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Role of TGF-β signaling in extracellular matrix production under high glucose conditions

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Role of TGF-β signaling in extracellular matrix production under high glucose conditions.

Background. Hyperglycemia has been shown to play an important role in diabetic renal and vascular complications. Some studies show that high glucose may mediate diabetic complications by stimulating extracellular matrix (ECM) production. We hypothesize that this may be mediated by activating transforming growth factor-β (TGF-β)/Smads signaling.

Methods. Renal and vascular cells were cultured under high glucose conditions in the presence or absence of a neutralizing TGF-β antibody and examined for activation of Smad signaling and collagen production. The regulating role of Smad signaling in high glucose–induced collagen synthesis was determined by inducing overexpression of the inhibitory Smad7 in a stable Smad7-expressing tubular cell line.

Results. Activation of Smad signaling, as evidenced by Smad2 and Smad3 nuclear translocation and phosphorylation, was found in renal and vascular cells at 24 hours after high glucose stimulation (up to 55% increased). This was associated with de novo synthesis of collagen I at day 3 by all cell types. High glucose–induced activation of Smad signaling and collagen synthesis were TGF-β–dependent since these were associated with a significant increase in TGF-β production at 24 hours ($P < 0.01$) and were blocked by a neutralizing TGF-β antibody. Importantly, overexpression of Smad7 resulted in marked inhibition of high glucose–induced Smad2 and Smad3 activation and type I collagen synthesis, suggesting that Smad signaling is a key pathway in high glucose–mediated renal and vascular scarring.

Conclusion. High glucose acts by activating the TGF-β dependent Smad signaling pathway to stimulate collagen synthesis by renal and vascular cells. Smad signaling plays a critical role in regulating high-glucose–mediated diabetic renal and vascular complications.

Diabetic nephropathy and vascular disease remain major complications of diabetes, accounting for up to 40% of cases with end-stage-renal disease (ESRD) and 75% of cases with cardiovascular disease [1, 2]. Many studies suggest a key role for hyperglycemia in the pathogenesis of these complications [3–6]. Indeed, elevated glucose is known to activate a variety of cells to stimulate extracellular matrix (ECM) synthesis, which has been shown to be mediated by stimulating transforming growth factor-β (TGF-β) production [7–14]. Furthermore, the pathogenic role of TGF-β in diabetic renal and vascular complications is supported by the ability of blocking TGF-β using a neutralizing antibody or antisense strategies in experimental models of diabetes [15–17]. However, the intracellular signaling pathway whereby high glucose induces renal and vascular sclerosis in diabetes remains unclear.

TGF-β is known to stimulate ECM in various cell types by activating its intracellular signaling pathway, Smads [18–20]. After binding to the receptors, TGF-β activates both receptor-associated Smads (Smad2 and Smad3). Activated Smad2 and Smad3 heteroligomerize with the common partner Smad4 (Co-Smad) to form the complexes and translocate into the nucleus to regulate target gene expression. Activation of the TGF-β signaling pathway can also result in the expression of inhibitory Smads (I-Smads), including Smad6 and Smad7. These I-Smads appear to act by specifically inhibiting Smad2 and Smad3 phosphorylation by blocking their access to TGF-β receptors via the negative-feedback loop [18–20]. Although high glucose is known to stimulate ECM synthesis in a variety of cell types, the role of TGF-β/Smad signaling in diabetic nephropathy and vascular disease under high glucose conditions has not been fully understood. In this study, we examined whether high glucose can activate the Smad signaling pathway and determined if this was dependent on TGF-β.

METHODS

Establishing doxycycline (Dox)-regulated Smad7 expressing tubular epithelial cell line

The doxycycline (Dox)-regulated Smad7 expressing renal tubular epithelial cell line (NRK52E) was estab-

Key words: high glucose, TGF-β signaling, Smad2 and Smad3, diabetic renal and vascular fibrosis.

