AMERICAN JOURNAL OF PHYSIOLOGY
LUNG CELLULAR AND MOLECULAR PHYSIOLOGY

Am J Physiol Lung Cell Mol Physiol. 2012 Sep 1; 303(5): L439–L448. PMCID: PMC3774493
Published online 2012 Jul 6. doi: 10.1152/ajplung.00408.2011: 10.1152/ajplung.00408.2011 PMID: 22773696

Glycogen synthase kinase-3β/β-catenin signaling regulates neonatal lung mesenchymal stromal cell myofibroblastic differentiation

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Received 2011 Dec 22; Accepted 2012 Jun 29.

Abstract

In bronchopulmonary dysplasia (BPD), alveolar septa are thickened with collagen and α-smooth muscle actin-, transforming growth factor (TGF)-β-positive myofibroblasts. We examined the biochemical mechanisms underlying myofibroblastic differentiation, focusing on the role of glycogen synthase kinase-3β (GSK-3β)/β-catenin signaling pathway. In the cytoplasm, β-catenin is phosphorylated on the NH_2 terminus by constitutively active GSK-3β, favoring its degradation. Upon TGF-β stimulation, GSK-3β is phosphorylated and inactivated, allowing β-catenin to translocate to the nucleus, where it activates transcription of genes involved in myofibroblastic differentiation. We examined the role of β-catenin in TGF-β1-induced myofibroblastic differentiation of neonatal lung mesenchymal stromal cells (MSCs) isolated from tracheal aspirates of premature infants with respiratory distress. TGF-β1 increased β-catenin expression and nuclear translocation. Transduction of cells with GSK-3β S9A, a nonphosphorylatable, constitutively active mutant that favors β-catenin degradation, blocked TGF-β1-induced myofibroblastic differentiation. Furthermore, transduction of MSCs with ΔN-catenin, a truncation mutant that cannot be phosphorylated on the NH_2 terminus by GSK-3β and is not degraded, was sufficient for myofibroblastic differentiation. In vivo, hyperoxic exposure of neonatal mice increases expression of β-catenin in α-smooth muscle actin-positive myofibroblasts. Similar changes were found in lungs of infants with BPD. Finally, low-passage unstimulated MSCs from infants developing BPD showed higher phospho-GSK-3β, β-catenin, and α-actin content compared with MSCs from infants not developing this disease, and phospho-GSK-3β and β-catenin each correlated with α-actin content. We conclude that phospho-GSK-3β/β-

catenin signaling regulates α-smooth muscle actin expression, a marker of myofibroblast differentiation, in vitro and in vivo. This pathway appears to be activated in lung mesenchymal cells from patients with BPD.

Keywords: bronchopulmonary dysplasia, myofibroblast, neonate

WITH IMPROVEMENTS IN NEONATAL CARE, the survival rates of very premature infants have increased. However, improvements in survival rates have been accompanied by a corresponding increase in the incidence of bronchopulmonary dysplasia (BPD), a fibrotic lung disease that requires supplemental oxygen for months or years (14). In BPD, alveolar septa are thickened with collagen and α-smooth muscle actin-, transforming growth factor (TGF)-β-positive myofibroblasts (6, 15, 16, 25). Adenoviral transfer of the TGF-β gene to newborn rat lungs induces changes consistent with BPD, including excess matrix deposition and large undeveloped prealveolar saccules (10). Overexpression of TGF-β in neonatal mouse lungs induces proliferation of α-actin-positive cells within the alveolar septal walls and hypoalveolarization (26). The accumulation of α-actin-positive cells and excess matrix deposition in the alveolar walls impairs gas exchange. Together, these data imply a critical role for the interstitial myofibroblasts in the development of BPD.

β-Catenin is a transcription factor that regulates the expression of many genes expressed by myofibroblasts (20). In the nucleus, β-catenin converts the T-cell factor/lymphoid enhancer factor repressor complex into a transcriptional activator, leading to the transcription of a number of target genes, including α-actin, collagen type I, Cyr61/CCN1, endothelin-1, fibronectin, laminin-γ2, matrix metalloproteinases, VEGF, and versican. Glycogen synthase kinase (GSK)-3β is a constitutively active kinase, which normally phosphorylates β-catenin in the cytoplasm (7). Phosphorylation of β-catenin targets it for degradation, thereby preventing gene expression. Phosphorylation of GSK-3β inactivates it, allowing β-catenin to accumulate in the cytoplasm, translocate to the nucleus, and activate gene expression.

We hypothesized that, in BPD, phosphorylation and inactivation of GSK-3β and accumulation of β-catenin in lung mesenchymal cells induce myofibroblastic differentiation. To test this, we examined the requirement and sufficiency of GSK-3β/β-catenin signaling for myofibroblastic differentiation of neonatal lung mesenchymal stromal cells (MSCs) obtained from the tracheal aspirates of premature infants undergoing mechanical ventilation. We also monitored activation of the GSK-3β/β-catenin signaling pathway in the lungs of infants dying of BPD and hyperoxia-exposed neonatal mice, an animal model of this disease.

MATERIALS AND METHODS

Cell culture. Isolation of neonatal lung MSCs was approved by the University of Michigan Institutional Review Board. Cells were isolated from tracheal aspirates of premature infants undergoing mechanical ventilation as described previously (12). Experiments were performed in the absence of serum. Unstimulated passage 2 or 3 MSCs were plated for 24 h in 10% fetal bovine serum and then serum starved for 2 h before harvesting.

For selected experiments, MSCs were serum starved for 24 h and treated with a combination of either TGF-β1 (10 ng/ml; PeproTech, Rocky Hill, NJ) and the selective phosphatidylinositol (PI) 3-kinase inhibitor LY294002 (10 μM; Cayman Chemical, Ann Arbor, MI).

