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Cultured tubule cells from TGF-β1 null mice exhibit impaired hypertrophy and fibronectin expression in high glucose

SHELDON CHEN, BRENDA B. HOFFMAN, JOSEPH S. LEE, YUKI KASAMA, BELINDA JIM, JEFFREY B. KOPP, and FUAD N. ZIYADEH

Renal-Electrolyte and Hypertension Division, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; Kidney Disease Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland

Cultured tubule cells from TGF-β1 null mice exhibit impaired hypertrophy and fibronectin expression in high glucose.

Background. To firmly establish the role of the transforming growth factor-β1 (TGF-β1) isoform in the pathophysiology of diabetic tubulointerstitial hypertrophy and fibrosis, we examined how the total absence of TGF-β1 would alter the effect of high glucose on cellular hypertrophy and matrix expression in tubuloepithelial cells cultured from TGF-β1 null mice.

Methods. Primary tubule cell cultures, obtained from kidneys of TGF-β1 knockout mice and their wild-type littermates, were treated with exogenous TGF-β1 or high glucose. The TGF-β system was characterized at the ligand and receptor levels using Northern and Western blotting. Cellular hypertrophy and growth were assessed by thymidine incorporation, cell counting, leucine incorporation, and protein content. Fibronectin expression was assessed by Northern analysis and enzyme-linked immunosorbent assay (ELISA).

Results. Knockout cells did not express TGF-β1 but did express TGF-β2, TGF-β3, and TGF-β type I and type II receptors. Exogenous TGF-β1 down-regulated the ligand-binding type II receptor but up-regulated type I receptor expression. Knockout cells proliferated more rapidly than wild-type cells, but restoring TGF-β1 to knockout cells slowed their proliferation. In wild-type cells, high glucose caused cellular hypertrophy, evidenced by greater leucine incorporation and protein content along with decreased thymidine incorporation. High glucose also increased fibronectin message and protein. However, in knockout cells, high glucose failed to induce hypertrophy and was severely limited in its capacity to stimulate fibronectin.

Conclusion. In tubular epithelial cells, TGF-β1 mediates the hypertrophic and fibronectin-stimulatory effects of high glucose, confirming the role of the TGF-β1 isoform in the pathogenesis of diabetic tubular hypertrophy and fibronectin overexpression.

Key words: diabetic nephropathy, TGF-β type I receptor, TGF-β type II receptor, knockout.

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Several manifestations of diabetic nephropathy are consequences of altered protein production in response to cytokines or growth factors [1]. Our group has focused on transforming growth factor-β (TGF-β) because it plays a central role in mediating the effects of high ambient glucose on renal cells [2]. High glucose promotes cellular hypertrophy and stimulates extracellular matrix production [3, 4] in cultured mesangial cells [5, 6], glomerular epithelial cells [7, 8], and proximal tubular cells [9, 10]. In turn, these high glucose effects are inhibited by blocking the autocrine activation of the TGF-β system, suggesting that high glucose acts through a TGF-β–dependent mechanism [6, 11]. Similarly, the deleterious effects of diabetes on the kidney may be mediated by TGF-β overactivity, and the increased renal expression of TGF-β1 and its type II signaling receptor serve to aggravate the renal injury in diabetes [12–14]. Further proof of TGF-β stimulation in the diabetic kidney can be found in the activation of the Smad pathway that transmits the TGF-β signal [15, 16].

If TGF-β overactivity is causal, then inhibition of TGF-β should successfully treat diabetic nephropathy. In the streptozotocin-diabetic mouse, panselective neutralizing anti-TGF-β antibodies decreased renal hypertrophy and prevented the overexpression of glomerular matrix proteins in the short-term (9 days) [17]. In a long-term study (8 weeks) of the type 2 diabetic *db/db* mouse, panselective anti-TGF-β antibodies also attenuated matrix overexpression, prevented mesangial glomerulosclerosis, and averted the development of renal insufficiency [18]. Lastly, anti-TGF-β antibodies can even reverse the established lesions of diabetic glomerulopathy [19]. Although anti-TGF-β therapy was started well after the onset of diabetes in the *db/db* mouse [19], glomerular basement membrane thickening and mesangial matrix expansion were both significantly improved with treatment, implicating TGF-β as the predominant cytokine effector of diabetic kidney disease.

In the above studies, the use of panselective anti-TGF-β antibodies that neutralize all three mammalian isoforms of TGF-β (TGF-β1, -β2, and -β3) does not allow for the elucidation of the relative roles of each of the three isoforms in the pathogenesis of diabetic nephropathy. Although there are indirect hints that the TGF-β1 isoform is the principal mediator of diabetic renal lesions [20, 21], direct proof is largely lacking. It would be desirable to use the TGF-β1 knockout mouse to confirm the selective role of the TGF-β1 isoform in diabetic kidney disease, but unfortunately these animals die at an early age from a diffuse inflammatory disorder, making the chronic study of diabetes in these animals impossible [22–25]. Instead, we established primary cultures of tubular epithelial cells from the kidneys of young TGF-β1 knockout mice (−/−) and their wild-type littermates (+/+) [26, 27].

Tubular cells were isolated because, in some respects, the tubulointerstitium plays the more significant role in the progression of diabetic renal disease. In fact, the extent of tubulointerstitial fibrosis correlates most closely with the prognosis for end-stage renal disease from any cause, including diabetes [28, 29], and tubular hypertrophy, with the attendant increase in reabsorption and decrease in glomerular filtrate reaching the macula densa, may activate tubuloglomerular feedback and beget glomerular hyperfiltration, which is detrimental in its own right [30]. This emphasis on the primary role of tubulointerstitial abnormalities in the genesis of glomerular functional defects has been aptly termed the "tubulocentric" view of diabetic nephropathy [30]. Thus, it remains important to investigate the mechanisms whereby tubular hypertrophy and fibrosis are caused by the metabolic features of diabetes. With this in mind, we examined how the pathobiologic effects of high ambient glucose are altered by the total absence of TGF-β1 in renal tubular cells with respect to cellular hypertrophy and matrix expression.

METHODS

Establishment of cell lines

Tubular epithelial cells were isolated from the kidneys of TGF-β1 knockout mice and their control littermates (strain background C57BL/6) by a modification of the method described by Taub and Sato [31]. The TGF-β1 knockout mouse breeding colony and the normal littermates were maintained at the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Briefly, mice were killed at 1 week of age. Kidney cortices were dissected from the medulla, sliced, and minced on a metal mesh. Glomeruli were removed by differential sieving, and the remaining tissue was digested in collagenase to isolate tubular cells. Primary cultures were grown in renal epithelial growth medium containing 130 mg/dL D-glucose, 0.5% fetal calf serum (FCS), human epidermal growth factor, insulin, hydrocortisone, gentamicin, amphotericin B, epinephrine, triiodothyronine, and transferrin (REGM Bullet Kit) (Cambrex BioScience, Walkersville, MD, USA). To exclude the outgrowth of fibroblasts, primary cell cultures were maintained for at least 10 days in serum-deprived media. Clonal cell lines were propagated from the kidneys of two TGF-β1 knockout mice, knockout 2.3 and knockout 2.4, and two wild-type littermates, wild-type 1.2 and wild-type 3.5. All of the cell lines express the epithelial cell markers, cytokeratin and uvomorulin (E-cadherin), and are negative for smooth muscle actin, desmin, and vimentin, consistent with a tubular epithelial phenotype [26, 27]. Further, the wild-type and knockout cells display lotus lectin binding, which is more characteristic of a proximal tubular cell [26].

Cell culture experiments

Cells were maintained at 37°C in a humidified incubator with 5% CO_2 and propagated in REGM/0.5% FCS containing 130 mg/dL D-glucose ("normal glucose"). The cells were passaged every 5 to 7 days by light trypsinization. Wild-type and knockout cells were carefully matched for passage number (between 3 and 17) and were plated at identical densities for the experiments outlined in this study. Experiments were initiated by resting the cells for 24 hours in REGM/0% FCS, and then the medium was changed to fresh, normal glucose REGM/0.5% FCS. In certain plates, the D-glucose concentration was increased to 450 mg/dL ("high glucose") by the addition of a small volume of 1.0 mol/L D-glucose. To control for a possible osmotic effect of high glucose, 0.5 mol/L D-mannitol was added to raise the mannitol concentration to 450 mg/dL. Finally, some plates were treated with exogenous recombinant human TGF-β1 at 1, 2, or 5 ng/mL (R&D Systems, Minneapolis, MN, USA). The cells were harvested after 24 to 48 hours, or as specifically indicated.