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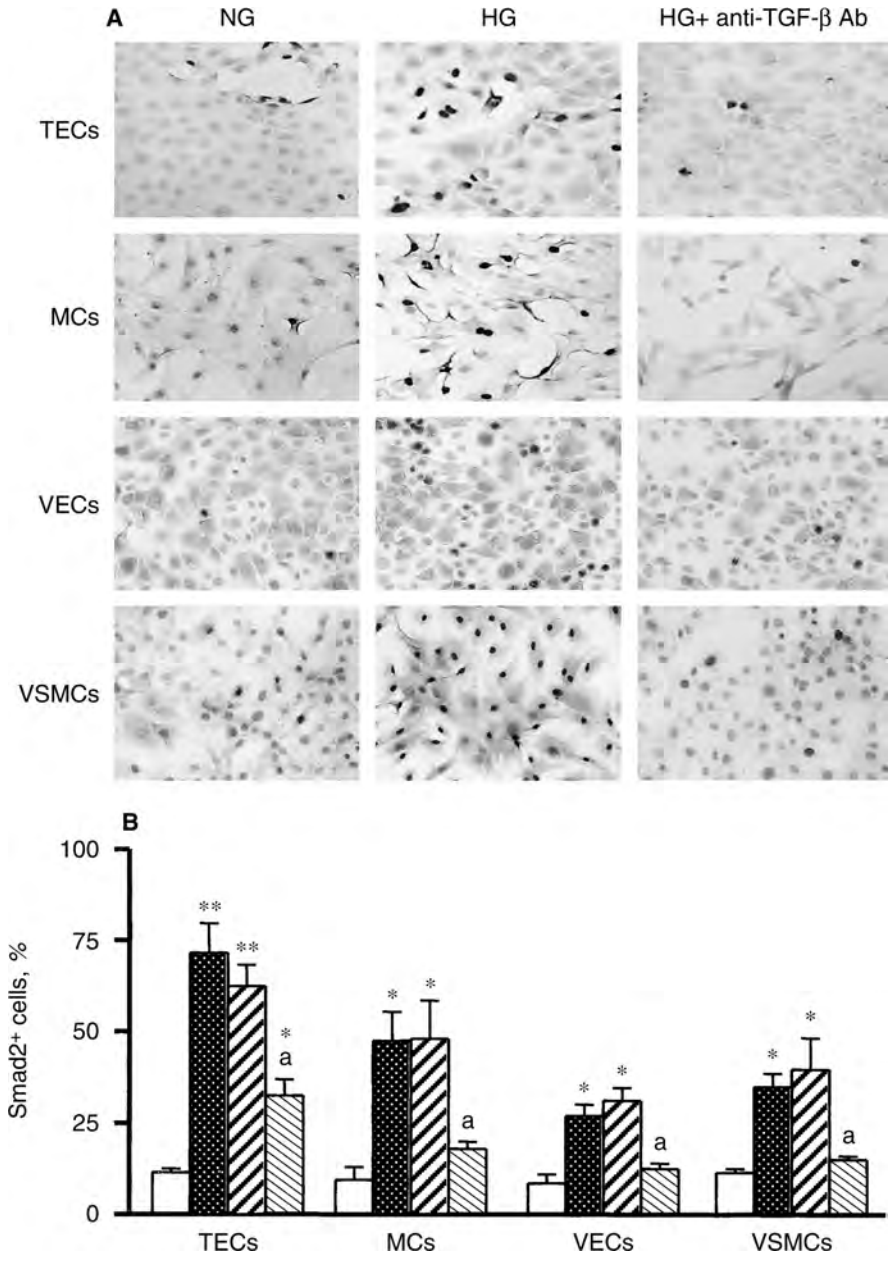
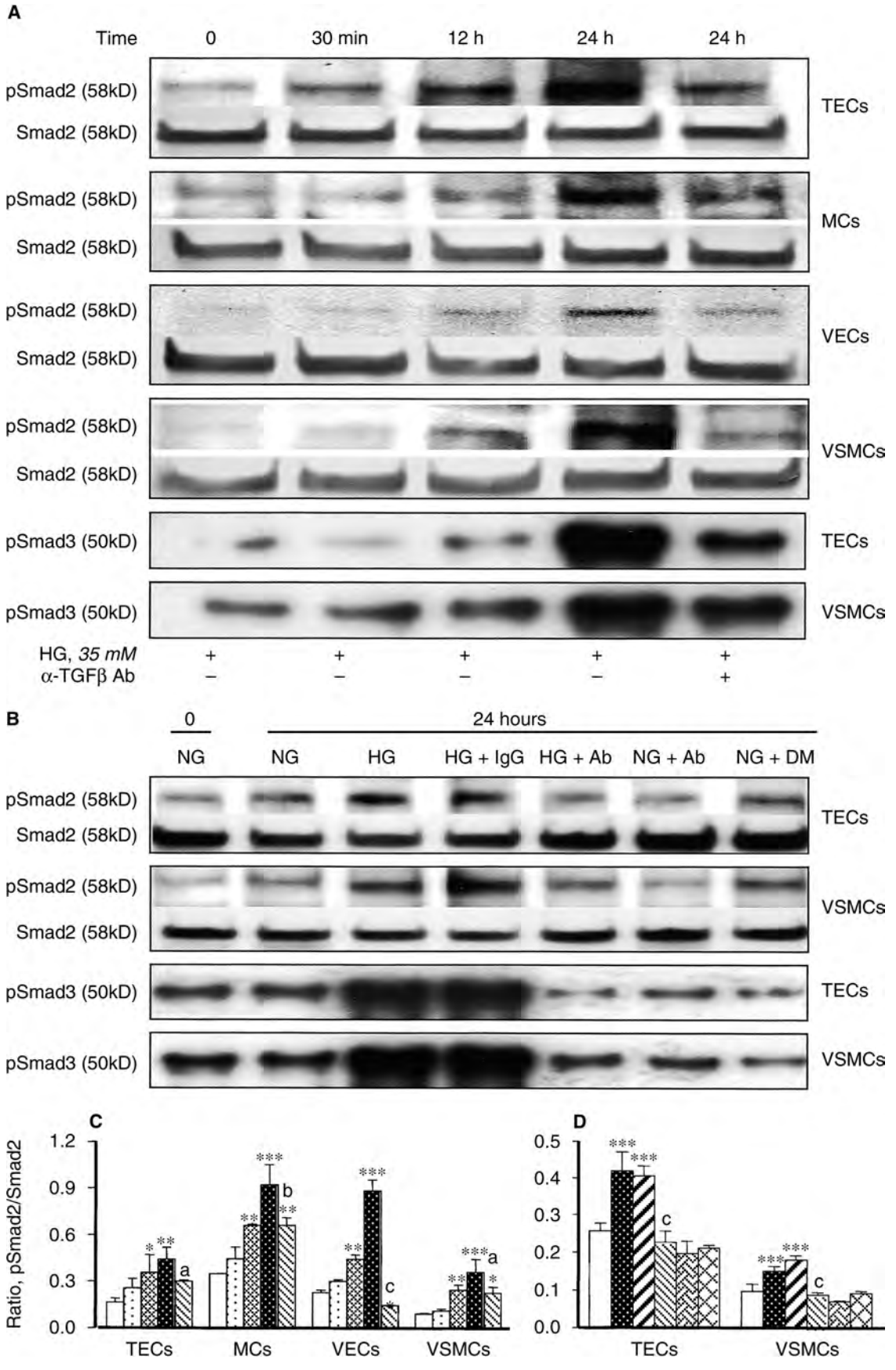