Retrovirus infection. Cultures were infected with retrovirus encoding a nonphosphorylatable GSK3-β (pMSCV-GSK3-β S9A), β-catenin (ΔN-β-catenin), or empty vector (pMSCV). cDNA encoding a constitutively active GSK-3β S9A mutant was subcloned into the pMSCVpuro retroviral vector (BD Biosciences, San Jose, CA) (8). ΔN89-β-catenin (13) was subcloned into the pSIN-EF1α-ΔN-bcat-IRES-PLAP vector and provided by C. M. Alexander (University of Wisconsin, Madison, WI) (18). The Phoenix-GP retrovirus packaging cell line, a 293 cell derivative that expresses only the *gag-pol* viral components (provided by G. Nolan, Stanford University) was transiently transfected with pHCMV-G, which contains the vesicular stomatitis virus envelope glycoprotein, and either pMSCVpuro-GSK-3β S9A, pMSCVpuro alone, or pSIN-EF1a-ΔN-bcat-IRES-PLAP. Viral supernatant was collected, filtered, and supplemented with polybrene (8 μg/ml). Neonatal lung MSCs were infected with viral supernatant (4 times for 4 h each). Infected cells were selected with puromycin (1 μg/ml). After selection, cells were grown to confluence, split into six-well plates, and cultured in the serum-free DMEM in the presence or absence of 10 ng/ml TGF-β1 or 10 mM LiCl (Mallinckrodt Chemical, St. Louis, MO).

Animal model. Two- to three-day-old wild-type C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were exposed to air or 75% oxygen for 14 days using a polypropylene chamber coupled to an oxygen controller and sensor (BioSpherix, Lacona, NY) (23). Dams were exchanged between air and hyperoxia daily. Animal work was approved by the Institutional Animal Care and Use Committee.

Mouse lung histology, fluorescence microscopy, and immunohistochemistry. Lungs were perfused with 5 mM EDTA and inflated to 30 cm H_2O pressure with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO). Slides were probed with FITC- or Cy3-labeled mouse anti-α-smooth muscle actin (clone 1A4, Sigma-Aldrich), AlexaFluor-conjugated rabbit anti-phospho-GSK-3β/mouse anti-β-catenin (Cell Signaling Technology, Beverly, MA), or rabbit anti-connective tissue growth factor (CTGF) (Abcam, Cambridge, MA). Primary antibodies were coupled to AlexaFluor dyes (Invitrogen, Carlsbad, CA) as described (5).

Immunocytochemistry. Neonatal lung MSCs were grown on collagen- or fibronectin-coated glass slides (BD Biosciences). Cells were fixed in 1% paraformaldehyde. Cells were permeabilized in 0.1% Triton X-100 in PBS and probed with antibodies against α-smooth muscle actin, phospho-GSK-3β, β-catenin, and phospho-SMAD3. Cells were imaged using either a Zeiss Apotome or Zeiss LSM 150 confocal microscope (Thornwood, NY).

Immunoblotting. Cell lysates were adjusted for protein concentration, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane. Membranes were blocked in 5% milk for 1 h in room temperature and probed with antibodies against α-smooth muscle actin (clone 1A4; Calbiochem, San Diego, CA), β-catenin, or phospho-GSK-3β.

Flow cytometry. Lungs were collagenase digested and erythrocyte lysed, and cells were fixed in ethanol and processed directly for flow cytometry. Cells were stained with isotype controls and AlexaFluor 488-conjugated anti-phosphoserine 9-GSK-3β, Cy3-conjugated anti-α-actin, AlexaFluor 633-conjugated anti-β-catenin, and AlexaFluor 750-conjugated anti-CD45. Cells were then analyzed in a flow cytometer (FACSCalibur; Becton-Dickinson, Franklin Lakes, NJ).

Quantitative real-time PCR. Whole-lung RNA was extracted with RNeasy (Qiagen, Valencia, CA) and analyzed by quantitative real-time PCR using specific primers and probes. The $2^{-\Delta CT}$ algorithm was used to analyze relative gene expression. The groups were compared by unpaired *t*-test.

Immunohistochemistry of lung tissue from infants with BPD. Human lung tissue was obtained from the University of Rochester Lung Biorepository under a protocol approved by the Institutional Review Board of Strong Memorial Hospital (Rochester, NY). Specimens were obtained from infants who died in the intensive care nursery. The diagnosis of BPD was based on premature delivery, need for chronic respiratory support, requirement for supplemental oxygen after 36-wk gestation, and consistent chest radiographs, as well as pathological tissue diagnosis at autopsy. Specimens were also obtained from infants succumbing to nonpulmonary disorders. Samples were processed within 6 h of death. Immunofluorescence of these sections was performed as described above. For immunohistochemistry, we used rabbit anti-CTGF (Abcam) and an anti-rabbit IgG horseradish peroxidase kit (Vector Laboratories, Burlingame, CA) with diaminobenzidine as a substrate.

RESULTS

Treatment with TGF-β1 increases α-actin, p-GSK-3β, and β-catenin content in neonatal lung MSCs. We have previously shown that treatment of neonatal lung MSCs with TGF-β1 increases the expression of contractile proteins typically found in myofibroblasts (22). To examine the effects of TGF-β1 on the expression of β-catenin and phosphorylation of GSK-3β, neonatal lung MSCs were treated with or without TGF-β1 10 ng/ml in serum-free medium. Protein phosphorylation or expression was monitored by immunofluorescence (Fig. 1, *A* and *B*) and immunoblotting (Fig. 1, *C* and *D*). MSCs treated with TGF-β1 showed increased α-actin, β-catenin, and phospho-GSK-3β. After treatment, the β-catenin signal was higher in the cell nucleus (Fig. 1*B*). Thus, TGF-β1-induced myofibroblastic differentiation of neonatal lung MSCs is associated with increased expression of α-actin, increased phosphorylation of GSK-3β, and increased expression and nuclear translocation of β-catenin.

Transduction of MSCs with GSK-3β S9A, a nonphosphorylatable constitutively active mutant, blocks TGF-β1-induced myofibroblastic differentiation. To determine whether β-catenin is required for neonatal lung MSC myofibroblastic differentiation, we expressed GSK-3β S9A, a constitutively active mutant that cannot be phosphorylated or inactivated, therefore favoring breakdown of β-catenin, in neonatal lung MSCs via retroviral gene transfer. Cells were treated with TGF-β1 to determine the effect of GSK-3β S9A on myofibroblastic differentiation. Unlike cells transduced with empty vector (Fig. 2, *A* and *B*), cells transduced with GSK-3β S9A failed to show β-catenin nuclear translocation in response to TGF-β1, consistent with the effect of constitutive GSK-3β on β-catenin abundance in the cell (Fig. 2, *D* and *E*). Furthermore, GSK-3β S9A-transduced cells failed to show

increased α-actin expression. Immunoblots of GSK-3β S9A-transduced cells confirmed reductions in β-catenin and α-actin, as well as overexpression of GSK-3β (Fig. 2*G*). These data suggest that GSK-3β phosphorylation and β-catenin are each required for TGF-β1-induced myofibroblastic differentiation of neonatal lung MSCs.

