, and ECs that infiltrate the plug become transduced and express the transgene. The expression of the HA-tagged GSK3β transgene products was shown by immunohistochemistry (Fig. 5A). HA-positive immunostaining was detectable in plugs formulated with adenoviral vectors encoding GSK-KM and GSK-S9A, but little or no signal was detected in plugs formulated with the β-galactosidase-expressing adenovirus. EC infiltration of these plugs was assessed by immunohistochemical analysis of CD31-positive cells. Plugs formulated with adenoviral vectors encoding GSK-KM and GSK-S9A displayed a lower density of CD31-positive cells than controls. These data were corroborated by analyzing the densities of alkaline phosphatase-positive capillaries within these plugs (Fig. 5, A and B).

GSK3β-mediated Activation of the Angiogenic Phenotype Is Not Associated with Elevated NO Production—Previous studies suggest that Akt activation can promote an angiogenic phenotype in ECs in part via its ability to promote NO production (11, 18). Therefore, the effects of GSK3β-mediated signaling on NO output in HUVEC were investigated. For this we measured the increase in intracellular levels of cGMP that is inhibitable by the NOS inhibitor L-NAME, an assay for endothelial cell-derived NO output (19). Table I shows that the expression of constitutively active Akt (Myr-Akt) increased NO output by endothelial cells, consistent with previous studies (20, 21). In contrast, the expression of either GSK3β-KM or GSK3β-S9A did not result in an increase in NO output. These treatments also did not detectably alter the level of eNOS protein expression (data not shown). Taken together, these results are consistent with the notion that changes in NO production are not required for GSK3β-mediated effects on migration, survival, or angiogenesis.

DISCUSSION

This study examined the regulation and function of GSK3β in EC biology and blood vessel growth. Protein kinase inhibitors were used to assess the signaling pathways that regulate GSK3β in ECs. The inhibitory effect of LY294002 on GSK3β phosphorylation suggests that PI3K-dependent pathways are a regulator of its activity in response to mitogen stimulation, and these data are consistent with the finding that GSK3β is directly phosphorylated by the PI3K-regulated protein kinase Akt (4). This hypothesis is supported further by the finding that serum-stimulated GSK3β phosphorylation is inhibited by transduction of dominant-negative Akt, whereas the transduction of constitutively active Akt is sufficient to promote GSK3β phosphorylation in the absence of mitogen stimulation. Our studies also revealed that GSK3β is regulated independent of PI3K/Akt signaling. For example, MAPK inhibition down-regulates GSK3β phosphorylation, whereas it has the opposite effect on Akt phosphorylation. Furthermore, PKA agonists were found to increase GSK3β phosphorylation, whereas PKA inhibition promoted the phosphorylation of Akt. Collectively, these data suggest that GSK3β is downstream from many signaling pathways in ECs (Fig. 6), suggesting that its activity may be regulated by a variety of angiogenic growth factors and cytokines.
**FIG. 3.** Effects of GSK3β signaling on cellular viability under conditions of prolonged serum deprivation in HUVEC. A, fluorescence-activated cell sorter analysis of DNA content in propidium iodide-stained HUVEC after transduction with the indicated adenoviral vectors (100 m.o.i.) and incubation in serum-free media for 4 days. The average proportion of subdiploid cells was calculated for each experimental group from five independent experiments. (*, $p < 0.01$ between β-galactosidase (β-gal) and GSK-KM; #, $p < 0.05$ between β-galactosidase and GSK-S9A). B, microscopic analysis of Hoechst 33342-stained HUVECs at 4 days after transduction with the indicated adenoviral vector (50 m.o.i.) and incubation in serum-free media for 4 days. Three separate experiments were performed in quadruplicate. In each slide, the average number of pyknotic nuclei per total nuclei was calculated from three randomly chosen microscopic fields. The data are presented relative to the value of the β-galactosidase-transduced cells. (*, $p < 0.01$ between β-galactosidase and GSK-KM, #, $p < 0.05$ between β-galactosidase and GSK-S9A). C, WST-1 assay of mitochondrial function in HUVEC cultures transduced with the indicated adenoviral vectors (100 m.o.i.) and incubated in the absence of serum for 4 days (*, $p < 0.01$ between β-galactosidase and GSK-S9A or GSK-KM).
EC survival is an important factor influencing angiogenesis and vessel integrity, and angiogenic growth factor withdrawal will lead to vessel regression in tumors (22). This study shows that GSK3β plays a key role in controlling EC survival in response to growth factor limitation. The activation of GSK3β signaling increased EC apoptosis in response to growth factor deprivation as shown by increased DNA fragmentation and decreased mitochondrial function. Conversely, the ablation of GSK3β signaling protected cells from apoptosis under conditions of mitogen deprivation. Although the modulation of GSK3β has been shown to influence apoptosis in response to mitogen deprivation in other cell types (8, 20), the data reported here are the first to show that GSK3β signaling can regulate cell death that results from the disruption of cell-matrix interactions (i.e., anoikis). When ECs were deprived of anchorage attachments, there were time-dependent decreases in the levels of phosphorylated GSK3β. Apoptosis under these conditions was markedly reduced by the expression of catalytically inactive GSK3β and increased by constitutively active GSK3β, demonstrating that GSK3β signaling is a critical modulator of EC anoikis. With regard to angiogenesis, the control of anoikis is critical because proper associations between cells and matrix are essential for neovascularization because they promote EC survival as they migrate toward the angiogenic source (17). Previous studies have shown that the pro-survival signals from cell surface-extraplacental matrix interactions can be mediated by Akt (9) and by integrin-linked kinase (24), which phosphorylates both Akt and GSK3β (25).

