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Sp1 and Smad3 are Required for High Glucose-Induced $p21^{WAF1}$ Gene Transcription in LLC-PK_1 Cells

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Abstract The cyclin-dependent kinase inhibitor $p21^{WAF1}$ is required for diabetic glomerular hypertrophy. High glucose-induced hypertrophy in proximal tubule cells is dependent on transforming growth factor-β (TGF-β). Many of the TGF-β-induced effects are dependent on Smad2/3. Thus, the molecular mechanisms of high glucose-induced $p21^{WAF1}$ and hypertrophy were studied in high glucose-cultured proximal tubule-like LLC-PK_1 cells. We found that high glucose (30 mM) induced hypertrophy at 72 h. High glucose also increased the expression of $p21^{WAF1}$ protein and $p21^{WAF1}$ mRNA transcription and abundance at 48 h. The DNA element in the 5′ regulatory region of $p21^{WAF1}$ gene essential for high glucose-induced $p21^{WAF1}$ gene transcription was identified as Sp1 by a series of the 5′ regulatory region of $p21^{WAF1}$ gene deletion mutants. Moreover, high glucose activated Smad2/3 while increasing the Sp1 DNA-binding activity. High glucose also increased the Sp1-dependent transcriptional activity of $p21^{WAF1}$ gene. High glucose-induced hypertrophy was attenuated by $p21^{WAF1}$ short interfering RNA and Smad3 dominant-negative plasmid transfection. We concluded that high glucose induced hypertrophy via Sp1-Smad2/3-dependent activation of $p21^{WAF1}$ gene transcription in LLC-PK_1 cells. J. Cell. Biochem. 102: 1190–1201, 2007.

Key words: diabetic nephropathy; hypertrophy; $p21^{WAF1}$; Sp1; Smad3

Tubulointerstitial disease is as important as glomerulopathy in diabetic nephropathy [Gilbert and Cooper, 1999; Chuang and Guh, 2001; Thomas et al., 2005]. A partial list of the pathogenesis of diabetic nephropathy includes: renal hypertrophy, hyperglycemia, transforming growth factor-β (TGF-β), derangements in signal transducers and cell cycles, etc. [Wolf, 2000; Chuang and Guh, 2001; Schrijvers et al., 2004; Wolf, 2004]. Thus, we and others have been using high glucose-cultured renal cells and experimental diabetic nephropathy to study the molecular mechanisms of diabetic nephropathy [Chuang and Guh, 2001].

Renal hypertrophy consists of glomerular and tubular hypertrophy [Wolf and Ziyadeh, 1999]. For example, high glucose induces hypertrophy in proximal tubule cells [Park et al., 2001; Chen et al., 2004; Fujita et al., 2004]. Thus, we have shown that high glucose induces hypertrophy concomitantly with increasing TGF-β and TGF-β receptors in the proximal tubule-like porcine LLC-PK_1 cells [Guh et al., 1996]. Moreover, high glucose-induced hypertrophy in proximal tubule cells is dependent on TGF-β [Park et al., 2001; Chen et al., 2004].

Many of the TGF-β-induced effects are dependent on Smads [Chuang and Guh, 2001], the signal transducers and transcription factors downstream of the TGF-β superfamily [Chuang and Guh, 2001]. Smads 2 and 3 are TGF-β receptor-associated Smads while Smads 6 and 7 are inhibitory Smads [Chuang and Guh, 2001]. Smads 2 and 3 are increased in experimental diabetic nephropathy [Huang and Preisig, 2000; Hong et al., 2001; Isono et al., 2002; Furuse et al., 2004; Kim et al., 2004]. Additionally, we have shown that β-hydroxybutyrate-inhibited

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mitogenesis in HK-2 (human proximal tubule) cells is dependent on Smad3 [Guh et al., 2003].