Fig. 1. Immunocytochemistry demonstrates that high glucose (HG) induces Smad2 nuclear translocation in tubular epithelial cells (TECs), mesangial cells (MCs), vascular smooth muscle cells (VSMCs), and vascular endothelial cells (VECs) via the transforming growth factor-β (TGF-β)-dependent mechanism. Activation of Smad signaling as evidenced by phosphorylated Smad2 (p-Smad2) nuclear location (dark black nucleus) is found at 24 hours under high glucose (35 mmol/L), but not in normal glucose (NG) (5.5 mmol/L) conditions. Note that high glucose–induced p-Smad2 nuclear translocation is inhibited by the addition of a neutralizing TGF-β antibody (5 μg/mL). (*A*) Immunohistochemical staining with the anti-p-Smad2 antibody (Ab). (*B*) Semiquantitative data. Magnification ×250. $*P < 0.05$; $**P < 0.01$ compared to NG; $P < 0.05$ compared to the anti-TGF-β antibody (Ab) or control rabbit immunoglobulin G (IgG) treatment. Data are expressed as the mean ± SD for four independent experiments. Symbols are: (□) normal glucose; (▩) high glucose; (▨), high glucose + IgG; and (▧) high glucose + antibody.

lished as previously described [21]. The stable transfected cells were selected in the presence of puromycin (2 μg/mL). Positive clones were confirmed by their ability to express Smad7 in the presence of Dox by Western blot analysis using an antiflag M2 antibody (IBI, Eastman Kodak, Rochester, NY, USA). The clone S7-7 was used in this study.

Cell culture

Four cell lines, including normal rat kidney epithelial cells (NRK52E), glomerular mesangial cells (1097), vascular smooth muscle cells (VSMCs), and human vascular endothelial cells (VECs) (EA.hy926), were used in this study. In addition, the stable, Dox-regulated Smad7 expressing NRK52E cell line was used. All cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, BRL, Gaithersburg, MD, USA) containing 0.5% fetal bovine serum (FBS) in 6-well plastic plates or 8-chamber glass slides (Nunc, Naperville, CT, USA) at 37°C. Cells were stimulated with D-glucose at normal (5.5 mmol/L) or high (35 mmol/L) levels for periods of 15, 30, 60 minutes and 12 and 24 hours for Smad2 and 3 detection and for periods of 1, 3, and 5 days for collagen matrix measurement. D-mannitol (35 mmol/L) was used as a control for osmolality. In addition, a rabbit anti-TGF-β neutralizing antibody and its isotype- and species-matched normal rabbit immunoglobulin G (IgG) (5 μg/mL, R&D Systems, Minneapolis, MN, USA) was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pSmad2 (58kD)
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added into the cells and cultured for periods as described above to block TGF-β function induced by high glucose. To induce Smad7 expression, cells were treated with Dox at an optimal concentration (2 μg/mL) for 24 hours in the medium containing normal glucose (5.5 mmol/L) and then were cultured under high glucose (35 mmol/L) conditions. Levels of secreted TGF-β1 within the medium were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems). Each experiment was repeated at least four times throughout the study.

Reverse transcription-polymerase chain reaction analysis

Total RNA from cultured cells was isolated and reverse transcription-polymerase chain reaction (RT-PCR) was performed using the method described previously [21]. Gene expression for collagen I was detected using published primers [21]. All samples were subjected to RT-PCR for housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as a positive control and as an internal standard [21]. RT-PCR products were resolved on 1.5% agarose gels in 1 × Tris-borate-ethylenediaminetetraacetic acid (EDTA) (TBE) buffer, visualized by ethidium bromide, photographed using a gel 1000 ultraviolet documentation system (Bio-Rad, Hercules, CA, USA), and analyzed by densitometry.

Western blotting and immunoprecipitation

Cells grown in 6-well plates with normal, high glucose, and D-mannitol in the presence or absence of a neutralizing TGF-β antibody or Dox (2 μg/mL) at various time points were analyzed for the activation of TGF-β Smad signaling and collagen I expression (Santa Cruz Biotechnology, Santa Cruz, CA, USA) by Western blotting as described previously [21]. Since activated Smads consist of the phosphorylated Smad2 and Smad3, thus, activation of TGF-β Smad signaling can be determined with the antiphosphorylated Smad2 (p-Smad2) antibody (Upstate Biotechnology, Lake Placid, NY, USA) by both immunohistochemistry and Western blotting. Detection of phosphorylated Smad3 (p-Smad3) was subjected to immunoprecipitation with the rabbit anti-Smad3 antibody (Zymed Laboratories, South San Francisco, CA, USA) followed by rabbit antiphosphoserine antibody (Zymed Laboratories). Total Smad2 and GAPDH were used as a protein loading control for p-Smad2 or collagen I, respectively.