For proliferation studies, 5×10^4 cells were propagated in 24-well plates (Nunclon, Roskilde, Denmark) in media with a normal D-glucose concentration. Some wells were treated with exogenous TGF-β1 at a concentration of 1 to 2 ng/mL or with a panselective neutralizing polyclonal chicken anti-TGF-β antibody (R&D Systems) at a concentration of 5 μg/mL over a period of 8 days. Cells were harvested in duplicate from the plates and counted with an automated cell counter (Coulter Electronics, Hialeah, FL, USA).

Northern analysis

At the end of the incubation period, cells were washed in RNAse-free phosphate-buffered saline (PBS) (pH 7.2) and directly lysed and denatured in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total cellular RNA

was extracted according to the manufacturer's instructions. Fifteen micrograms of RNA per lane were separated on a 1.0% agarose gel containing 0.67 mol/L formaldehyde, transferred to a Gene-Screen nylon membrane (NEN Research Products, Boston, MA, USA) by capillary blotting, and cross-linked with ultraviolet light. The integrity and equal loading of RNA samples were assessed by methylene blue staining of the transferred RNA. The cDNA probes used in the studies were separated from their plasmids in low-melt agarose and labeled with ^{32}P-deoxycytidine triphosphate (dCTP) (Amersham, Piscataway, NJ, USA) using a random-priming kit (Ready-to-Go) (Amersham). The cDNA probes encoding murine TGF-β1, TGF-β types I and II receptors, and fibronectin were used for Northern hybridization as previously described [17, 32]. The cDNA probes encoding murine TGF-β2 and TGF-β3, kindly provided by Dr. Harold L. Moses, were a 442 bp *Eco*RI-*Xho*I fragment from pmTGF-β2-9A11C and a 609 bp *Bam*HI-*Xho*I fragment from pmTGF-β3-11C [33, 34]. Labeled cDNAs were separated from unincorporated nucleotides by passage over a Nensorb purification cartridge (NEN Research Products), and membranes were hybridized with 10^6 cpm/mL of probe. Hybridization, washing, and autoradiography were performed as previously reported [8]. Afterward, Northern membranes were probed for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data on another housekeeping gene, 28S rRNA, were obtained from the positive image of the methylene blue–stained nylon membrane [8, 35]. Autoradiograms were then quantitated by computer-assisted video densitometry. Using NIH Image 1.62c, the mRNA level was calculated relative to that of GAPDH or 28S. Measurements of this ratio in cells cultured in "normal glucose" REGM were assigned a relative value of 100%.

Western immunoblotting

Wild-type or knockout cells were washed in ice-cold 1× PBS and then lysed in RIPA buffer [50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethylsulfonyl fluoride, and 5 μg/mL each of aprotinin and leupeptin]. The cell lysate was spun at 12,000 × *g* for 10 minutes, and the supernatant containing total cellular protein was saved. The concentration of protein was measured by the Lowry assay (Bio-Rad, Hercules, CA, USA), and afterwards the protein was prepared in Laemmli sample buffer and boiled for 5 minutes. Equal amounts of protein (20 to 25 μg) were subjected to SDS 4% to 12% gradient polyacrylamide gel electrophoresis (PAGE) using the NuPAGE system (Invitrogen). Electrophoresed proteins were transferred to a nitrocellulose membrane (Bio-Rad). After blocking with 5% nonfat milk in Tris-buffered saline-0.1% Tween-20 (TBS-T), pH 7.6, the membrane was probed for 16 hours at 4°C with one of the following primary antibodies: rabbit anti-TGF-β1 (Promega, Madison, WI, USA), rabbit anti-TGF-β2, rabbit anti-TGF-β3, rabbit anti-TGF-β type I receptor, or rabbit anti-TGF-β type II receptor (all from Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA). After three washes in TBS-T, membranes were probed with a donkey antirabbit antibody conjugated to horseradish peroxidase (Amersham Biosciences, Buckinghamshire, England). The peroxidase-catalyzed chemiluminescence reaction was developed with SuperSignal West Pico substrate (Pierce Biotechnology, Rockford, IL, USA), allowing the detection of immunoreactive protein bands. Finally, the membrane was probed with mouse anti-β-actin antibody (Sigma Chemical Co., St. Louis, MO, USA) to serve as a loading control.

Flow cytometry

Single-cell suspensions were prepared by harvesting wild-type and knockout cells from subconfluent T-75 flasks. Cells were washed in ice-cold PBS/1% bovine serum albumin (BSA) and 5 mmol/L EDTA, pH 8.0 (staining buffer), and aliquots of 1 × 10^6 cells were resuspended in 50 μL staining buffer. Indirect staining for TGF-β type II receptor was performed for 20 minutes on ice with a primary anti-type II receptor antibody (Santa Cruz Biotechnologies, Inc.) or an isotype-matched control antibody (rabbit IgG) (Pierce). Washed cells were then incubated a second time on ice with goat anti-rabbit IgG-fluorescein isothiocyanate (FITC) (Boehringer Mannheim, Mannheim, Germany). Stained cells were washed and resuspended in staining buffer. Propidium iodide (Sigma Chemical Co.) at 2 μg/mL was added to cell suspensions, and fluorescence was recorded on a FACScan cytofluorograph (Becton Dickinson; Mountain View, CA, USA). Data were analyzed with CELL Quest software. In each run, at least 10,000 gated events (propidium iodide excluded) were analyzed.

Tritiated thymidine incorporation

Wild-type and knockout cells (1 × 10^4 cells/well) were subcultured in flat-bottomed, 96-well plastic plates (Nunclon) and were rested overnight in serum-free REGM with a D-glucose concentration of 130 mg/dL. Fresh REGM/0.5% FCS with a normal (130 mg/dL) or a high (450 mg/dL) D-glucose concentration or an equivalent D-mannitol concentration (450 mg/dL) was then added. Cells were grown for an additional 48-hour period, and in the last 6 hours of growth, 1 μCi of [^{3}H]-thymidine (5 Ci/mmol) (Amersham) was added to each well. Cells were lysed with trypsin at 37°C and the nuclei

were harvested on glass-fiber paper and counted for scintillations as previously described [9, 36]. Data are shown as percentages of the control.

Tritiated leucine incorporation

Wild-type and knockout cells (1×10^5 cells/well) were seeded onto 24-well culture plates and at 50% to 60% confluence were rested overnight in serum-free, normal glucose REGM. Afterward, the medium was changed to fresh REGM/0.5% FCS with a normal (130 mg/dL) or a high (450 mg/dL) D-glucose concentration or an equivalent D-mannitol concentration (450 mg/dL). In the last 16 hours of culture, 1 μCi of L-[4,5-^{3}H] leucine (73.0 Ci/mmol) (Amersham) was applied to each well. After a total of 48 hours in normal glucose, high glucose, or mannitol, cells were washed and leucine incorporation was assayed as previously described [20]. Leucine incorporation was divided by the cell number per well, determined from a 24-well plate incubated in parallel and counted using a Bright-Line Hemacytometer (Hausser Scientific, Horsham, PA, USA). Data are presented as percentages of the control.

Protein content measurement

For determination of protein content, wild-type and knockout cells grown in 24-well plates were exposed to REGM/0.5% FCS containing normal glucose (130 mg/dL), high glucose (450 mg/dL), or mannitol (450 mg/dL) for 48 hours. The cells were then lysed in equal volumes of RIPA buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1% Nonidet P-40, 0.1% SDS, 1 mmol/L EDTA, 0.5 mmol/L DTT) per well to extract the total cellular protein. After a 10-minute spin at 4°C and $12{,}000 \times g$ to pellet the cellular debris, the concentration of the cellular proteins in the supernatant was measured by the DC Protein Assay (modified Lowry assay), according to the manufacturer's instructions (Bio-Rad). The total protein content was then normalized for the cell number, counted as above. Data are shown as percentages of the control.

Fibronectin enzyme-linked immunosorbent assay (ELISA)

The cell culture supernatants from wild-type and knockout cells exposed to 24 hours of normal glucose, high glucose, mannitol, or TGF-β1 were assayed for fibronectin protein content by a commercial ELISA kit, according to the manufacturer's instructions (Exocell, Philadelphia, PA, USA). In brief, culture media were added for 30 minutes to the wells of a microtiter plate precoated with a gelatin to adsorb the fibronectin. After several washes, an antifibronectin antibody directly conjugated to horseradish peroxidase was added to each well for an additional 30 minutes. After further washes, the horseradish peroxidase-catalyzed colorimetric reaction was developed with a tetramethyl benzidine (TMB) substrate and stopped with an acid solution. The absorbance was read on a microplate reader at 450 nm. The fibronectin concentrations of the supernatants were determined from the standard curve, plotted as the log of fibronectin concentration against the absorbance at 450 nm. For each experimental condition, fibronectin concentrations were corrected for the respective total RNA content, and the results are expressed as percentages of the control.