To examine the sufficiency of GSK-3β inactivation for myofibroblastic differentiation, MSCs transduced with empty vector or GSK-3β S9A were treated with an inhibitor of GSK-3β, LiCl. Cells transduced with empty vector and treated with LiCl showed increased nuclear accumulation of β-catenin and expression of α-actin (Fig. 2*C*). In contrast, cells transduced with nonphosphorylatable GSK-3β and treated with LiCl failed to show β-catenin nuclear accumulation or increased α-actin expression (Fig. 2*F*). These experiments show that inhibition of GSK-3β is indeed sufficient for differentiation of MSCs to myofibroblasts.

Transduction of MSCs with ΔN-β-catenin, a nonphosphorylatable β-catenin, is sufficient for myofibroblastic differentiation. To assess whether β-catenin is necessary for neonatal lung MSCs myofibroblastic differentiation, we expressed ΔN-β-catenin, a truncation mutant that cannot be phosphorylated on the NH_2 terminus by GSK-3β and therefore is not degraded, in neonatal lung MSCs via retroviral gene transfer. Compared with cells transduced with empty vector (Fig. 3, *A*–*D*), MSCs transduced with ΔN-β-catenin increased expression and nuclear translocation of β-catenin (Fig. 3, *E*–*H*). Furthermore, cells showed increased expression of α-actin and incorporation of actin into contractile filaments. Immunoblots of cell lysates showed similar results (Fig. 3*I*). These data indicate that accumulation and nuclear translocation of β-catenin is sufficient for neonatal lung MSC myofibroblastic differentiation.

Hyperoxic exposure of neonatal mice increases expression of β-catenin and α-actin in alveolar walls. Two- to three-day-old wild-type C57BL/6J mice were exposed to air or 75% oxygen for 14 days. Lungs were either perfused and harvested for morphology or collagenase digested and processed for flow cytometry. Compared with lungs from air-exposed mice (Fig. 4, *A*–*D*), hyperoxic exposure caused the development of fewer and larger airspaces and thickened alveolar walls (Fig. 4, *E*–*H*). Fluorescence microscopy showed increased expression of β-catenin and deposition of α-actin. β-Catenin and α-actin colocalized with phospho-GSK-3β in the interstitial cells of thickened alveolar walls. Flow cytometry on lung cells from hyperoxia-exposed mice, after gating out CD45-positive cells, showed increased levels of α-actin, phospho-GSK-3β, and β-catenin (Fig. 4*I*). These data demonstrate that hyperoxic exposure of neonatal mice increased phosphorylation of GSK-3β and expression of α-actin and β-catenin in lung cells.

Increased expression of PAI-1 mRNA in lungs of hyperoxia-exposed mice. As noted above, TGF-β treatment induces phosphorylation of GSK-3β and expression of β-catenin in vitro. In addition, we have shown that MSCs produce TGF-β1 in an autocrine manner (22). Accordingly, we examined whole lung lysates from air- and hyperoxia-exposed mice for evidence of TGF-β signaling. We measured plasminogen activator inhibitor-1 (PAI-1) expression, a surrogate marker for TGF-β signaling pathway activity (1). Compared with age-matched controls exposed to ambient air, PAI-1 mRNA expression was significantly higher in the lungs from 2-day-old mouse pups exposed to 75% O_2.

Lungs of infants with BPD show increased expression of β-catenin and α-smooth muscle actin in the thickened alveolar interstitium. Lung sections from full-term infants (Fig. 5, *A*–*E*) were immunostained and compared with lungs from infants dying of BPD (Fig. 5, *F*–*J*). Immunofluorescent staining of the full-term infant lung showed α-actin-positive cells in airway smooth muscle and at the tips of alveolar septa distribution. Epithelial cells (and red blood cells) were stained for phospho-GSK-3β. The α-actin signal did not colocalize with the phospho-GSK-3β or β-catenin signals. Immunofluorescent staining of lungs from infants dying of BPD showed increased expression of α-actin and β-catenin, which colocalized with phospho-GSK-3β in the thickened alveolar interstitium.

Potential role of CTGF. CTGF has recently been shown to be sufficient (30) and required for murine hyperoxia-induced neonatal lung injury (2). We tested whether CTGF could be responsible for GSK-3β/β-catenin-mediated myofibroblastic differentiation in our model, as well in infants with BPD. First, we examined the effect of CTGF on cultured neonatal lung MSCs. Treatment with CTGF increased p-GSK-3β/β-catenin and α-smooth muscle actin content (Fig. 6, *A* and *B*). Hyperoxic exposure of neonatal mice increased CTGF whole lung mRNA (Fig. 6*C*) and protein expression (Fig. 6, *D*–*G*). Hyperoxia appeared to increase CTGF content in the epithelium, mesenchyme, and thickened interstitia. Finally, lungs of infants with BPD show increased CTGF expression compared with controls (Fig. 6, *H*–*K*).

Phospho-GSK-3β and β-catenin correlate with α-actin content in unstimulated neonatal lung MSCs and are increased in MSCs from infants developing BPD. We measured phospho-GSK-3β/β-catenin and α-actin content by immunoblotting in unstimulated low-passage neonatal lung MSCs isolated from the tracheal aspirates of 18 premature infants with respiratory distress, 10 of whom went on to develop BPD and 8 who did not. MSCs from infants developing BPD showed higher phospho-GSK-3β, β-catenin, and α-actin content compared with MSCs from infants not developing this disease, consistent with the notion that this signaling pathway is activated in patients with BPD (Fig. 7*A*). In addition, phospho-GSK-3β and β-catenin content each significantly correlated with α-actin content (Fig. 7*B*). These data suggest that phospho-GSK-3β/β-catenin signaling regulates α-smooth muscle actin expression, a marker of myofibroblast differentiation, in vivo.

TGF-β1-induced GSK-3β phosphorylation is dependent on PI3-kinase. We examined the signaling pathway upstream of GSK-3β, focusing on PI3-kinase/Akt signaling. TGF-β1 treatment induced phosphorylation of Akt and GSK-3β (Fig. 8). Pretreatment with LY294002, a chemical inhibitor of PI3-kinase, sharply decreased basal and TGF-β-stimulated phosphorylation of Akt and GSK-3β. These data show that TGF-β1-induced phosphorylation is dependent on PI3-kinase/Akt signaling.