Immunocytochemistry

To detect the activation of TGF-β Smad signaling, cells cultured in 8-chamber glass slides were stained with rabbit polyclonal antibodies to p-Smad2 (Upstate Biotechnology) or Smad3 (Zymed Laboratories) using a peroxidase antiperoxidase method as previously described [21]. An irrelevant isotype rabbit IgG1 (R&D Systems) was used as the negative control.

Quantitative analysis

Quantitative analysis of p-Smad2 nuclear translocation for cultured cells was determined as described previously [21]. The number of cells with nuclear staining for p-Smad2 or Smad3 was counted at least 1000 cells under high power (×400) in each well. All scoring was performed blind on coded slides.

Statistic analysis

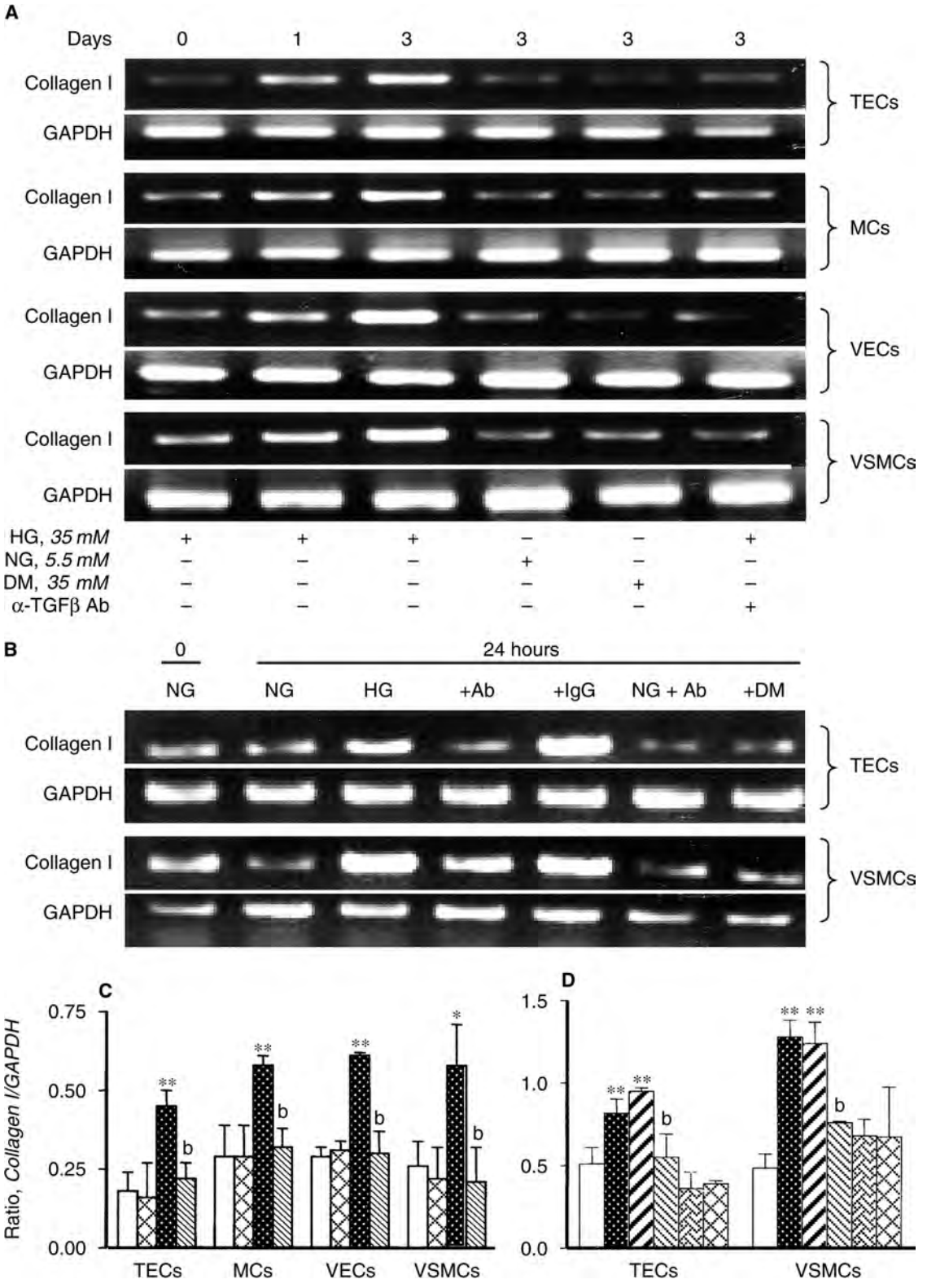
Data obtained from this study are expressed as the mean ± SD and analyzed by one-way analysis of variance (ANOVA) or by unpaired Student *t* test using GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

High glucose activates Smad signaling by renal and vascular cells

First, we examined whether the TGF-β Smad signaling pathway is activated in renal and vascular cells under high glucose conditions as determined by Smad2 phosphorylation and transnuclear location. As shown in Figure 1, immunocytochemistry demonstrated that it was not until 24 hours after high glucose stimulation that TGF-β Smad signaling became activated in all cell types as evidenced by p-Smad2 nuclear translocation, which