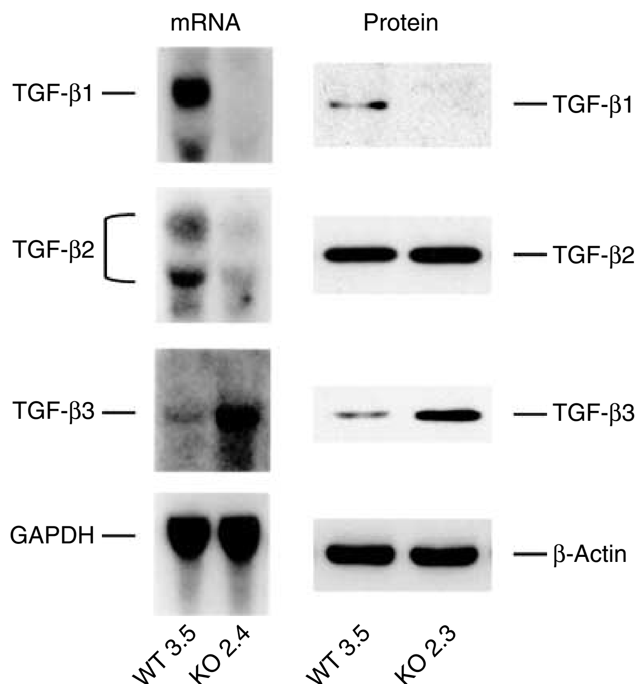

Fig. 1. Knockout cells from transforming growth factor-β1 (TGF-β1) knockout mice lack TGF-β1 mRNA and protein. Northern analysis and Western blotting performed on wild-type (WT) and knockout (KO) cells demonstrate that wild-type cells express all three mammalian isoforms of TGF-β. Although knockout cells lack TGF-β1, they do express message and protein for TGF-β2 and TGF-β3. GAPDH is glyceraldehyde-3-phosphate dehydrogenase.

RESULTS

Knockout cells lack TGF-β1 but express the other components of the TGF-β system

Northern analysis performed on total cell RNA from wild-type and knockout cells demonstrates that knockout cells lack transcripts for TGF-β1, as expected, but do express transcripts for TGF-β2and TGF-β3 (Fig. 1). TGF-β2 mRNA is resolved as two bands, which is likely due to alternative splicing [33, 37]. The expression of TGF-β3 appears to be greater in knockout cells (Fig. 1).

By Western immunoblotting, TGF-β1 protein is absent in knockout cells, whereas TGF-β2 and TGF-β3 are both

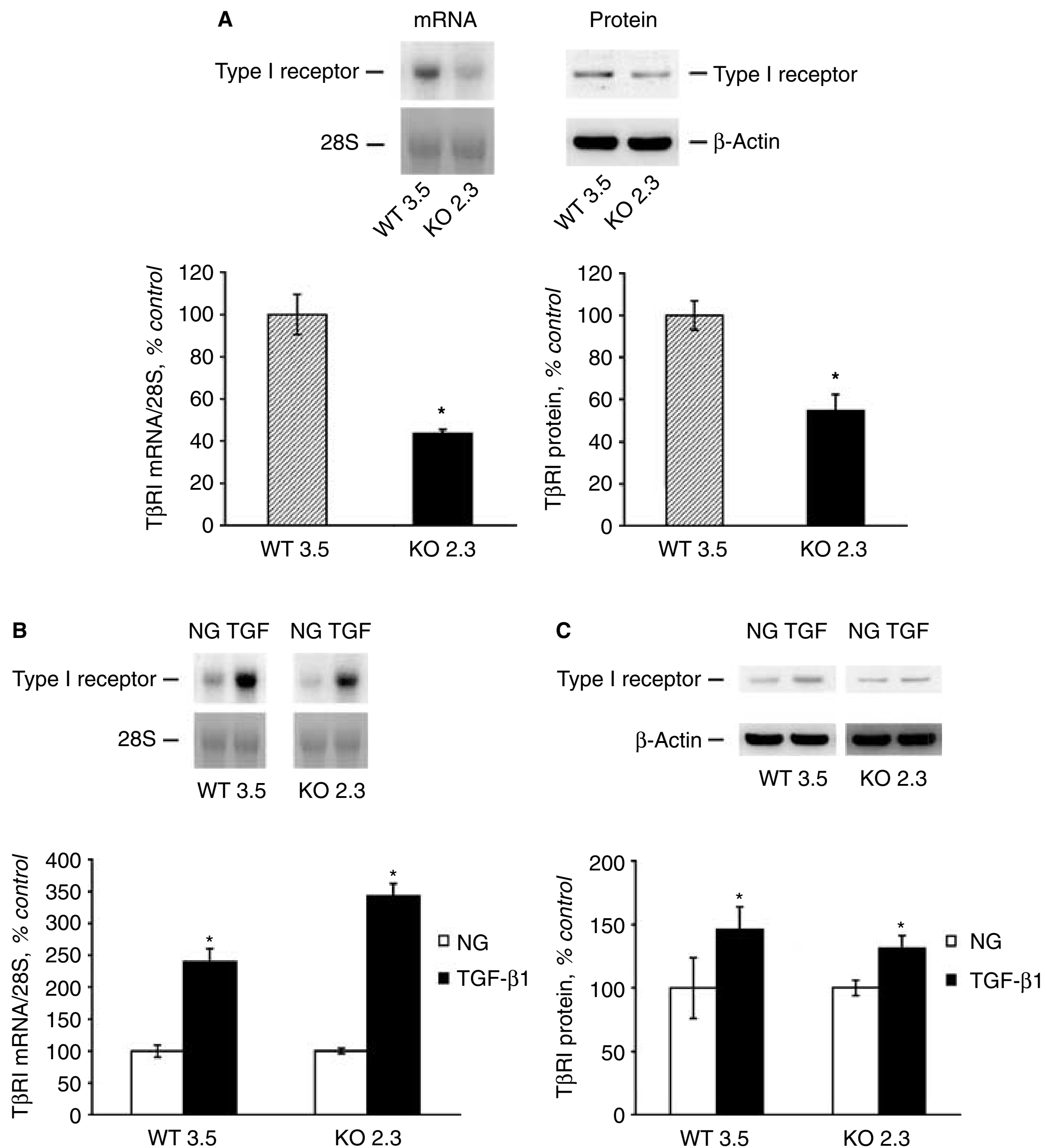

Fig. 2. Constitutive expression of the transforming growth factor-β (TGF-β) type I receptor (TβRI) that is increased by exogenous TGF-β1. Northern analysis and Western blotting show (*A*) greater gene expression and protein production of the type I receptor at baseline in wild-type (WT) compared with knockout (KO) cells, all under normal glucose conditions. (*B*) mRNA expression of the type I receptor in both wild-type and knockout cells is stimulated by treatment with 2 ng/mL TGF-β1 for 24 hours. (*C*) Quantities of type I receptor protein are stimulated in both wild-type and knockout cells by 2 ng/mL TGF-β1 for 48 hours. Representative autoradiograms and immunoblots are shown, and graphs depict the mean ± SEM of the relative densities of the type I receptor bands corrected for 28S rRNA or β-actin, with control values arbitrarily set to 100% ($N = 3$ to 5 independent experiments). $^*P < 0.05$ vs. wild-type or normal glucose (NG) control.

present in knockout cells (Fig. 1). The amount of TGF-β3 protein is visibly increased in knockout cells compared to wild-type, consistent with the mRNA data by Northern analysis.