DISCUSSION

In this study, we show that treatment of neonatal lung MSCs with TGF-β1-induced myofibroblast differentiation, as evidenced by expression of α-smooth muscle actin, is accompanied by increases in phospho-GSK-3β and β-catenin content. Transduction of MSCs with GSK-3β S9A, a nonphosphorylatable, constitutively active mutant of GSK-3β that promotes breakdown of β-catenin, blocked TGF-β1-induced myofibroblastic differentiation. Transduction of neonatal lung MSCs with ΔN-β-catenin, which cannot be phosphorylated on the NH_2 terminus by GSK-3β and therefore is not de-

graded, showed increased expression of α-actin and incorporation of actin into contractile filaments. Together, these data demonstrate for the first time that β-catenin is required and sufficient for myofibroblastic differentiation. To further characterize this process in vivo, we examined the lungs of neonatal mice exposed to hyperoxia, which show arrested alveolar development and thickened alveolar walls reminiscent of BPD. Hyperoxic exposure was associated with increased expression of α-actin and β-catenin, which colocalized with phospho-GSK-3β in interstitial cells of thickened alveolar walls. Similarly, immunofluorescent staining of lungs from infants dying of BPD showed increased expression of α-actin and β-catenin in the thickened alveolar interstitium, which colocalized with phospho-GSK-3β. Most importantly, we found that low-passage unstimulated MSCs from infants developing BPD showed higher phospho-GSK-3β, β-catenin, and α-actin content compared with MSCs from infants not developing this disease, and phospho-GSK-3β and β-catenin each correlated with α-actin content. These findings suggest that GSK-3β/β-catenin signaling pathway is activated in lung mesenchymal cells from patients with BPD and may play an important role in the pathogenesis of neonatal lung injury.

β-Catenin is a multifunctional protein that may act in the cytoplasm to link cadherins to the actin cytoskeleton or enter the nucleus and function as a transcription factor (4, 20). In the resting state, β-catenin is maintained at a low level in the cytosol and nucleus through rapid turnover of free β-catenin. This turnover is activated through the multiprotein β-catenin destruction complex, which is anchored by Axin1/2 and adenomatous polyposis coli. Casein kinase I-α and GSK-3β, a serine/threonine kinase that is constitutively active and inactivated upon phosphorylation at Ser^{9} (7), sequentially phosphorylate β-catenin, which in turn leads to its ubiquitination by the E3 ligase complex. On the other hand, phosphorylation and inactivation of GSK-3β stabilizes β-catenin, allowing nuclear translocation. In our study, GSK-3β phosphorylation and β-catenin were required for myofibroblastic differentiation of neonatal lung MSCs. TGF-β1-induced GSK-3β phosphorylation was dependent on PI3-kinase/Akt signaling. Phosphorylation of GSK-3β and accumulation and stabilization of β-catenin have previously been associated with hypertrophic responses in cardiomyocytes and skeletal muscle cells (3, 11). These data suggest GSK-3β/β-catenin signaling plays a key role in progenitor cell response to profibrotic stimuli in vitro.

To examine the role of GSK-3β/β-catenin signaling in vivo, we sought evidence of pathway activation in the lungs of hyperoxia-exposed neonatal mice and the lungs of infants with BPD. We also correlated pGSK-3β, β-catenin, and α-actin content with clinical outcome. We found that expression of β-catenin was increased in the lungs of hyperoxic neonatal mice and infants with BPD and that β-catenin colocalized with pGSK-3β in α-actin-positive interstitial myofibroblasts. Finally, we found in unstimulated low-passage neonatal lung MSCs that pGSK-3β, β-catenin, and α-actin content were all higher in MSCs isolated from patients developing BPD. Taken together, these data strongly suggest that abnormal differentiation of MSCs to myofibroblasts, mediated via the GSK-3β/β-catenin pathway, is a feature of BPD pathogenesis.

In BPD, alveolar septa are thickened with collagen and α-smooth muscle actin-, transforming growth factor (TGF)-β-positive myofibroblasts (6, 15, 16, 25). Adenoviral transfer of the TGF-β gene to newborn rat lungs induces changes consistent with BPD, including excess matrix deposition and large undeveloped prealveolar saccules (10). Overexpression of TGF-β in neonatal mouse lungs induces proliferation of α-actin-positive cells within the alveolar septal walls and hypoalveo-

larization (26). It is therefore likely that TGF-β plays an important causal role in the development of BPD. However, although we stimulated GSK-3β/β-catenin signaling and myofibroblastic differentiation of MSCs with TGF-β in vitro, we did not determine whether TGF-β stimulates GSK-3β/β-catenin signaling in vivo. We have previously shown that tracheal aspirates from which MSCs are isolated contain higher levels of TGF-β than those without MSCs (21), and we now show that lungs from hyperoxia-exposed mice contain higher amounts of PAI-1 mRNA, consistent with increased TGF-β bioactivity (1). However, because we have also shown that neonatal lung MSCs produce TGF-β1 in an autocrine manner (22), it is therefore possible that TGF-β is the effect, not the stimulus, of GSK-3β/β-catenin signaling.

The GSK-3β pathway may be initiated (and GSK-3β kinase activity suppressed) by a number of physiological stimuli beside TGF-β, including Wnts, sonic hedgehog, bone morphogenic proteins, cardiotrophin, endothelin, serotonin (8, 9), and CTGF. CTGF is a cysteine-rich, nonstructural extracellular matrix protein that regulates cell-matrix interactions, thereby influencing fiber deposition, adhesion, migration, proliferation, and survival. Ample evidence exists for CTGF involvement in lung fibrosis. Serum levels of CTGF correlate with the extent of pulmonary fibrosis in patients with systemic sclerosis (24). High tidal volume ventilation upregulates CTGF expression in the newborn rat lung (29). CTGF mRNA expression increases within 30 min of mechanical ventilation in preterm lambs (27). Overexpression of CTGF in respiratory epithelial cells during the neonatal period induces thickening of the alveolar septa, decreased secondary septal formation, myofibroblast differentiation (30), and pulmonary hypertension (28). Overexpression of CTGF in mouse lungs also increases whole lung Ser^9 GSK-3β phosphorylation and nuclear localization of β-catenin, and CTGF induces β-catenin nuclear translocation in primary alveolar type II epithelial cells (28). We therefore examined the role of CTGF in our model systems. We found that CTGF induces GSK-3β phosphorylation and β-catenin accumulation in neonatal lung MSCs and that hyperoxic exposure increases lung CTGF mRNA and protein expression, not only in epithelial cells, but in lung mesenchyme and thickened alveolar interstitia. CTGF expression was also increased in the lungs of infants with BPD. Together, these data are consistent with the notion that CTGF, perhaps in combination with TGF-β, is responsible for GSK-3β/β-catenin signaling and myofibroblastic differentiation observed in neonatal mice, as well as in human infants with BPD.