Fig. 2. Western blot or immunoprecipitation analyses demonstrate that high glucose induces Smad2 and Smad3 phosphorylation in tubular epithelial cells (TECs), mesangial cells (MCs), vascular smooth muscle cells (VSMCs), and vascular endothelial cells (VECs) via the transforming growth factor-β (TGF-β)-dependent mechanism. (*A*) High glucose–induced Smad2 (by Western blotting) and Smad3 (by immunoprecipitation) phosphorylation at 24 hours is blocked by a neutralizing anti-TGF-β antibody (5 μg/mL). (*B*) High glucose (HG) (35 mmol/L), but not normal glucose (NG) (5.5 mmol/L) in the presence or absence of anti-TGF-β antibody (Ab) or D-mannitol control (35 mmol/L), induces Smad2 and Smad3 phosphorylation at 24 hours in tubular epithelial cells and vascular smooth muscle cells. Note that high glucose–induced TGF-β–dependent Smad phosphorylation was blocked by the addition of a neutralizing TGF-β antibody, but not by an isotype control rabbit immunoglobulin G (IgG). (*C*) Semiquantitative data (a ratio between p-Smad2/Smad2) from Western blot data in (A). Symbols are: (□) 0 minutes; (▩) 30 minutes; (▨) 12 hours; (▩) 24 hours; and (▧) 24 hours + antibody. (*D*) Semiquantitative data (a ratio between p-Smad2/Smad2) from Western blot data in (B). Symbols are: (□) normal glucose; (▩) high glucose; (▨) high glucose + IgG; (▧) high glucose + antibody; (▨) normal glucose + antibody; and (⊠) normal glucose + D-mannitol. Results represent the mean ± SD for three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to normal glucose $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ compared to high glucose and high glucose + control rabbit IgG treatment.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was detected with the rabbit anti-p-Smad2 antibody (Fig. 1A), accounting for up to 55% of cells (Fig. 1B). The specificity of the antibody staining for p-Smad2 was confirmed by the control antibody staining in which no positive cells were found (not shown). In contrast, cells cultured at the normal level of glucose exhibited no increase in Smad2 nuclear translocation (Fig. 1). Consistent with these observations, Western blot analysis showed that high glucose induced significant Smad2 phosphorylation in all cell types examined at 24 hours (Fig. 2). Similarly, immunoprecipitation also demonstrated that high glucose induced marked Smad3 phosphorylation at 24 hours by both renal tubular epithelial cells and VSMCs (Fig. 2). In contrast, no significant Smad2 and Smad3 phosphorylation at 24 hours was found in cells cultured under normal glucose or stimulated by D-mannitol (Fig. 2 B and D).

High glucose activates Smad signaling and induces type I collagen synthesis via the TGF-β–dependent mechanism

Next, we determined the mechanism by which high glucose activates TGF-β Smad signaling. We postulate that high glucose may activate Smad signaling and induce collagen synthesis via a TGF-β–dependent mechanism. We tested this hypothesis by measuring the TGF-β secretion within the medium and by adding a neutralizing TGF-β antibody or an isotype control normal rabbit IgG into the culture. Results in Figures 1 to 4 showed that high glucose–induced Smad2 and Smad3 activation and collagen I mRNA and protein expression in tubular epithelial cells, mesangial cells, VECs, and VSMCs were TGF-β dependent. This is supported by the findings that there was an increase in TGF-β production in the medium at 24 hours (122 ± 7.8 pg/mL vs. 21 ± 5.3 pg/mL in normal glucose, $P < 0.01$) and because the addition of a neutralizing TGF-β antibody, but not the isotype control antibody, blocked high glucose–induced Smad 2 nuclear translocation (Fig. 1) and Smad2 and Smad3 phosphorylation (Fig. 2), and collagen type I mRNA and protein expression (Figs. 3 and 4). In contrast, cells exposed to normal glucose in the presence or absence of the neutralizing TGF-β antibody or D-mannitol showed no significant increase in Smad2 and Smad3 phosphorylation and collagen type I mRNA (by RT-PCR) and protein (by Western blotting) expression in both tubular epithelial cells and VSMCs (Figs. 2 B and D, 3 B and D, and 4 B and D). It is also possible that high glucose–induced activation of Smad signaling may be partially attributed to its osmotic effect since mannitol seems to induce Smad phosphorylation above the background level as shown in Figure 2B.