We have also shown that high glucose-induced hypertrophy in LLC-PK$_1$ cells is cell cycle-dependent with cells arresting in the G_0/G_1 phase of the cell cycle [Yang et al., 1997]. In this regard, diabetic renal hypertrophy is associated with an increase of the cyclin-dependent kinase inhibitors: p21^{WAF1} and p27^{kip1} [Kuan et al., 1998; Shankland and Wolf, 2000; Wolf, 2000]. The role of p21^{WAF1} in diabetic renal hypertrophy has been shown in glomerular and mesangial cell hypertrophy. Thus, glomerular hypertrophy is absent in p21^{WAF1} knockout mice [Al-Douahji et al., 1999]. Moreover, p21^{WAF1} antisense oligodeoxynucleotide attenuates high glucose-induced mesangial cell hypertrophy [Fan and Weiss, 2004]. However, the role of p21^{WAF1} in diabetic tubular hypertrophy and the molecular mechanisms of the regulation of p21^{WAF1} in diabetic nephropathy remain unexplored.

Therefore, we studied the effects of high glucose on p21^{WAF1} protein and mRNA expression in LLC-PK$_1$ cells in terms of cell hypertrophy. To elucidate the molecular mechanisms of the regulation of p21^{WAF1}, we further assessed the role of Sp1 and Smad2/3 in high glucose-induced p21^{WAF1} gene transcription.

METHODS

Reagents

Fetal bovine serum (FBS), DMEM, antibiotics, trypsin-EDTA, trypan blue stain, and all medium additives were obtained from Life Technologies Inc. (Glasgow, UK). p21^{WAF1} and β-actin antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). α-tubulin antibody was purchased from Lab Vision Corporation (Fremont, CA). Smad2/3 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Sp1 antibody was purchased from Bethyl Laboratories (Montgomery, TX). Horseradish peroxidase-conjugated donkey anti-goat or goat anti-mouse and anti-rabbit secondary antibodies were also obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Phospho-Smad2/3 antibody was obtained from BioSource International Inc. (Camarillo, CA). ^{3}H-leucine, γ-^{32}P-ATP, and the enhanced chemiluminescence kit were obtained from PerkinElmer Life Sciences Inc. (Norwalk, CT). Streptozotocin, *N*,*N*-methylene-bisacrylamide, acrylamide, SDS, ammonium persulfate, Temed, Tween 20, DMSO, and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Plasmids

The full-length 5′ regulatory region of p21^{WAF1} (p21P) and the deletion mutants (p21P1.1, p21psma, p21psma△1, p21p93-S, p21P93-S mut#2 and p21P93-S mut#3) were gifts of Dr. Xiao-Fan Wang [Datto et al., 1995b]. The pGL2 luciferase reporter plasmid was purchased from Promega Corp. (Madison, WI). The dominant-negative Smad3 plasmid (Smad3△C) was a gift of Dr. R. Derynck [Zhang et al., 1997].

Cell Culture

Like our previous study [Guh et al., 1996]. LLC-PK$_1$ cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in culture flasks and maintained in DMEM (5.5 mM glucose) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FBS in a humidified 5% CO_2 incubator at 37°C. Cell viability was assessed by the trypan blue exclusion test and was routinely >92%.

Imunoblotting

This was performed as described in our previous studies [Huang et al., 2005]. Briefly, a 30 μg sample of cell lysate was subjected to electrophoresis on 12% SDS-polyacrylamide gels. The samples were then electroblotted on polyvinylidene difluoride membranes. After blocking, blots were incubated with primary antibody in blocking solution for 1 h followed by two 5-min washes in PBS within 0.1% Tween 20. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min. Enhanced chemiluminescence reagents were employed to depict the protein bands on membranes. The membranes were washed and reprobed with β-actin or α-tubulin antibody to control for protein loading. Relative densities were shown as the ratio between the specific proteins and β-actin (or α-tubulin).

Measurement of Cell Protein and DNA

This was performed as described in a previous study [Fujita et al., 2004]. Briefly, cells were trypsinized and pelleted, resuspended in 1 ml

buffer (50 mM Na_2PO_4, pH 7.4), and cells were lysed on ice by repeated passage through a 27-gauge needle. The lysate was aliquoted for protein (Bio-Rad protein assay kit, Bio-Rad, Hercules, CA) and DNA determination. DNA was measured using the fluorescent compound bisbenzimide H-33258 fluorochrome.