The two cell types engage in gene expression and protein production of the TGF-β type I receptor (Fig. 2) and the type II receptor (Fig. 3), both belonging to the family of transmembrane serine-threonine kinase receptors. Type II is the primary or ligand-binding receptor, because it forms a high-affinity complex with TGF-β and then binds to the type I receptor. The type II receptor phosphorylates and activates the type I receptor, which can then transduce a signal. While wild-type cells have a higher steady-state mRNA level of the type I receptor

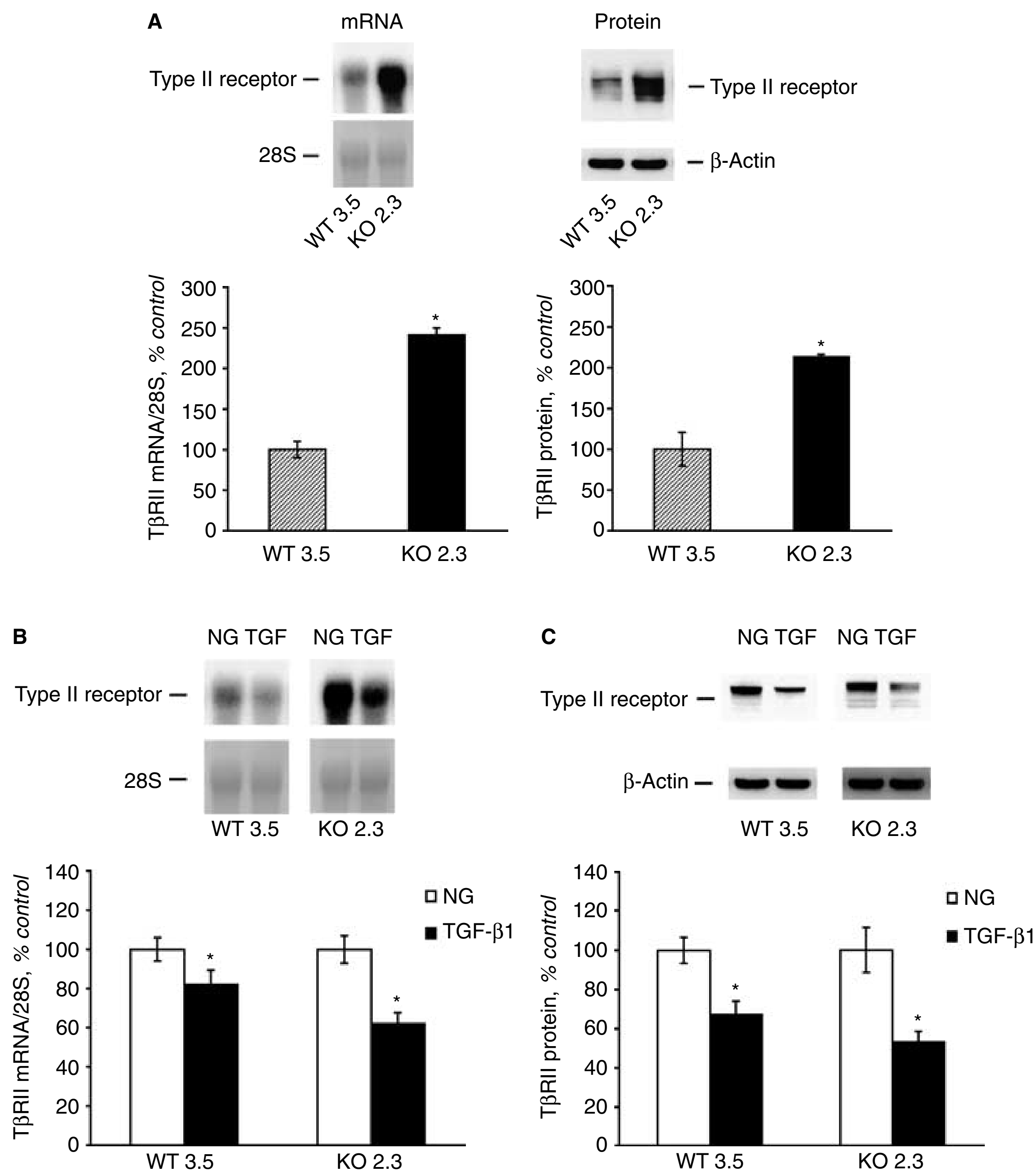

Fig. 3. Constitutive expression of the transforming growth factor-β (TGF-β) type II receptor (TβRII) that is decreased by exogenous TGF-β1. (*A*) Northern analysis and Western blotting show greater gene expression and protein production of the type II receptor at baseline in knockout (KO) compared with wild-type (WT) cells, all under normal glucose conditions. (*B*) mRNA expression of the type II receptor in both wild-type and knockout cells is inhibited by treatment with 2 ng/mL TGF-β1 for 24 hours. (*C*) Quantities of type II receptor protein are reduced in both wild-type and knockout cells by treatment with 2 ng/mL TGF-β1 for 48 hours. Representative autoradiograms and immunoblots are shown, and graphs depict the mean ± SEM of the relative densities of the type II receptor bands corrected for 28S rRNA or β-actin, with control values arbitrarily set to 100% ($N = 3$ to 4 independent experiments). $*P < 0.05$ vs. wild-type or normal glucose (NG) control.

(wild-type 100% ± 9.5% vs. knockout 44% ± 1.7% of wild-type control, $N = 3$, $P < 0.005$) (Fig. 2A), knockout cells demonstrate a significantly greater expression of the type II receptor (wild-type 100% ± 10% vs. knockout 241% ± 9% of wild-type control, $N = 3$, $P < 0.005$) (Fig. 3A). Likewise, by Western blotting, wild-type cells contain more type I receptor protein (wild-type 100% ± 7.0% vs. knockout 55% ± 7.4% of wild-type control, $N = 5$, $P < 0.005$) (Fig. 2A), but knockout cells possess more type II receptor protein (wild-type 100% ± 20.6% vs. knockout 213% ± 3.3% of wild-type control, $N = 4$, $P < 0.005$) (Fig. 3A).

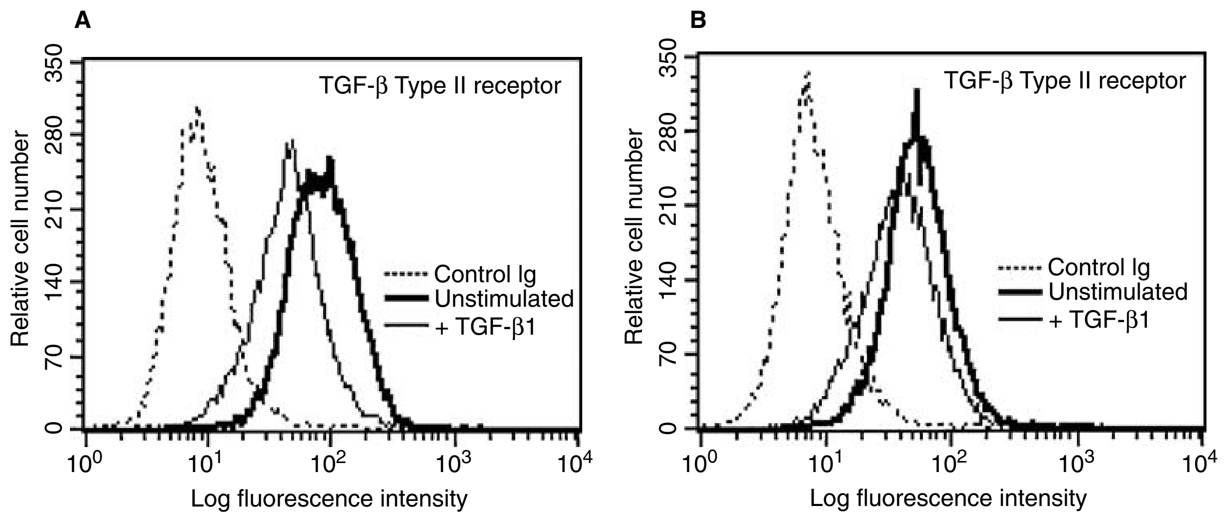

Fig. 4. Cell surface expression of the transforming growth factor-β (TGF-β) type II receptor protein evaluated by the use of an anti-type II receptor antibody in fluorescence-activated cell sorting (FACS). Treatment with 5 ng/mL of TGF-β1 for 72 hours (thin solid line) decreases the type II receptor cell surface expression from baseline (thick solid line) in both wild-type (*A*) and knockout cells (*B*). Control Ig (dashed line), matched for the species and isotype of the anti-type II receptor antibody, shows the degree of nonspecific binding of antibodies.