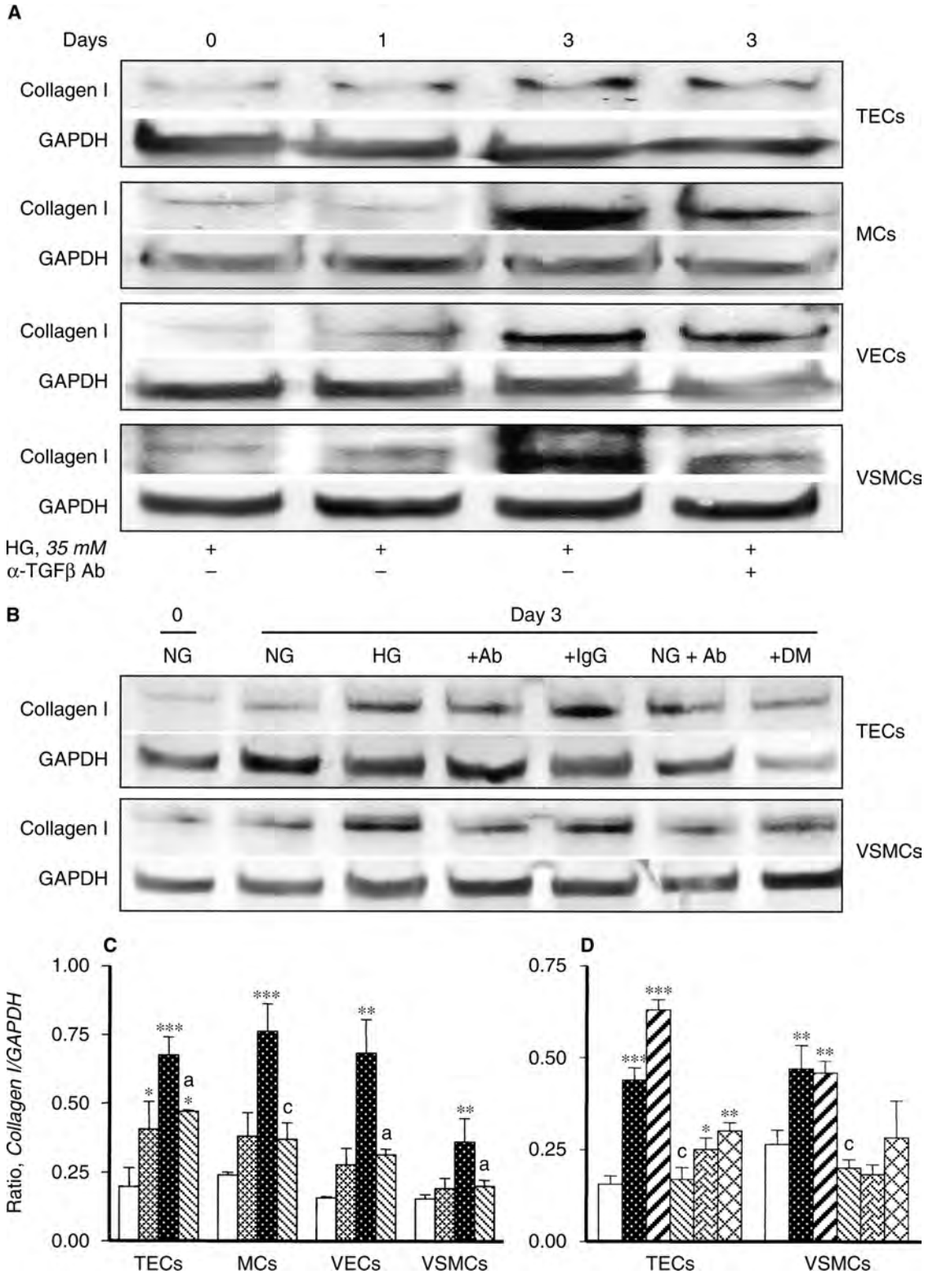
Overexpression of the inhibitory Smad7 blocks high glucose–induced activation of Smad signaling and collagen type I mRNA and protein expression

The regulating role of Smad signaling in the high glucose–induced fibrotic process was further demonstrated in a normal rat tubular epithelial cell line (NRK52E) that was stably transfected with a Dox-regulated Smad7 gene as previously described [21]. We have shown that Smad7 expression is tightly controlled by the concentrations of Dox and that overexpression of Smad7 induced by Dox at 2 μg/mL blocked TGF-β (10 ng/mL)–induced Smad2 activation and collagen matrix expression [21]. Similarly, Dox (2 μg/mL)-induced overexpression of Smad7 prevented high glucose–induced Smad2 and Smad3 nuclear translocation and phosphorylation (Fig. 5). This was associated with inhibition of collagen type I mRNA and protein expression (Fig. 5 B and C).

DISCUSSION

Hyperglycemia is thought to play a pivotal role in the pathogenesis of diabetic nephropathy and vascular complications by stimulating TGF-β production [7–14]. The present study demonstrates that this involves activation of the Smad signaling pathway. Indeed, high glucose was shown to activate the Smad signaling pathway as demonstrated by Smad2 and Smad3 phosphorylation and nuclear translocation and this was associated with the induction of collagen I mRNA and protein expression in tubular epithelial cells, mesangial cells, VECs, and VSMCs. Activation of the Smad signaling pathway is TGF-β dependent since this was associated with an increased TGF-β production at 24 hours and because high glucose activated the TGF-β Smad signaling path-

Fig. 3. Reverse transcription-polymerase chain reaction (RT-PCR) demonstrates that high glucose (HG) induces collagen I mRNA expression by tubular epithial cells (TECs), mesangial cells (MCs), vascular smooth muscle cells (VSMCs), and vascular endothelial cells (VECs) via a transforming growth factor-β (TGF-β)-dependent mechanism. (*A* and *B*) RT-PCR. (*C*) Semiquantitative data [a ratio between mRNA/glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] from (A). Symbols are: (□) normal glucose; (▨) normal glucose + D-mannitol; (▩) high glucose; and (▧) high glucose + antibody. (*D*) Semiquantitative data (a ratio between mRNA/GAPDH) from (B). Note that collagen I mRNA is up-regulated in all renal and vascular cells under high glucose (HG), but not in normal glucose (NG) in the presence or absence of the anti-TGF-β antibody (Ab) or D-mannitol (DM) conditions. Up-regulation of collagen I mRNA by high glucose is inhibited by the addition of a neutralizing TGF-β antibody (α-TGF-β antibody, 5μg/mL), but not by the isotype control rabbit immunoglobulin G (IgG). Symbols are: (□) normal glucose; (▩) high glucose (▨) high glucose + IgG; (▧) high glucose + antibody; (▨) normal glucose + antibody; and (▨) normal glucose + D-mannitol. Results represent the mean ± SD for three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ compared to normal glucose; $^{b}P < 0.01$ compared to high glucose and high glucose + control rabbit IgG treatment.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way and collagen matrix expression was blocked by a neutralizing TGF-β antibody. Furthermore, the finding that overexpression of the inhibitory Smad7 blocked high glucose–induced activation of TGF-β–dependent Smad signaling and collagen I mRNA and protein expression demonstrates that TGF-β Smad signaling plays a critical role in the regulation of renal and vascular scarring under high glucose conditions.