^{3}H-Leucine Incorporation Assay

Protein/DNA ratio and ^{3}H-leucine incorporation (de novo protein synthesis) were used to measure cellular hypertrophy [Fujita et al., 2004]. Briefly, cells were subcultured in a 24-well dish at a density of 1×10^4/ml. After 24 h, cells were incubated with normal (5.5 mM) or high glucose (30 mM) media with daily exchange. After 48 h, cells were pulsed with 1 μCi ^{3}H-leucine. After further incubation for 24 h, cells were washed with ice-cold PBS and 5% trichloroacetic acid, solubilized in 0.5 N NaOH, and counted by a liquid scintillation counter.

Short Interfering RNA (siRNA)

To create $p21^{WAF1}$ si-RNA-pSUPER expression vectors, the following oligonucleotides (MDBio. Inc., Taipe, Taiwan) were designed by the Dharmacon siDESIGN Center (http://www.dharmacon.com/sidesign/default.aspx) and annealed, ligated into pSUPER (OligoEngine Inc., Seattle, WA) between the BglII and HindIII sites. siRNA was designed according to the underlined pig $p21^{WAF1}$ sequence [Uenishi et al., 2007]: 5′-gatcccc<u>CCAGCATGACAGATTTCTA</u>ttcaagagaTAGAAATCTGTCATGCTGGttttta-3′ and 5′-agcttaaaaa CCAGCATGACAGATTTCTAtctcttgaaTAGAAATCTGTCATGCTGG ggg-3′. Scrambled $p21^{WAF1}$ siRNA, 5′-gatcccc AGACGTTCTATACCCGAAT ttcaagaga ATTCGGGTATAGAACGTCT-3′ and 5′-agcttaaaaa AGACGT-TCTATACCCGAAT tctcttgaaATTCGGGTATAGAACGTCT ggg-3′. All plasmids were sequenced to confirm that they were correct and transiently transfected to LLC-PK1 cells using LipofectAMINE (Invitrogen, Carlsbad, CA).

Real Time RT- PCR

Two micrograms of total RNA extracted by using Trizol method was used to synthesize cDNA with the reverse transcription system (Promega Corp., Madison WI). $p21^{WAF1}$ gene expression were analyzed by real-time quantitative RT-PCR in triplicate using the SYBR system based on the real-time detection of accumulated fluorescence (ABI Prism 7900; Perkin-Elmer, Foster City, CA) as in our previous study [Guh et al., 2003]. Briefly, 18S ribosomal RNA was used as an internal control. Primers were constructed with the use of Primer Express (Perkin-Elmer, Foster City, CA). For $p21^{WAF1,}$ the forward primer was 5′-CAGGACTGCGATGCACTGAT-3′ and the reverse primer was 5′-ACACGTTCCCAGGCGAA GT-3′. For 18S, the forward primer was: 5′-CGAGCCGCCTGGATACC-3′ and the reverse primer was 5′-CAGTTCCGAAAACCAACAAAATAGA-3′. Amplification was performed with the following time course: 95°C for 10 min and 40 cycles of 94°C for 20 s and 60°C for 1 min. Results are expressed relative to values in the control group, which were arbitrarily assigned a value of 1.

Luciferase Activity Assay

LLC-PK_1 cells were plated into 6-well plates at a density of 5×10^4 cells/well in DMEM and grown for 1 day. Cells were transfected with 1 μg of plasmid construct using a standard lipofectAMINE transfection protocol. Cells were also transfected with 0.5 μg of CMV-SPORT-β-galactosidase (Gibco-BRL, Rockville, MD, USA) to control for transfection efficiency [Guh et al., 2003]. Five hours after transfection, cells were treated with either normal or high glucose for the indicated times. Cells were lysed and luciferase activity was assayed by integrating the total light emission over 10 s by using the Dynatech ML1000 luminometer. Luciferase activities were normalized to β-galactosidase activity [Guh et al., 2003] performed in triplicate.