Recent studies implicate GSK-3β and β-catenin in the pathogenesis of other fibrotic lung diseases. GSK-3β phosphorylation and β-catenin expression are increased in lung tissue lysates from patients with idiopathic pulmonary fibrosis (IPF) (19). Lung tissue from patients with IPF also shows tyrosine phosphorylated-β-catenin in myofibroblasts (17). In our study, lung tissue from patients with BPD showed colocalization of phospho-GSK-3β and β-catenin in α-actin-positive interstitial myofibroblasts. Therefore, members of the GSK-3β/β-catenin pathway may represent targets in the prevention or treatment of BPD and other fibrotic lung diseases.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A.P.P., J.K.B., G.S.P., and M.B.H. conception and design of research; A.P.P., J.K.B., A.C.A., M.N.R., M.J.L., J.L., E.J.W., and A.M.G. performed experiments; A.P.P., J.K.B., G.S.P., and M.B.H. analyzed data; A.P.P., J.K.B., G.S.P., and M.B.H. interpreted results of experiments; A.P.P., J.K.B., and M.B.H. prepared figures; A.P.P., J.K.B., and M.B.H. drafted manuscript; A.P.P., J.K.B., and M.B.H. edited and revised manuscript; A.P.P. and M.B.H. approved final version of manuscript.

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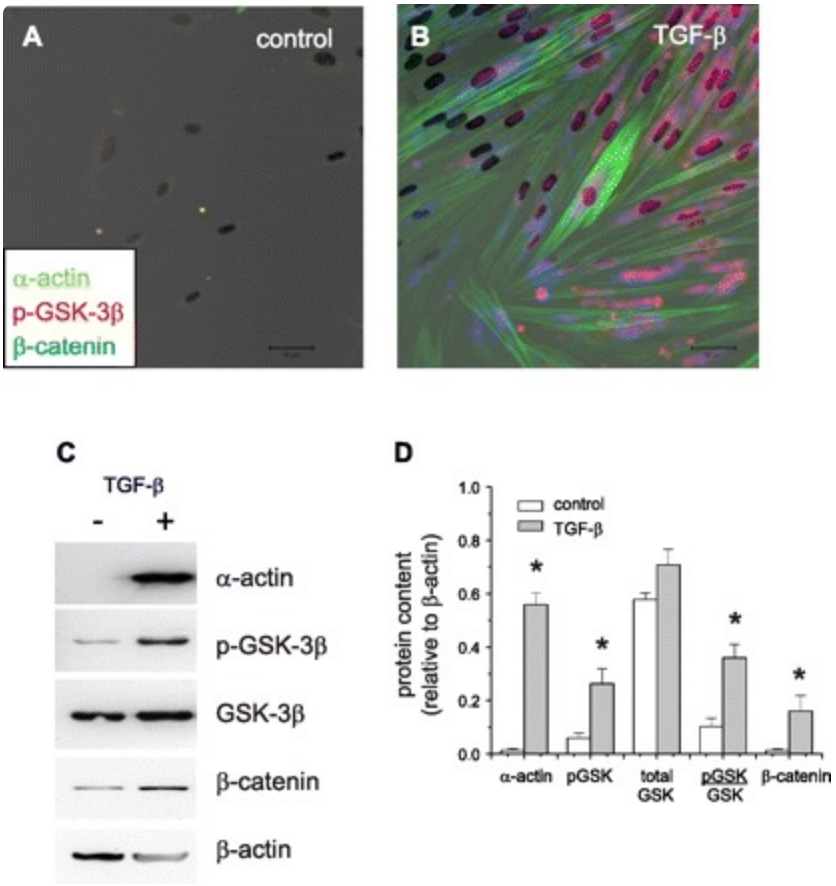
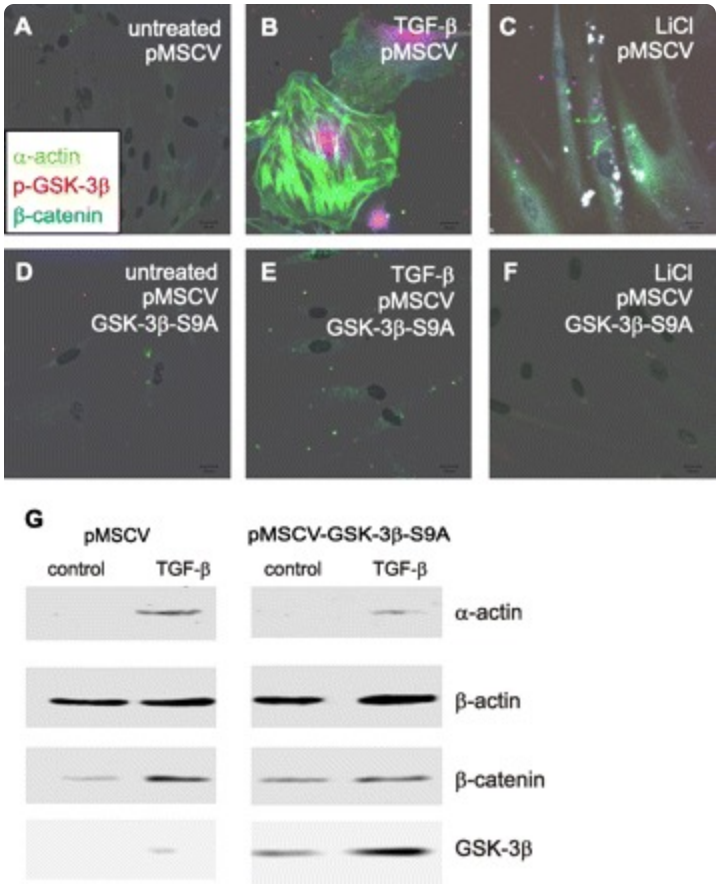
Figures and Tables

Fig. 1.