TGF-β is a secreted signaling molecule with fibrotic properties and regulates a diverse range of cellular responses, including proliferation, differentiation, migration, and apoptosis [11–14]. There is increasing evidence that TGF-β is a key mediator of fibrosis in both experimental and human kidney diseases, including diabetic nephropathy and vascular disease [11–14]. Recent studies have suggested a pivotal role of Smads as intracellular effector molecules of the TGF-β family members [19, 20]. The involvement of Smads in TGF-β–mediated renal fibrosis has been recently demonstrated by a number of studies. Indeed, Smad signaling has been shown to play a role in TGF-β–induced collagen matrix synthesis in human mesangial cells and rat tubular cells [21–23], glomerular podocyte apoptosis during glomerulosclerosis [24], and tubular epithelial-myofibroblast transdifferentiation [21]. Particularly, recent studies demonstrate that Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice [25] and Smad3 overexpression increases fibronectin promoter activity, an effect that is enhanced by high glucose or treatment with TGF-β1 [26]. These findings implicate that Smad3 plays a critical role in fibrosis. The present findings that high glucose activates Smad2 and Smad3 and mediates collagen matrix synthesis by stimulating TGF-β production in renal and vascular cells demonstrate that Smad signaling plays an important role in diabetic complications. These in vitro findings presented in the current study support the potential role of Smad activation in diabetic db/db mice and streptozotocin-induced diabetic mice [26, 27].

A critical role for Smad signaling in diabetic renal and vascular sclerosis was further demonstrated in the studies involving inducible gene transfer of the inhibitory Smad7 in vitro. Smad7 is an endogenous inhibitor of Smad2/3 and blocks TGF-β signaling via either the negative-feedback loop or the extracellular signal-related protein kinase-(ERK)/p38 mitigen-activated protein (MAP) kinase cross-talk pathways [18–20, 28, 29]. In addition, Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF-β receptor for degradation [30]. We have previously shown that TGF-β is able to induce endogenous Smad7 expression, but that the low grade of endogenous Smad7 expression is unable to overcome the fibrogenic effect of TGF-β on renal tubular epithelial cells associated with the activation of Smad2 [21]. However, overexpression of Smad7 by gene transfer is able to inhibit Smad2/3 activation in TGF-β–dependent context, including bleomycin-induced lung fibrosis and in a rat obstructive nephropathy [31, 32]. In addition, we and other investigators have also demonstrated that overexpression of Smad7 blocks heme oxygenase-1 expression and the fibrogenic effects of TGF-β on renal tubular epithelial cells and mesangial cells [21, 33, 34]. In the present study, the negative regulatory role of Smad7 in high glucose–induced renal and vascular fibrosis was studied using an improved tetracycline-inducible system in which the expression of Smad7 transgene is under the tight control of Dox [21]. Consistent with the known role of Smad7, Dox-induced overexpression of Smad7 blocked high-glucose–induced Smad activation as demonstrated by marked inhibition of Smad2 and Smad3 phosphorylation and transnuclear location and collagen matrix synthesis. These data further emphasize that Smad signaling plays a regulating role in hyperglycemia-induced diabetic renal and vascular sclerosis.

CONCLUSION

We demonstrate a key role for the TGF-β Smad signaling pathway in ECM production induced by high glucose in a variety of cell types. We further show that high glucose–activated Smad signaling is TGF-β dependent. Importantly, the observation that inhibition of Smad signaling by overexpression of Smad 7 can block high glucose–mediated collagen matrix production indicates that targeting the TGF-β Smad signaling pathway may provide a novel therapeutic strategy for prevention and treatment of diabetic complications.

Fig. 4. Western blot analysis demonstrates that high glucose (HG) induces collagen I protein synthesis by tubular epithelial cells (TECs), mesangial cells (MCs), vascular smooth muscle cells (VSMCs), and vascular endothelial cells (VECs) via a transforming growth factor-β (TGF-β)–dependent mechanism. (*A*) High glucose–induced collagen I protein synthesis by renal and vascular cells is inhibited by the addition of a neutralizing TGF-β antibody (5μg/mL). GAPDH is glyceraldehyde-3-phosphate dehydrogenase. (*B*) Note that collagen I protein synthesis is up-regulated in tubular epithelial cells and vascular smooth muscle cells under high glucose, but not in normal glucose (NG) in the presence or absence of the anti-TGF-β antibody (Ab) or D-mannitol (DM) conditions, and is blocked by the neutralizing TGF-β antibody, but not by the isotype control rabbit immunoglobulin G (IgG). (*C*) Semiquantitative analysis (a ratio between collagen I and GAPDH proteins) for (A). Symbols are: (□) day 0; (▩) day 1; (▩) day 3; and (▧) day 3 + antibody. (*D*) Semiquantitative analysis (a ratio between collagen I and GAPDH proteins) for (B). Symbols are: (□) normal glucose; (▩) high glucose (▨) high glucose + IgG; (▧) high glucose + antibody; (▨) normal glucose + antibody; and (▩) normal glucose + D-mannitol. Results represent the mean ± SD for four independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to NG; $^{a}P < 0.05$; $^{b}P < 0.001$ compared to high glucose and high glucose + control rabbit IgG treatment.