Preparation of Nuclear Extracts

Nuclear extracts were prepared as in our previous study [Huang et al., 1999]. Briefly, cells were harvested and vortexed. Nuclei were pelleted and the supernatants containing cytosolic proteins were collected. The pellet containing nuclei was suspended, collected by centrifugation and stored at −70°C. Protein concentration was determined using a colorimetric assay by a Bio-Rad assay kit (Bio-Rad, Hercules, CA).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay was performed as in our previous study [Huang

et al., 1999]. Briefly, complementary oligonucleotides corresponding to bases −71 through −86 in the 5′ regulatory region of wild type p21^{WAF1} were synthesized (GGTCCCGCCTCC-TTGA and TCAAGGAGGCGGGACC) as the Sp1 binding element probe [Milutinovic et al., 2004]. The single-stranded oligonucleotides were annealed as temperature descended from 95°C to room temperature. ^{32}P-labeling of Sp1 probe was carried out using T4-polynucleotide kinase (New England BioLabs, Beverly, MA) and γ-^{32}P-ATP (3000 Ci/mmol). Labeled DNA was separated from the unincorporated radioactivity. Binding reactions were carried out by adding 10 μg of nuclear protein to 20 μl of binding buffer and γ-^{32}P-ATP labeled Sp1 probes. Where indicated, cold competitive oligonucleotides were included during the preincubation periods. One μg of Sp1 antisera (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with the nuclear extract for 30 min at room temperature before the binding reaction. Samples were incubated at room temperature for 25 min and fractionated by electrophoresis. After electrophoresis, gels were transferred to filter paper, dried, and exposed to X-ray on the Hyperfilm-MP (Amersham Pharmacia Biotech) using an intensifying screen. The results were quantified by a scanning densitometer.

Detection of Sp1 in the nucleus was quantized using the NoShift transcription factor assay kit (EMD Biosciences Inc., San Diego, CA) according to the manufacturer's instructions. To assess sequence-specific binding activity, 50 μg of nuclear extract were incubated with various combinations of biotinylated Sp1 wild-type dsDNA, specific Sp1 competitor dsDNA lacking biotin end labels, and nonspecific, nonbiontinylated dsDNA with a mutant Sp1 consensus binding motif (GGTCCCGGATCCT-TGA and TCAAGGATCCGGGACC). The blank contained all assay components except the nuclear extract. All assays were performed in triplicate. Binding activity was measured via colorimetric absorbance at 450 nm using TMB (3,3′,5,5′-tetramethylbenzidine) as substrate.

Statistical Analyses

The results were expressed as the mean ± standard errors of the mean (S.E.M.). Unpaired Student's *t*-tests were used for the comparison between two groups. One-way ANOVA followed by unpaired *t*-test was used for the comparison among more than three groups. $P < 0.05$ was considered as statistically significant.

RESULTS

Effects of Glucose on p21^{WAF1} Expression and Gene Transcription in LLC-PK$_1$ Cells

The effect of glucose on p21^{WAF1} protein expression was studied by immunoblotting. Thus, high glucose (30 mM) increased p21^{WAF1} protein expression at 48 h (Fig. 1A). The effect of high glucose on p21^{WAF1} mRNA expression and transcriptional activity was studied by real-time RT-PCR and luciferase activity. Thus, high glucose (30 mM) time-dependently (48–72 h) increased p21^{WAF1} mRNA expression (Fig. 1B). Moreover, glucose dose (30–40 mM) (Fig. 2A) and time (48 h) (Fig. 2B)-dependently increased p21^{WAF1} gene transcriptional activity.