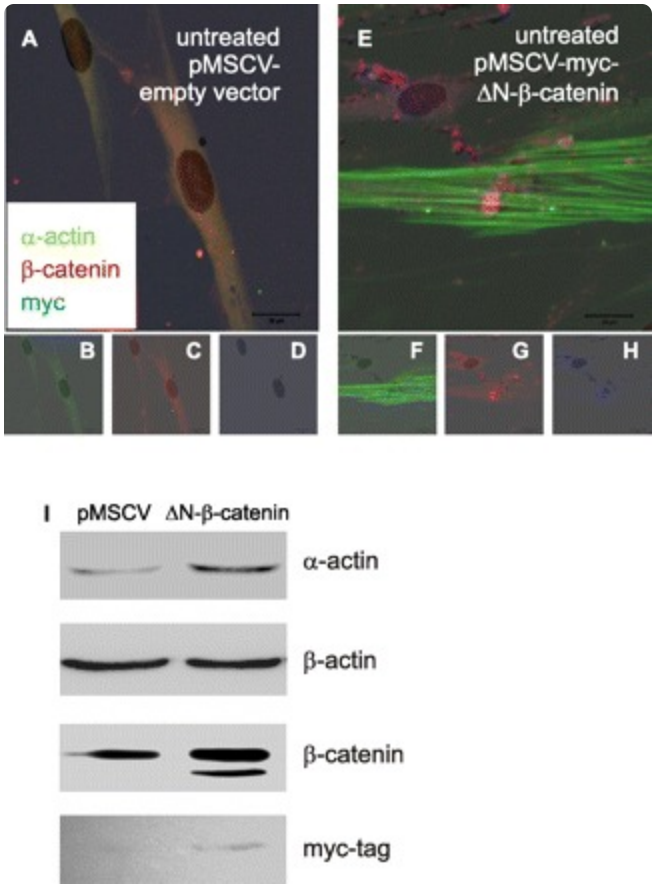
Treatment with TGF-β1 increases α-actin, p-glycogen synthase kinase (GSK)-3β, and β-catenin content in neonatal lung mesenchymal stromal cells (MSCs). Compared with unstimulated MSCs (*A*), MSCs treated with 10 ng/ml TGF-β1 for 48 h (*B*) show increases for α-actin (green), β-catenin (blue), and phospho-GSK-3β (red). β-Catenin and phospho-GSK-3β appear to be colocalized (purple) in the perinuclear space. Experiments are representative of 3 separate experiments. *C*: immunoblotting analysis for α-actin, β-catenin, phospho-GSK-3β, and GSK-3β in cells incubated with and without 10 ng/ml TGF-β1 for 48 h. *D*: group mean densitometry data for 4 experiments (*different from control, $P < 0.05$, 1-way ANOVA).

Fig. 2.



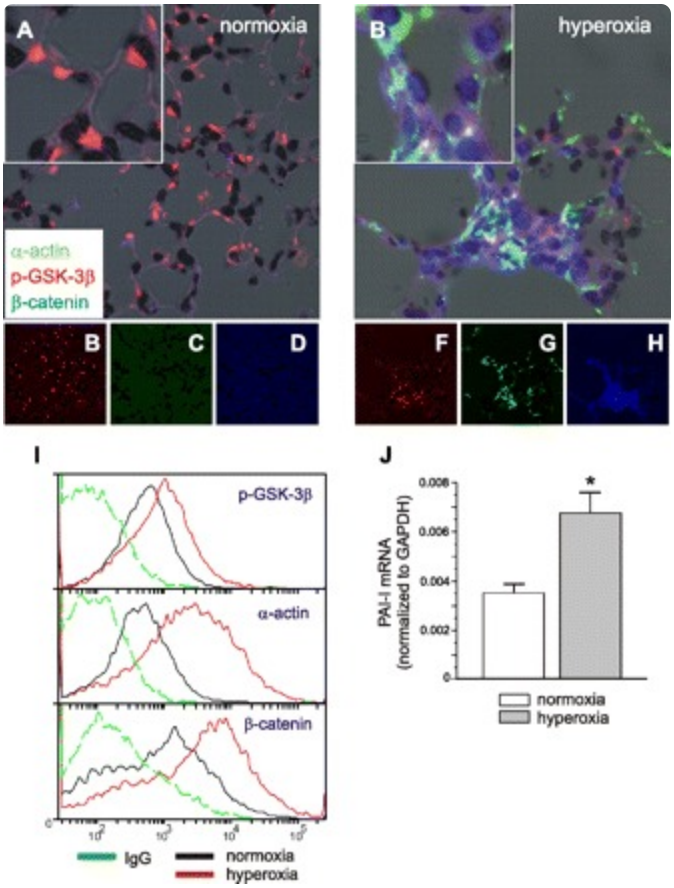
Transduction of MSCs with GSK-3β S9A, a nonphosphorylatable constitutively active mutant, blocks TGF-β1-induced β-catenin nuclear accumulation and myofibroblastic differentiation. Following TGF-β1 treatment (10 ng/ml for 48 h), cells transduced with empty vector (*A* and *B*) show increased phospho-GSK-3β content in the perinuclear space (green) and localization of β-catenin in the nucleus (blue). α-Actin staining shows increased expression in the cell cytoplasm and stress fibers indicative of incorporation of actin into contractile filaments. Cells transduced with nonphosphorylatable GSK-3β (*D* and *E*) fail to show increased α-actin expression or β-catenin nuclear translocation in response to TGF-β1. Following treatment with GSK-3β inhibitor, LiCl (10 mM for 48 h), cells transduced with empty vector (*C*) showed increased nuclear accumulation of β-catenin (blue) and expression of α-actin (green). In contrast, cells transduced with nonphosphorylatable GSK-3β failed to show β-catenin nuclear accumulation or increased α-actin expression following treatment with LiCl (*F*). *G*: immunoblotting of cells transduced with empty vector shows increased α-actin and β-catenin and no change in total GSK-3β content. In contrast, cells transduced with nonphosphorylatable GSK-3β fail to increase α-actin and β-catenin in response to TGF-β1 stimulation. Results are representative of 3 individual experiments.

Fig. 3.