Modulation of type I and type II receptor expression by TGF-β1

To examine the role of TGF-β1 in regulating its type I and type II receptors, further analyses were performed on TGF-β1–treated wild-type and knockout cells. As shown in Figure 2B, exogenous TGF-β1 markedly stimulates the gene expression of the type I receptor in both wild-type and knockout cells, with knockout cells displaying the larger relative increase. Specifically, in response to 2 ng/mL of TGF-β1 for 24 hours, type I receptor mRNA increases by 2.4-fold in wild-type cells (239% ± 20% of control, $N = 3$, $P = 0.01$) (Fig. 2B) and by 3.4-fold in knockout cells (343% ± 20% of control, $N = 3$, $P < 0.005$) (Fig. 2B). At the protein level, type I receptor increases by nearly 50% in wild-type cells (146% ± 18% of control, $N = 4$, $P < 0.05$) (Fig. 2C) and by ~30% in knockout cells (131% ± 10% of control, $N = 3$, $P < 0.05$) (Fig. 2C) when stimulated with 2 ng/mL of TGF-β1.

In stark contrast, exposure to exogenous TGF-β1 significantly inhibits the mRNA expression of the type II receptor in both wild-type and knockout cells. By densitometric analysis, the steady-state mRNA level of the type II receptor is decreased by ~20% in wild-type cells (TGF-β1 82% ± 7% of control, $N = 4$, $P < 0.05$) (Fig. 3B) and by ~40% in knockout cells (TGF-β1 62% ± 6% of control, $N = 4$, $P < 0.005$) (Fig. 3B). TGF-β1 treatment also decreases the quantity of type II receptor protein. In wild-type cells, type II receptor declines by ~30% (67% ± 6.9% of control, $N = 4$, $P < 0.01$) (Fig. 3C), and in knockout cells, type II receptor decreases by almost 50% (53% ± 5.4% of control, $N = 4$, $P < 0.001$) (Fig. 3C). The inhibitory effect of TGF-β1 on the type II receptor is clearly seen in the representative Western blots (Fig. 3C). Additionally, the effect of TGF-β1 on the quantity of the ligand-binding type II receptor protein located on the cell membrane was evaluated by fluorescence-activated cell sorting (FACS), both at baseline (unstimulated) and after 72 hours of treatment with 5 ng/mL TGF-β1 (Fig. 4). Compared with the respective unstimulated controls, wild-type cells show a 45% decline in surface type II receptor protein, and knockout cells manifest a 30% decrease in type II receptor on the cell surface (Fig. 4).

Knockout cells demonstrate a rapid basal rate of proliferation that is slowed by TGF-β1 addition

TGF-β normally inhibits the replication of proximal tubular cells [38], so the absence of TGF-β1 may be expected to increase the proliferation rate. Serial cell counting demonstrates that the two knockout cell lines have a doubling time between 1 and 2 days vs. a doubling time of 5 days seen in the two wild-type cell lines (Fig. 5). By day 5, both of the knockout cell lines showed significantly greater fold increases in cell number than either of the wild-type cell lines (Fig. 5).

In a separate experiment, the effects of TGF-β blockade and TGF-β1 restoration were tested on the proliferation rates of wild-type and knockout cells. Treatment with a neutralizing, panselective chicken polyclonal anti-TGF-β antibody (5 μg/mL) significantly increases the rate of proliferation of wild-type cells (Fig. 6). Although they proliferate more rapidly with TGF-β deactivated, the wild-type cells still do not divide as quickly as the knockout cells (Fig. 6). The addition of anti-TGF-β antibody does not increase the proliferation of knockout cells any further. On the other hand, addition of exogenous

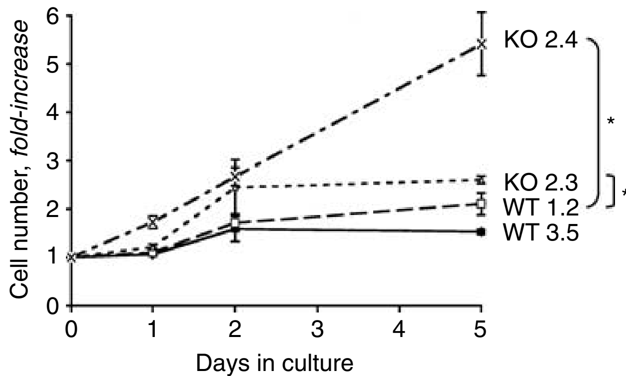

Fig. 5. Knockout (KO) cells demonstrate more rapid rates of proliferation than wild-type (WT) cells under normal glucose conditions. Equal numbers of each of the wild-type and knockout cell lines were seeded at day 0. Serial cell counts (using a Coulter counter) reveal that the two knockout cell lines, KO 2.3 and KO 2.4, have a doubling time between 1 and 2 days, compared with ~5 days for the two wild-type cell lines, WT 1.2 and WT 3.5. Graph shows the mean ± SEM of the fold increase in cell number over 5 days ($N = 3$ independent experiments). $^{*}P < 0.05$ vs. WT 1.2 at day 5.

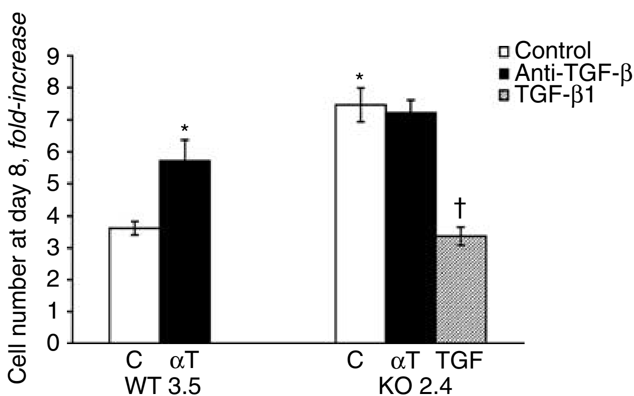

Fig. 6. Antiproliferative effect of endogenous transforming growth factor-β1 (TGF-β1) in wild-type (WT) tubular epithelial cells. Equal numbers of WT 3.5 and KO 2.4 cells were seeded at day 0. Blockade of endogenous TGF-β in wild-type cells with a panselective, chicken polyclonal, neutralizing anti-TGF-β antibody (αT) increases the wild-type proliferation rate, although this still lags behind the knockout (KO) proliferation rate. Knockout cells divide much more rapidly than wild-type cells, and the addition of neutralizing anti-TGF-β antibody (αT) has no discernible effect on the knockout cell count at day 8. However, supplying exogenous TGF-β1 to the knockout cells retards their replication. Graph depicts the mean ± SEM of the fold increase in cell number at day 8 ($N = 3$ independent experiments). $^{*}P < 0.05$ vs. WT 3.5 control; $^{\dagger}P < 0.05$ vs. KO 2.4 control.

TGF-β1 (1 ng/mL) inhibits the replication of knockout cells (Fig. 6).

In high glucose, knockout cells do not undergo hypertrophy, contrary to the wild-type cells

The accepted definition of cellular hypertrophy is an increase in protein synthesis or protein content without a concurrent increase in the DNA content or cell number. Tritiated thymidine incorporation and cell counting were used to gauge DNA replication and proliferation, while tritiated leucine incorporation and a modified Lowry assay were used to evaluate protein synthesis rate and cellular protein content. Wild-type cells treated with high glucose for 48 hours decrease their incorporation of thymidine by 8% (92% ± 1.9% of control, $N = 5$, $P < 0.05$) (Fig. 7A), while wild-type cells exposed to an equivalent concentration of mannitol display a similar decrease in thymidine incorporation (87% ± 2.5% of control, $N = 5$, $P < 0.05$) (Fig. 7A). On the other hand, knockout cells exposed to high glucose for 48 hours react by increasing their thymidine incorporation (126% ± 9.5% of control, $N = 5$, $P < 0.05$) (Fig. 7A). Mannitol has no effect on thymidine incorporation in knockout cells (100% ± 4.5% of control, $N = 5$, $P =$ NS) (Fig. 7A).

The thymidine data are corroborated by the cell counts. The number of wild-type cells after 48 hours in high glucose declines significantly by 11% (89% ± 3.1% of control, $N = 4$, $P < 0.05$) (Fig. 7B) and by 16% in mannitol (84% ± 3.1% of control, $N = 4$, $P < 0.05$) (Fig. 7B). In contrast, the number of knockout cells increases by 15% after 48 hours of high glucose (115% ± 4.1% of control, $N = 4$, $P < 0.05$) (Fig. 7B), and the knockout cell count is not influenced by mannitol (99% ± 4.0% of control, $N = 4$, $P=$ NS) (Fig. 7B).