Role of p21^{WAF1} in High Glucose-Induced Hypertrophy in LLC-PK$_1$ Cells

We have previously shown that high glucose induces hypertrophy in LLC-PK$_1$ cells [Guh et al., 1996]. To study the role of p21^{WAF1} in this effect, p21^{WAF1} siRNA was used to knock-down endogenous p21^{WAF1}. Thus, p21^{WAF1} siRNA, but not scrambled siRNA, attenuated high glucose (30 mM)-induced p21^{WAF1} protein expression at 48 h (Fig. 3A) and hypertrophy at 72 h (Fig. 3B and C).

Sp1 Binding Elements are Required for the Induction of p21^{WAF1} Gene Transcription in LLC-PK$_1$ Cells

Transient transfection of LLC-PK$_1$ cells with the 5′ regulatory region of p21^{WAF1} gene constructs was used to identify the elements required for p21^{WAF1} mRNA transcription. The 5′ regulatory region of p21^{WAF1} contains 6 Sp1 binding elements: Sp1-1 (−119 to −114), Sp1-2 (−109 to −104), Sp1-3 (−82 to −77), Sp1-4 (−69 to −64), Sp1-5 (−60 to −55) and Sp1-6 (−55 to −50) [Koutsodontis et al., 2002]. As shown in Figure 4A, p21P is a full-length 2.4-kilobase pair of the 5′ regulatory region of p21^{WAF1} containing the p21^{WAF1} cDNA start site at its 3′ end [Datto et al., 1995b]. p21P△1.1 is a deletion mutant of approximately 1.1-kilobase from the 5′ end of the 5′ regulatory region of p21^{WAF1} gene by removing the two consensus p53-responsive elements [Gartel and Radhakrishnan, 2005]. p21PSma contains the

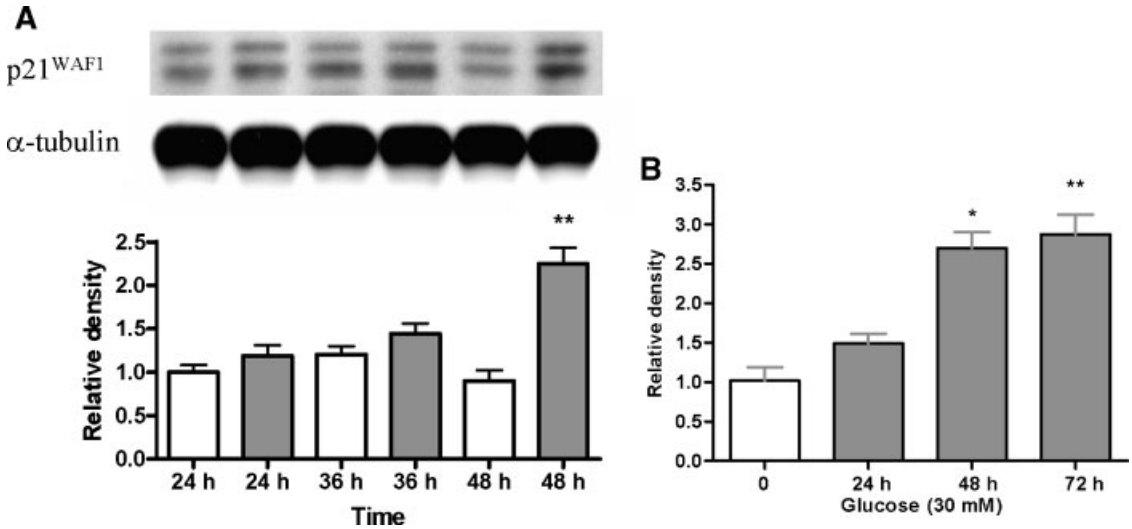

Fig. 1. Effects of glucose on $p21^{WAF1}$ protein and mRNA expression in LLC-PK_1 cells. Cells were treated with either 5.5 mM glucose (empty bars) or 30 mM glucose (gray bars). $p21^{WAF1}$ protein normalized to β-actin was measured by immunoblotting while $p21^{WAF1}$ mRNA was measured by real-time RT-PCR as described in Methods. (**A**) High glucose (30 mM) increased $p21^{WAF1}$ protein expression at 48 h. (**B**) High glucose time-dependently (48–72 h) increased $p21^{WAF1}$ mRNA expression. Data were expressed as the mean±S.E.M. of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ vs. 5.5 mM glucose.