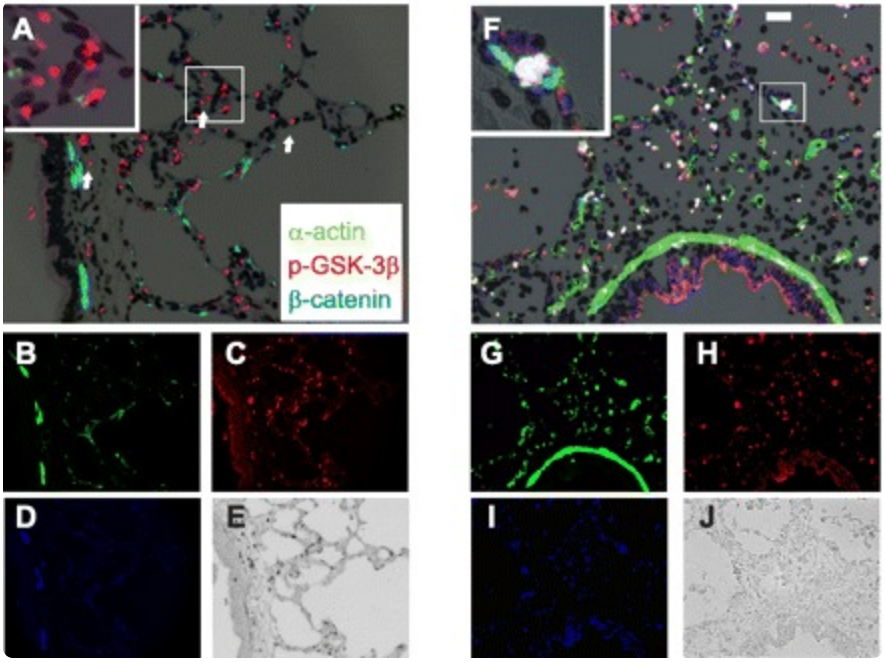
Transduction of MSCs with ΔN-β-catenin, a nonphosphorylatable β-catenin, is sufficient for myofibroblastic differentiation. Compared with MSCs transduced with empty vector, (*A*–*D*), MSCs transduced with ΔN-β-catenin (*E*–*H*) show increases for α-actin (green) and β-catenin (red). α-Actin staining shows stress fibers indicative of incorporation of actin into contractile filaments. Anti-myc tag staining is blue (*H*). *I*: immunoblots for α-actin, β-catenin, myc, and β-actin are shown. MSCs were transduced with empty vector- or ΔN-β-catenin. These results are representative of 3 individual experiments.

Fig. 4.



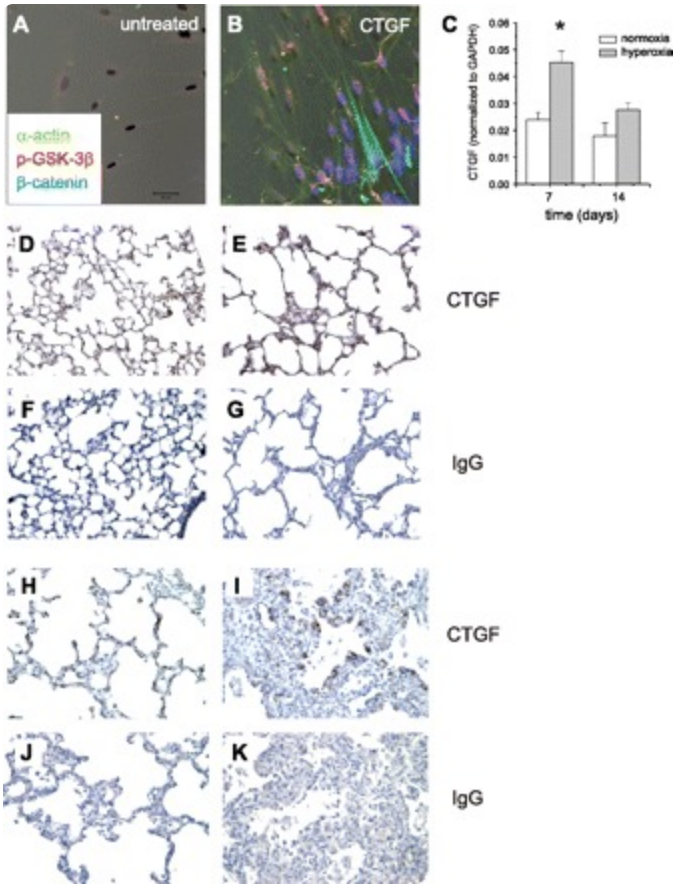
Hyperoxic exposure of neonatal mice increases lung cell β-catenin and α-actin expression. Wild-type C57BL/6J mice (2–3 days old) were exposed to air or 75% oxygen for 14 days. Compared with air-exposed mice (*A*–*D*), hyperoxic exposure (*E*–*H*) caused the development of fewer and larger airspaces and thickened alveolar walls. Fluorescence microscopy showed basal phospho-GSK-3β content (red) in the epithelium of air-exposed mice (*A*–*D*), and increased deposition of α-actin (green) and β-catenin (blue) in the lung interstitial of hyperoxia exposed lungs (*E* and *F*). α-Actin (green) and β-catenin colocalized with phospho-GSK-3β (white). DAPI staining of nuclei is depicted as black superimposed upon a visible image in the gray background. *I*: collagenase-digested erythrocyte-lysed lungs from 2–3-day-old wild-type C57BL/6J mice, exposed to air or 75% oxygen for 14 days, were fixed in ethanol and processed directly for flow cytometry. Hematopoietic cells were gated out with anti-CD45-AF750. Fluorescence for anti-pGSK-3β-AF488 (*top*), anti-α-actin-Cy3 (*middle*), and anti-β-catenin-AF633 (*bottom*) is shown (green, IgG immunoreactivity in the 3 channels; black, lung cells from normoxia-exposed animals; red, lung cells from hyperoxia exposed mice). These results are representative of 3 individual experiments. *J*: lung plasminogen activator inhibitor (PAI)-1 mRNA expression from 2-day-old mouse pups exposed to ambient air or 75% O_2 for 14 days is shown ($n = 9$ for each group, $^*P < 0.01$, unpaired *t*-test).

Fig. 5.