As a marker of protein synthetic rate, tritiated leucine incorporation per cell increases by a significant amount in wild-type cells exposed to high glucose for 48 hours, rising by 50% over the control value (150% ± 2.4% of control, $N = 6$, $P < 0.05$) (Fig. 8A). Mannitol exerts an effect similar to high glucose on wild-type cells, increasing the leucine incorporation per cell by 55% (155% ± 4.2% of control, $N = 6$, $P < 0.005$) (Fig. 8A). However, knockout cells behave differently from wild-type and respond to high glucose with a decrease in leucine incorporation per cell (69% ± 1.9% of control, $N = 6$, $P < 0.005$) (Fig. 8A). Unlike high glucose, though, mannitol does not significantly affect the leucine incorporation per knockout cell (92% ± 3.3% of control, $N = 6$, $P=$ NS) (Fig. 8A).

Consistent with the leucine incorporation data, the protein content per cell increases by 42% in wild-type cells exposed to high glucose (142% ± 2.2% of control, $N = 3$, $P < 0.05$) (Fig. 8B) and by 40% in wild-type treated with mannitol (140% ± 2.4% of control, $N = 3$, $P < 0.05$) (Fig. 8B). On the other hand, with knockout cells, high glucose causes the protein content per cell to decrease by 15% (85% ± 1.7% of control, $N = 3$, $P < 0.05$) (Fig. 8B), whereas mannitol does not significantly alter the amount of total protein per cell (104% ± 1.2% of control, $N = 3$, $P=$ NS) (Fig. 8B). Taken together with the thymidine and cell number figures from above, the protein data indicate that high glucose and mannitol can induce cellular hypertrophy in wild-type cells but not in knockout cells.

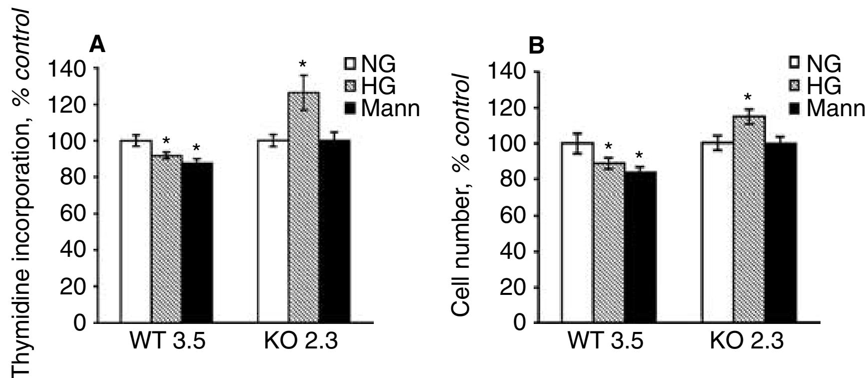

Fig. 7. Divergent effects of high glucose on thymidine incorporation and cell count in wild-type (WT) and transforming growth factor-β1 (TGF-β1) knockout (KO) cells. (*A*) Wild-type and knockout cells were grown in 96-well plates for 48 hours in media containing normal glucose (NG) (130 mg/dL), high glucose (HG) (450 mg/dL), or mannitol (Mann) (450 mg/dL) and then were labeled with [^{3}H]-thymidine in the last 6 hours. Scintillations (cpm) were counted and results are shown as the mean ± SEM of the thymidine incorporation as percentages of the control, assigned a value of 100%. In wild-type cells both high glucose and mannitol reduced thymidine incorporation, a measure of DNA replication, and in knockout cells high glucose but not mannitol stimulated thymidine incorporation. (*B*) Wild-type and knockout cells were seeded in 12-well plates and exposed to media containing normal glucose, high glucose, or mannitol for 48 hours. After trypsinization, the cells were scraped and resuspended in a fixed volume of media containing 10% fetal calf serum (FCS). The number of cells per well was counted in a Bright-Line Hemacytometer. Data are presented as the mean ± SEM of the cell counts as percentages of the control, assigned a value of 100%. Both high glucose and mannitol decreased the number of wild-type cells, and high glucose but not mannitol increased the number of knockout cells ($N = 5$ independent experiments for thymidine and $N = 4$ independent experiments for cell count). *$P < 0.05$ vs. control.

Knockout cells exhibit decreased fibronectin at baseline but respond exuberantly to exogenous TGF-β1

At baseline under normal glucose conditions, Northern analysis shows that knockout cells have a significantly reduced steady-state mRNA level of fibronectin compared with wild-type cells (Fig. 9A). Nevertheless, exogenous treatment with TGF-β1 (2 ng/mL) for 24 hours significantly increases fibronectin gene expression in both wild-type and knockout cells. In wild-type cells, TGF-β1 causes fibronectin mRNA to increase by more than sixfold (635% ± 94% of wild-type control, $N = 6$, $P < 0.005$) (Fig. 9A), and in knockout cells, TGF-β1 raises fibronectin mRNA by more than eightfold (832% ± 106% of knockout control, $N = 5$, $P < 0.005$). The enormous fold increase for knockout cells is a bit misleading, however, because knockout cells express much less fibronectin than wild-type cells in general. Not until knockout cells are stimulated with TGF-β1 does fibronectin expression become greater than in wild-type controls (147% ± 19% of wild-type control, $N = 5$, $P < 0.01$) (Fig. 9A). In other words, the relative increase in fibronectin with TGF-β1 may be greater for knockout cells (~eightfold vs. ~sixfold), but the absolute amount of fibronectin mRNA in knockout cells, even with TGF-β1 stimulation, just barely surpasses the fibronectin level of wild-type cells at baseline (Fig. 9A). From the same experiments as above, cell culture supernatants were assayed for fibronectin protein by ELISA. TGF-β1 treatment for 24 hours causes wild-type cells to enrich their media by ~1.7-fold more fibronectin (171% ± 12% of control, $N = 4$, $P < 0.001$) (Fig. 9B) and knockout cells to increase their media level of fibronectin protein by 1.24-fold (124% ± 16% of control, $N = 5$, $P < 0.05$). Again, to provide the proper perspective, the absolute quantity of fibronectin protein in knockout cells still lags behind that of wild-type cells (Fig. 9B).

High glucose-induced fibronectin expression is severely blunted in knockout cells

Exposure to high glucose for 24 hours causes the wild-type cell to increase its quantity of fibronectin mRNA by ~70% (169% ± 23% of control, $N = 6$, $P < 0.05$) (Fig. 9A). An equal concentration of mannitol, to control for an osmotic effect of high glucose, also stimulates fibronectin expression in wild-type cells, but only by ~35% (134% ± 16% of control, $N = 6$, $P = 0.05$) (Fig. 9A). At the protein level, fibronectin in the cell culture supernatant increases by ~30% during treatment with high glucose for 24 hours (133% ± 3% of control, $N = 4$, $P = 0.05$) (Fig. 9B). Mannitol, however, does not significantly raise the fibronectin protein content in the culture media of wild-type cells (115% ± 10% of control, $N = 4$, $P=$ NS) (Fig. 9B).

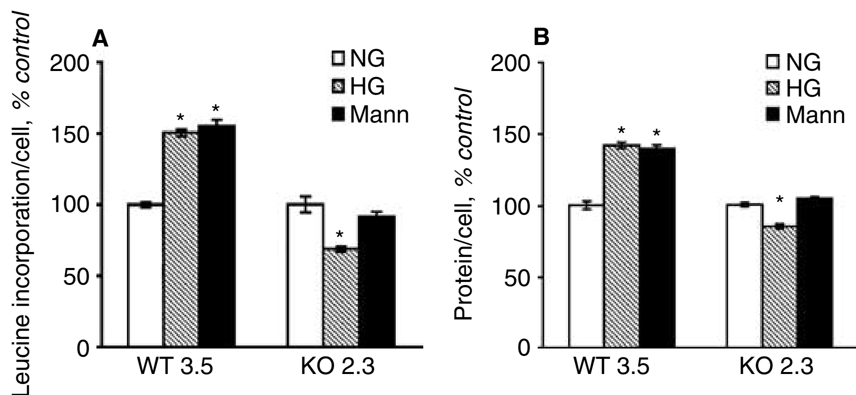

Fig. 8. Divergent effects of high glucose on leucine incorporation and protein content in wild-type (WT) and transforming growth factor-β1 (TGF-β1) knockout (KO) tubuloepithelial cells. (*A*) Wild-type and knockout cells were grown for 48 hours in media containing normal glucose (NG) (130 mg/dL), high glucose (HG) (450 mg/dL), or mannitol (Mann) (450 mg/dL) and then were labeled with [^{3}H]-leucine in the last 16 hours. Scintillations (cpm) were adjusted for the cell number and results are shown as the mean ± SEM of the leucine incorporation per cell as percentages of the control, assigned a value of 100%. In wild-type cells, both high glucose and mannitol significantly stimulate leucine incorporation, a measure of protein synthetic rate, and, in knockout cells, high glucose but not mannitol inhibits leucine incorporation. (*B*) Wild-type and knockout (KO) cells were seeded onto 24-well plates and exposed to media containing normal glucose, high glucose, or mannitol for 48 hours. The cells were then lysed in standardized volumes of RIPA buffer, and the cellular protein concentrations were measured by a modified Lowry assay. Total protein content was calculated and corrected for the cell number, counted in identically treated wells of the 24-well plate. Results are presented as the mean ± SEM of the protein content per cell as percentages of the control, assigned a value of 100%. In wild-type cells, both high glucose and mannitol increased the protein content per cell, and, in knockout cells, high glucose but not mannitol lowered the protein content per cell ($N = 6$ independent experiments for leucine and $N = 3$ independent experiments for protein content). $^*P < 0.05$ vs. control.