This study also revealed that GSK3β signaling controls EC migration toward VEGF or bFGF and controls smooth muscle cell migration toward PDGF or bFGF. These effects on migration were not the result of cytotoxic or cytoprotective actions of the different GSK3β vectors because these assays were performed for short periods (5 h) in the presence of a chemotactant, whereas significant apoptosis did not occur unless cells were deprived of serum for 2 or more days. Because migration of ECs is an essential component of the angiogenic response, a mouse angiogenesis assay was employed to assess the consequences of adenovirus-mediated GSK3β gene transfer on capillary formation in Matrigel plugs containing bFGF. Unlike other angiogenesis models, the Matrigel plug assay can be adapted to study the consequences of adenovirus-gene transfer on vessel growth because the Matrigel serves as a reservoir for the viral vector, leading to high efficiency transduction of ECs as they infiltrate the plug. In these experiments, the kinase mutant GSK3β increased capillary density in the plug, whereas constitutively active GSK3β markedly reduced capillary formation. These data show that GSK3β signaling functions in ECs negatively regulate angiogenesis, and they are consistent with the inhibitory effects of GSK3β signaling on EC migration and survival in vitro.

Previous studies have shown that the PI3K/Akt signaling pathway is a key regulator of the angiogenic phenotype in ECs (26). The results of this study extend our knowledge of the signaling pathways that regulate angiogenesis by documenting that phosphorylation of GSK3β, one of many substrates for Akt, is essential for EC survival and migration in vitro and angiogenesis in vivo. Of note, GSK3β-mediated regulation of the angiogenic phenotype was not associated with changes in NO production, suggesting that GSK3β signaling can regulate vessel growth independent of the Akt-eNOS regulatory axis. It is also shown that MAPK- and PKA-dependent signaling pathways promote changes in GSK3β phosphorylation that favor angiogenesis. These observations are significant because some angiogenic factors such as bFGF are efficient activators of MAPK but do not activate PI3K/Akt signaling (data not

**Fig. 4. Effect of GSK3β signaling on EC anoikis.** A, time-dependent decreases in Akt and GSK3β phosphorylation upon detachment of HUVEC from culture plates. Immunoblot analysis of HUVEC that were harvested at different times following transfer to poly-HEMA-coated Petri dishes, which do not permit cellular attachment. B, representative phase-contrast and fluorescent microscopic view of HUVECs, which were deprived with the indicated adenoviral vectors overnight prior to transfer to poly-HEMA-coated plates for 1 day. Cells were double-stained with annexin V and propidium iodide. C, representative phase-contrast microscopic views of HUVECs, which were deprived of anchorage attachment for 1 day and then transferred to a standard cell culture plate where they were permitted to reattach for 1 or 2 days. D, quantitative data corresponding to assays in B and C for 1 day (*, p < 0.01 between β-galactosidase and GSK-KM or between β-galactosidase and GSK-S9A, n = 12).
shown). GSK3β phosphorylation is also controlled by Wnt through a mechanism that differs from that employed by mitogenic factors (2), and recent studies have shown that Wnt signaling can regulate EC growth (27). Taken together, these data indicate that GSK3β functions at a convergence of multiple signaling pathways to coordinate EC responses to different angiogenic inputs (Fig. 6) and that it can modulate the angiogenic phenotype of ECs in an eNOS-independent manner.

**FIG. 5.** GSK3β signaling regulates angiogenesis in a Matrigel plug assay in mice. Matrigel containing bFGF and the indicated adenoviral vector were injected subcutaneously in mice. After sacrifice, plugs were excised and immunostained for the GSK3β transgene protein expression with anti-HA antigen antibodies (A, upper panels). Adjacent sections were stained for infiltrating ECs using anti-CD31 antibody (A, middle panels). Sections were also stained for alkaline phosphatase activity to assess capillary infiltration (A, bottom panel). B, quantitative assessment of alkaline phosphatase-positive cells or CD31-positive cells within the Matrigel plugs. Three randomly chosen microscopic fields were counted from each section analyzed. 12 sections from three animals were analyzed for each experimental group. Data were presented as the percentage of positive cells relative to the β-galactosidase (β-gal)-transduced group. *, p < 0.01 between β-galactosidase and GSK-KM or between β-galactosidase and GSK-S9A, n = 12. The Matrigel plug and excised abdominal muscle (Abd Mus) are indicated in some of the photomicrographs.

**TABLE I**

| Treatment          | cGMP  | pmol/mg cell protein |
|--------------------|-------|----------------------|
| GFP                |       | 1.7 ± 0.2            |
| MyrAkt            |       | 11.3 ± 6.5*          |
| GSK3β-S9A          |       | 1.6 ± 0.2            |
| GSK3β-KM           |       | 1.7 ± 0.2            |
| l-NAME             |       | 1.4 ± 0.2            |

**FIG. 6.** Hypothetical scheme illustrating the regulation of GSK3β by MAPK, PI3K/Akt, and Wnt signaling pathways. Not included in this scheme is the potential regulation of GSK3β by PKA-dependent pathways. Inactivation of GSK3β promotes angiogenesis through mechanisms that do not involve changes in NO production by eNOS.

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