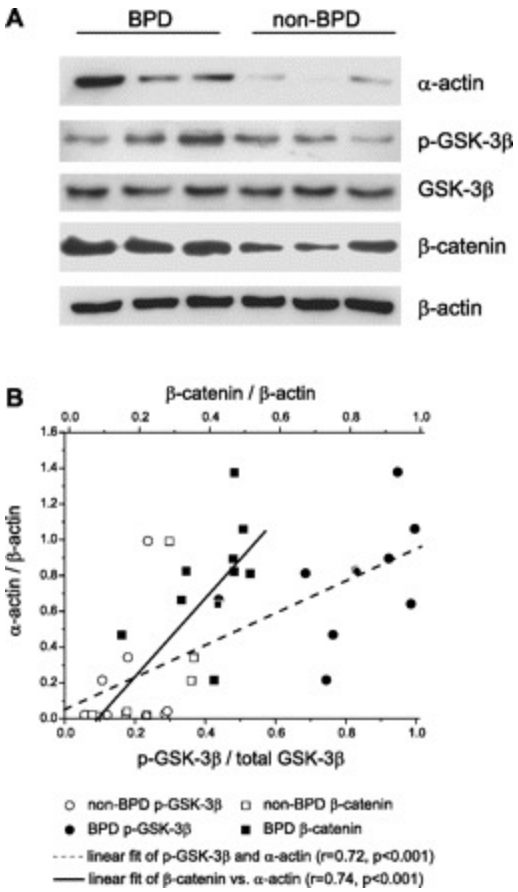
Lungs of infants with bronchopulmonary dysplasia (BPD) show increased expression of β-catenin and α-smooth muscle actin, which colocalizes with phospho-GSK-3β in the thickened alveolar interstitium. The lung of a full-term infant dying of a nonpulmonary cause is shown in (*A*–*E*). Immunofluorescent staining shows α-actin positive (green) cells in the airway smooth muscle and at the tips of alveolar septa. Epithelial cells and red blood cells (arrows) stain positive for phospho-GSK-3β (red). The α-actin signal (green) does not colocalize with phospho-GSK-3β (red), and little or no β-catenin signal (blue) is present. Lung sections from infants dying with BPD (*F*–*J*) show abnormal architecture with widened alveolar spaces and thickened alveolar walls. Immunofluorescence staining shows increased expression of α-actin (green) and β-catenin (blue), which colocalize with phospho-GSK-3β in the thickened alveolar interstitium. Colocalization of α-actin, β-catenin, and phospho-GSK-3β appears white. DAPI staining of nuclei is depicted as black superimposed upon a visible image in the gray background. Phase contrast images depict differences in lung architecture between the normal (*E*) and BPD (*J*) lung.

Fig. 6.



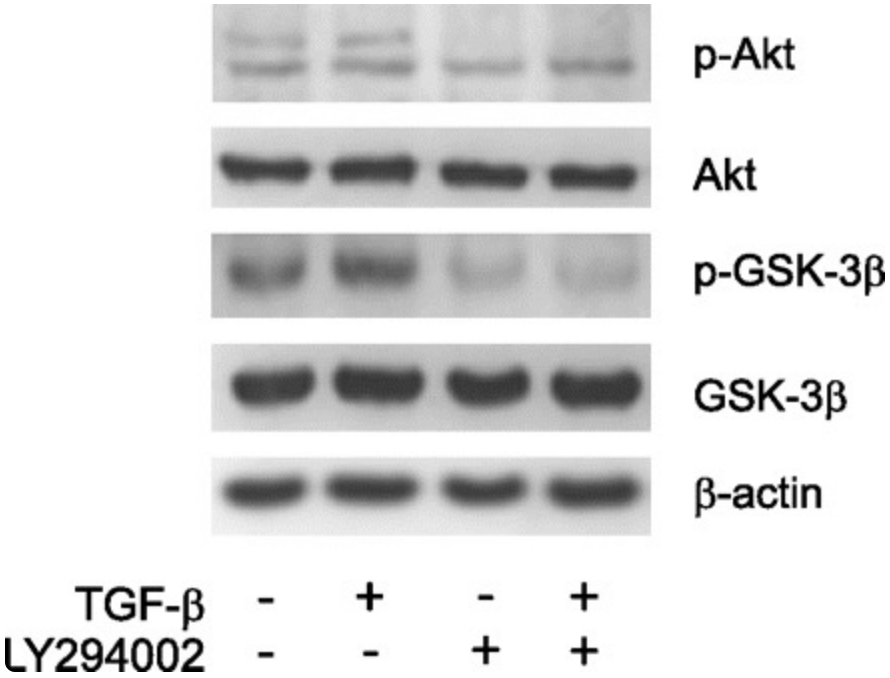
Connective tissue growth factor (CTGF) induces phosphorylation of GSK-3β and accumulation of β-catenin in cultured neonatal lung MSCs and is increased in the lungs of hyperoxia-exposed neonatal mice and human infants with BPD. Compared with unstimulated MSCs (*A*), MSCs treated with 5 μg/ml CTGF for 48 h (*B*) show increases in α-actin (green), p-GSK-3β (red), and β-catenin (blue). *C*: lung CTGF mRNA expression from 2-day-old mouse pups exposed to ambient air or 75% O_2 for 7 or 14 days is shown ($n = 3$, *different from air-exposed, $P < 0.05$). Lung sections from 14-day air-exposed (*D* and *F*) and hyperoxia-exposed (*E* and *G*) neonatal mice were immunostained for CTGF or isotype control and counter-stained with hematoxylin (×200 original magnification). Lung sections from a normal-term infant (*H* and *J*) and terminal case of BPD (*I* and *K*) were immunostained for CTGF (×200 original magnification).

Fig. 7.



Phospho-GSK-3β, β-catenin, and α-actin content are increased in low-passage unstimulated neonatal lung MSCs from patients developing BPD. *A*: immunoblotting analysis shows higher levels of α-actin, phospho-GSK-3β, GSK-3β, and β-catenin in low-passage, unstimulated MSCs from infants developing BPD compared with MSCs from infants not developing this disease. *B*: phospho-GSK-3β and β-catenin content correlate with α-actin content in unstimulated neonatal lung MSCs.

Fig. 8.



TGF-β1 stimulation induces phosphatidylinositol (PI)3-kinase-dependent phosphorylation of Akt and GSK-3β. Immunoblotting analysis for phospho-Akt, Akt, phospho-GSK-3β, and GSK-3β in cells incubated with 10 ng/ml TGF-β1 and LY 294002.