On the other hand, the TGF-β1 knockout cell manages only a 23% increase in fibronectin mRNA in response to high glucose (123% ± 5% of knockout control, $N = 5$, $P < 0.01$). Considering that knockout cells have a much lower basal expression of fibronectin than wild-type cells (Fig. 9A), the relatively small effect of high glucose on knockout cell expression of fibronectin is probably inconsequential. This is borne out by the ELISA data showing minimal effect of high glucose on fibronectin protein in the cell culture media of knockout cells (104% ± 6% of control, $N = 5$, $P=$ NS) (Fig. 9B). Mannitol fails to increase the level of either fibronectin mRNA (110% ± 22% of control, $N = 5$, $P=$ NS) (Fig. 9A) or fibronectin protein (101% ± 7% of control, $N = 5$, $P=$ NS) (Fig. 9B) in knockout cells.

DISCUSSION

Interventional studies using panselective anti-TGF-β therapy have elucidated the critical role that TGF-β plays in the pathogenesis of diabetic nephropathy. By specifically blocking the effects of all of the TGF-β isoforms, neutralizing anti-TGF-β antibodies have prevented mesangial matrix expansion and the development of renal isufficiency in mouse models of diabetes, even though hyperglycemia and proteinuria were persistent [17, 18]. Other strategies that have been employed to inhibit the TGF-β system in animal models and in cell culture include the use of antisense oligonucleotides, decorin, and a dominant-negative TGF-β receptor [39–41]. The ability to inhibit the TGF-β system with biologic molecules represents an important advance, but techniques to create gene knockouts allow one component of the TGF-β system to be obliterated entirely. Of the three mammalian TGF-β isoforms, TGF-β1 was targeted because it alone circulates in the bloodstream, is the most highly expressed isoform in the kidney, and has been most closely linked to the pathophysiology of diabetic nephropathy [21]. However, the TGF-β1 knockout mouse dies at an early age so studying the role of TGF-β1 in diabetic kidney disease would be nearly impossible in this animal model.

To circumvent the longevity problem, kidney cells from the TGF-β1 knockout mouse were established as primary cell culture lines, yielding knockout 2.3 and knockout 2.4. These knockout cells and their wild-type counterparts appear to be derived from the proximal tubular epithelium. As expected, cells from TGF-β1 knockout mice do not

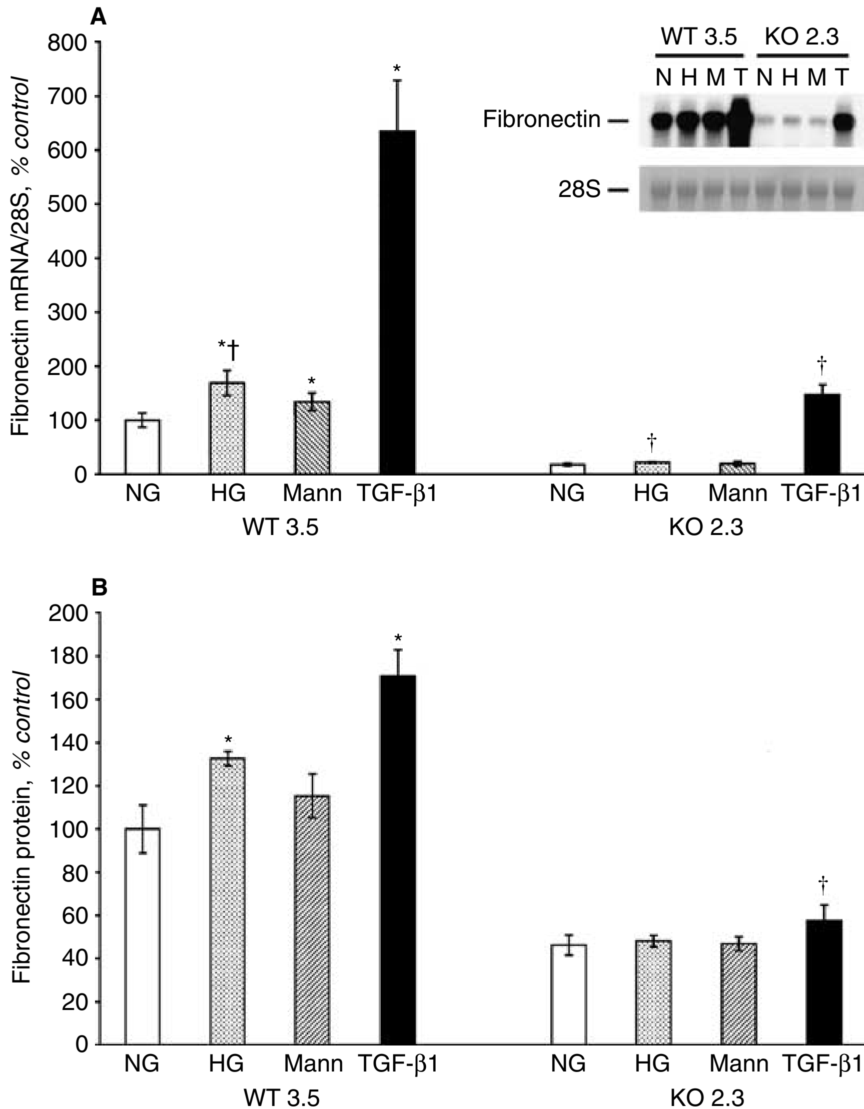

Fig. 9. Markedly decreased constitutive expression of fibronectin and attenuated stimulation of fibronectin by high glucose in knockout (KO) compared with wild-type (WT) cells. (*A*) Northern analysis of equal amounts of total RNA from wild-type and knockout cells demonstrates vastly greater quantities of fibronectin mRNA in wild-type vs. knockout cells under all conditions, normal glucose (N or NG), high glucose (H or HG), mannitol (M or Mann), and transforming growth factor-β1 (TGF-β1) (T) for 24 hours. The 28S band was generated from the positive image of the Northern membrane stained with methylene blue. High glucose significantly stimulates fibronectin expression by 69% in wild-type cells but only by 23% in knockout cells, relative to their own baseline levels. Mannitol, an osmotic control for high glucose, produces a 34% increase of fibronectin mRNA in wild-type cells but does not alter fibronectin expression in knockout cells. Exogenous TGF-β1 dramatically stimulates fibronectin expression in both wild-type and knockout cells. A representative autoradiogram is shown, and the graph depicts the mean ± SEM of the relative densities of the fibronectin bands corrected for 28S rRNA, all in relation to the wild-type control, which is assigned a value of 100% ($N = 5$ to 6 independent experiments). $^*P < 0.05$ vs. wild-type control (NG); $^+P = 0.05$ vs. mannitol (Mann); and $^†P < 0.05$ vs. knockout control (NG). (*B*) Enzyme-linked immunosorbent assay (ELISA) for fibronectin protein performed on the cell culture media of wild-type and knockout cells treated with normal glucose (NG), high glucose (HG), mannitol (Mann), or TGF-β1 for 24 hours. Under all conditions, fibronectin protein levels are significantly lower in knockout cells than in wild-type cells. High glucose increases fibronectin protein in the media of wild-type cells by 33% but fails to stimulate fibronectin production by knockout cells. Mannitol has no significant effect in either cell line. Finally, exogenous TGF-β1 increases fibronectin in both wild-type and knockout cells. Graph displays the mean ± SEM of the fibronectin protein corrected for total RNA content, all as percentages of the wild-type control, which is assigned a value of 100% ($N = 4$ to 5 independent experiments). $^*P \leq 0.05$ vs. wild-type control; $^†P < 0.05$ vs. knockout control.

express TGF-β1, but they still express message and protein for the TGF-β2 and -β3 isoforms. These three isoforms share similar actions in vitro but not necessarily in vivo [42]. It is unclear if TGF-β2 and TGF-β3 can "compensate" for the loss of the TGF-β1 isoform in the knockout cells. However, the fact that a panselective, neutralizing anti-TGF-β antibody had little effect on the proliferation rate of knockout cells (Fig. 6) supports the notion that the TGF-β1 isoform exerts the predominant effect on cell proliferation. In addition, the markedly decreased levels of fibronectin mRNA and protein in knockout cells compared with wild-type proves that TGF-β1 is essential for maintaining even a normal degree of fibronectin expression (Fig. 9). Among the three isoforms, TGF-β1 appears to be the most potent stimulator of extracellular matrix expression.