5′ regulatory region of $p21^{WAF1}$ gene sequences from base −111 through the transcriptional initiation site. p21Psma△1 lacks the Sp1-1, Sp1-2, Sp1-3 and Sp1-4 elements and contains 61 base pairs proximal to the transcriptional initiation site with only Sp1-5 and Sp1-6.

After high glucose (30 mM) treatment for 48 h, there was a 3.1-, 2.9-, 2.6- and 1.0-fold induction of $p21^{WAF1}$ gene transcriptional activity for p21P, p21P△1.1, p21PSma and p21PSma△1, respectively (Fig. 4B). Thus, Sp1-3 and Sp1-4 elements are required for high glucose-induced $p21^{WAF1}$ gene transcriptional activity.

To delineate the relative importance of Sp1-3 and Sp1-4 elements, p93-S mut#2 (mutated at

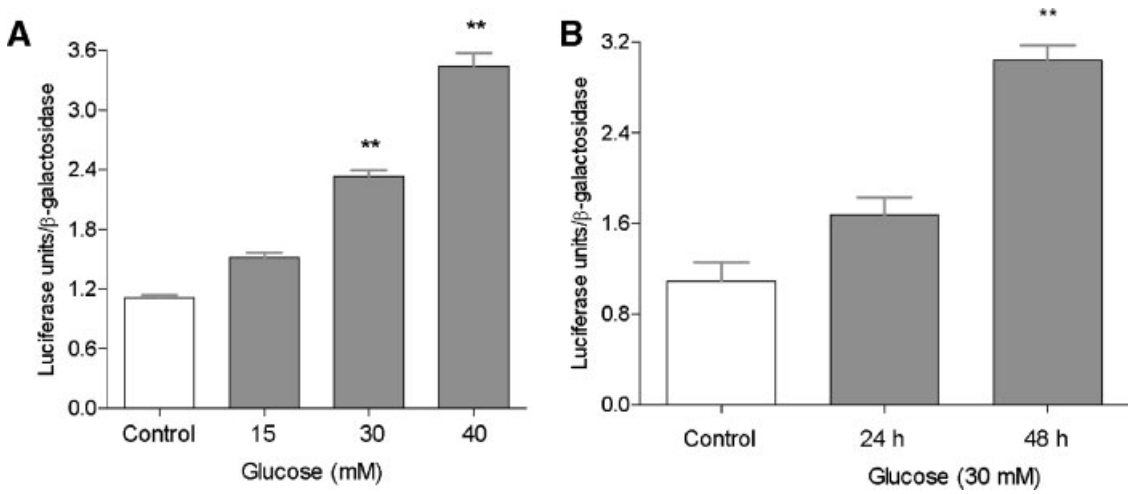

Fig. 2. Dose- and time-dependent effects of glucose on $p21^{WAF1}$ gene transcriptional activity in LLC-PK_1 cells. Cells were treated with either 5.5 mM glucose (empty bars) or high glucose (15–40 mM glucose, gray bars). $p21^{WAF1}$ gene transcriptional activity was studied by luciferase activity assay using the full-length $p21^{WAF1}$ 5′ regulatory region (p21P). Gene transcriptional activity was expressed as luciferase units normalized to β-galactosidase activity. (**A**) Glucose dose-dependently (30–40 mM) increased $p21^{WAF1}$ gene transcriptional activity at 48 h. (**B**) High glucose (30 mM) time-dependently (48 h) increased $p21^{WAF1}$ gene transcriptional activity compared to 5.5 mM glucose at 48 h. Data were expressed as the mean ± S.E.M. of three independent experiments. $^{**}P < 0.01$ vs. 5.5 mM glucose.