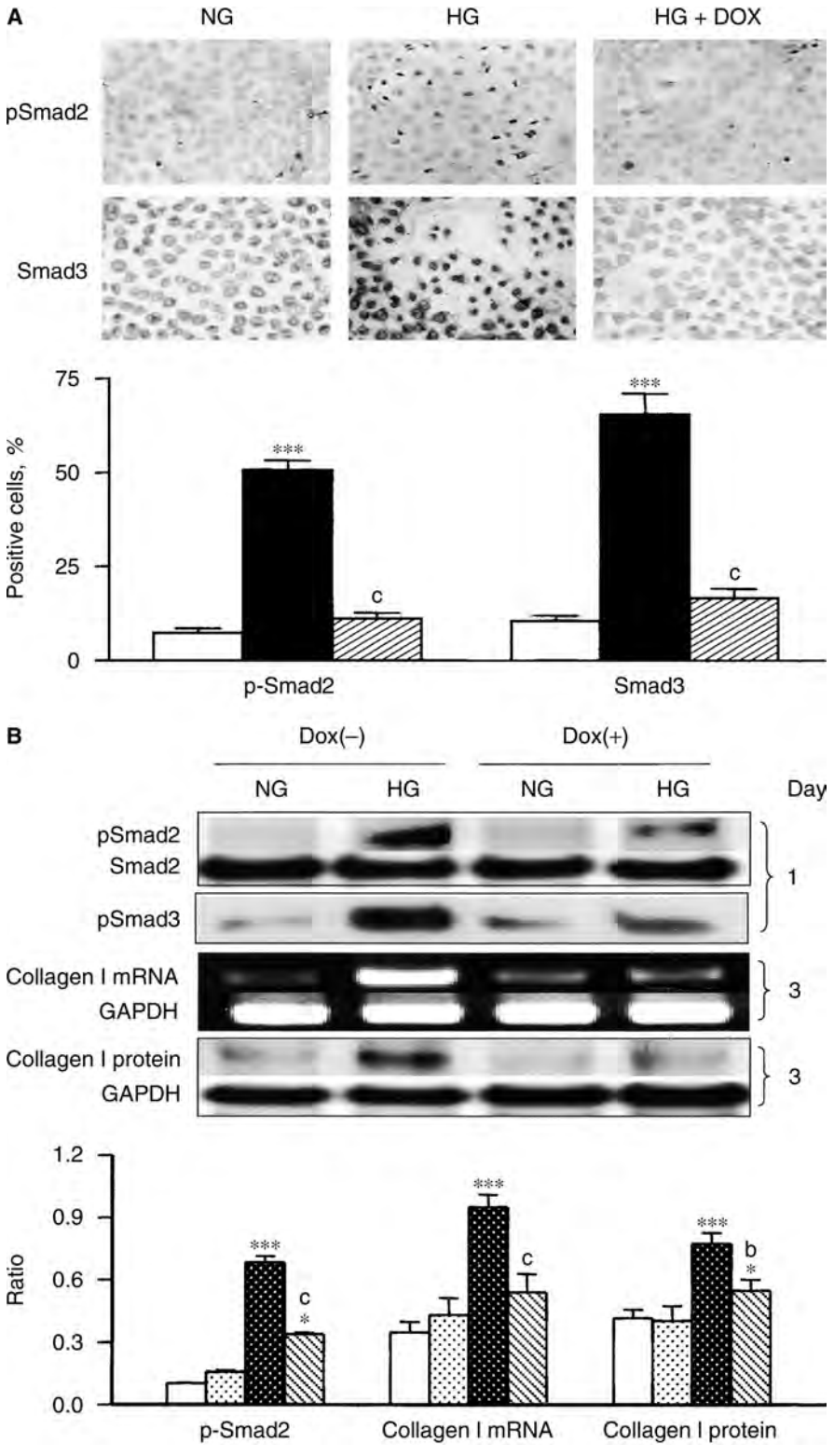

Fig. 5. Overexpression of Smad7 inhibits high glucose–induced Smad2 activation and collagen matrix synthesis in a stable Smad7 expressing tubular epithelial cell (TEC) line (NRK52E). (*A*) Immunocytochemistry demonstrates that doxycycline (Dox) (2 μg/mL)-induced overexpression of Smad7 completely blocks high glucose–induced phospho-Smad2 (p-Smad) and Smad3 transnuclear location (black nuclei). Note that Smad3 protein is primarily located within cytoplasm with negative nuclear staining in both normal glucose (NG) (□) and high glucose + Dox (▨), but it translocates into nuclei when becomes activated at 24 hours after high glucose (■) stimulation. (*B*) Western blot, immunoprecipitation, and reverse transcription-polymerase chain reaction (RT-PCR) analyses demonstrate that Dox (2 μg/mL)-induced overexpression of Smad7 results in substantial inhibition of Smad2 (by Western blotting) and Smad3 (by immunoprecipitation) phosphorylation and collagen I mRNA and protein expression. Values shown in (B) are a ratio between p-Smad2/Smad2 or collagen I mRNA (protein)/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (protein). Symbols are: (□) normal glucose; (▩) normal glucose + Dox; (▩) high glucose; (▨) high glucose + Dox. Results represent four independent experiments and are expressed as the mean ± SD. $^{*}P < 0.05$; $^{***}P < 0.001$ compared to NG. $^{b}P < 0.01$; $^{c}P < 0.001$ compared to high glucose without Dox treatment. Magnification ×250.

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