The knockout cells also express message and protein for the signaling type I and type II receptors and are able to respond to exogenous TGF-β1 with decreased cellular proliferation (Fig. 6) and increased fibronectin expression (Fig. 9). Compared with wild-types, however, knockout cells express significantly less type I receptor, but interestingly, more of the ligand-binding type II receptor. In both cell types, exogenous TGF-β1 down-regulates the type II

receptor, at the mRNA and protein levels and at the cell surface (Figs. 3B and C and 4), consistent with a known negative feedback physiology [43–45]. Accordingly, the increased expression of the type II receptor in knockout cells can be explained by the complete absence of TGF-β1, which would remove its inhibitory effect on the type II receptor (Fig. 3A). On the other hand, exogenous TGF-β1 up-regulates type I receptor mRNA and protein (Fig. 2B and C). This interesting result has been reported in human prostate smooth muscle cells [43], keloid fibroblasts [46], and ocular fibroblasts [47], but to our knowledge this has not been previously shown in renal tubule cells. Because TGF-β1 stimulates type I receptor expression, a complete lack of TGF-β1 in knockout cells probably explains their reduced type I receptor message and protein at baseline vs. wild-type cells (Fig. 2A).

Given that knockout cells have decreased type I receptor but increased type II receptor, it is difficult to predict how the balance of TGF-β receptors will affect the function of exogenously provided TGF-β1. There may be discrete subsets of TGF-β–mediated responses (e.g., mitogenesis vs. fibrogenesis) that are differentially regulated by the two receptors [43]. For instance, the type I receptor may be more important in promoting fibrotic responses [41, 43], while the type II receptor may be more involved with inhibiting cellular proliferation [43, 48]. Consistent with these postulates, the knockout cells with their decreased type I receptor do not attain the same degree of TGF-β1–induced fibronectin mRNA or protein as the wild-type cells (Fig. 9), and with their increased type II receptor the knockout cells appear to be exquisitely sensitive to the antiproliferative effects of TGF-β1 (Fig. 6).

Compared with the wild-type cell lines, both of the knockout clones demonstrate significantly greater rates of basal proliferation that may be partially due to the absence of TGF-β1 expression in these cells. This finding is consistent with the known antiproliferative effect of TGF-β1 on tubuloepithelial cells [9, 11]. Restoring TGF-β1 in the culture media of the knockout cells markedly diminishes their proliferation such that the cell number drops to the level of the wild-type cells (Fig. 6). Conversely, shielding the wild-type cells from the effects of ambient TGF-β (with anti-TGF-β antibody) increases their proliferation so that the cell count surpasses the number of untreated wild-type cells but still lags behind the number of TGF-β1 knockout cells (Fig. 6). Perhaps the knockout cells outgrow the wild-type cells treated with anti-TGF-β therapy because the gene knockout completely deletes TGF-β1, whereas the neutralizing antibody may only partially intercept TGF-β1.

We had previously shown that high glucose induces hypertrophy in cultured renal proximal tubule cells [9]. In the current study, high glucose causes the wild-type cells to increase their protein synthesis, assayed by leucine incorporation and measurement of total protein per cell, and to decrease their proliferation, determined by thymidine incorporation and cell counts (Figs. 7 and 8). Since these effects on wild-type cells were replicated by mannitol, the mechanism of high glucose-induced cellular hypertrophy is likely related to hyperosmolarity. However, the TGF-β1 knockout cells respond to high glucose in just the opposite manner. They show decreased rates of protein synthesis (by leucine incorporation per cell) and decreased protein content per cell (Fig. 8) and exhibit increased thymidine incorporation and cell number (Fig. 7), a profile inconsistent with cellular hypertrophy but compatible with hyperplasia. Moreover, unlike the case with wild-type cells, the high glucose effects on knockout cells were *not* reproduced by mannitol (Figs. 7 and 8). Thus, high glucose causes cellular hypertrophy in wild-type cells likely by an osmotic effect, that itself is dependent on the expression of TGF-β1 in these cells. In the absence of TGF-β1, high glucose in knockout cells stimulates cellular proliferation rather than hypertrophy, and this phenomenon is not related to hyperosmolarity since mannitol had no demonstrable effect.

As for extracellular matrix expression, the TGF-β1-knockout cells can increase their mRNA and protein levels of fibronectin in response to exogenous TGF-β1. At baseline, the knockout cells have significantly lower levels of fibronectin compared with wild-type cells, suggesting that the absence of TGF-β1 decreases the basal rate of matrix expression (Fig. 9). After treatment with TGF-β1, the knockout cells display a striking increase in fibronectin mRNA and protein, proving that these cells have an otherwise competent TGF-β signaling system that can stimulate matrix production. The wild-type cells also respond to TGF-β1 treatment with a vigorous increase in fibronectin. Although the absolute amount of TGF-β1–induced fibronectin message and protein are greater in wild-type cells (Fig. 9), the percentage increase in fibronectin mRNA from baseline is greater for the knockout cells (Fig. 9A). Our findings are similar to those of another paper looking at wild-type and TGF-β1 knockout cells that showed greater basal production of collagen IV in wild-type cells but a greater relative increase in TGF-β1–stimulated collagen IV expression in knockout cells [27].

Whereas knockout cells react exuberantly to exogenous TGF-β1, they respond to high glucose with a tiny but statistically significant increase in fibronectin mRNA (Fig. 9A). However, the biologic impact of this finding is questionable since high glucose does not stimulate fibronectin protein production significantly in knockout cells (Fig. 9B). In contrast, the wild-type cells respond to high glucose with a significant increment in both fibronectin message and protein (Fig. 9). This reinforces the concept that high glucose induces matrix expression

by a TGF-β–dependent mechanism and perhaps more specifically by a TGF-β1–dependent mechanism. The effect of high glucose to stimulate fibronectin does not appear to be mediated much by either TGF-β2 or TGF-β3, because knockout cells possess both of these isoforms but do not show any change in fibronectin protein when treated with high glucose (Fig. 9B). This supports our previous findings in cultured kidney cells that high glucose increases matrix by stimulating the production of TGF-β1 that then acts in autocrine fashion to induce matrix [6, 9, 11, 20, 49]. A minor component of the profibrotic effect of high glucose in wild-type cells may be related to hyperosmolarity, since exposure to an equivalent concentration of mannitol produced a small but significant increase in fibronectin mRNA (Fig. 9A). Mannitol treatment also produced a tiny rise in fibronectin protein, but this did not reach statistical significance (Fig. 9B). A genuine effect of mannitol cannot be ruled out, however, as others have reported mannitol increasing fibronectin message and protein in mesangial cells, perhaps attributable to transcriptional activation at the activated protein-1 (AP-1) site of the fibronectin promoter [50]. Regardless of the exact contribution of an osmotic nature, high glucose requires the participation of the TGF-β system, and in particular TGF-β1, to mediate its fibrogenic impact in diabetic nephropathy.

CONCLUSION

Cultured tubuloepithelial cells from the TGF-β1 knockout mouse provide an interesting in vitro tool to study the specific role of TGF-β1 in renal diseases such as diabetic nephropathy. The knockout cells appear to have an otherwise intact TGF-β system in that they express TGF-β2, TGF-β3, and the TGF-β type I and type II receptors. However, the mere absence of TGF-β1 in knockout cells confers an increased proliferative capacity, a resistance to the hypertrophic effects of high glucose, and a muted sclerotic response to high glucose. Therefore, TGF-β1 appears to be the most prominent isoform in the pathogenesis of tubular hypertrophy and tubulointerstitial fibrosis, which are characteristic of and maladaptive in diabetic nephropathy.

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Requests for reprints to Fuad N. Ziyadeh, M.D., 700 Clinical Research Building, 415 Curie Boulevard, Philadelphia, PA 19104. E-mail: ziyadeh@mail.med.upenn.edu

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