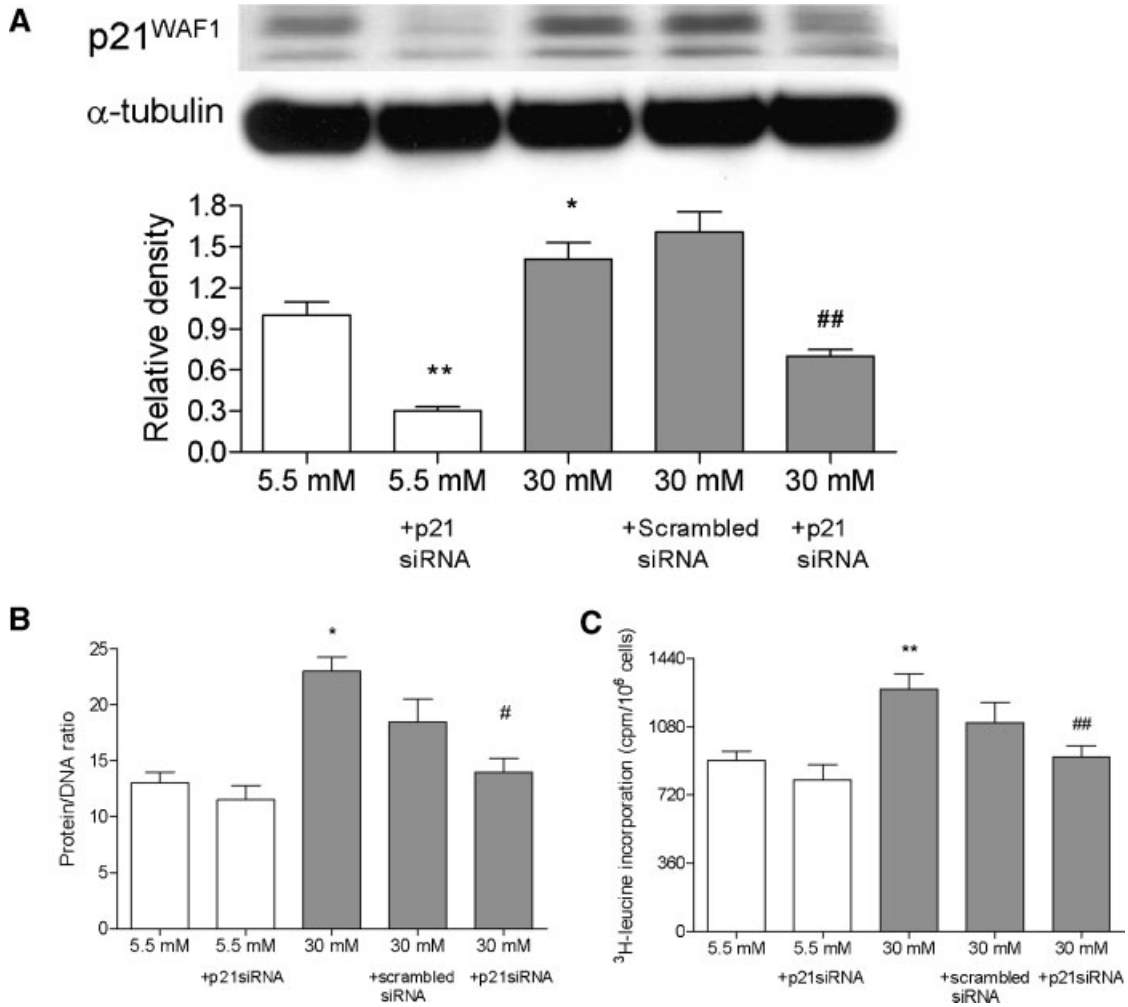

Fig. 3. Role of p21^{WAF1} in high glucose-induced hypertrophy in LLC-PK$_1$ cells. Cells were treated with either 5.5 mM glucose (empty bars) or 30 mM glucose (gray bars) for 72 h. p21^{WAF1} protein normalized to α-tubulin was measured by immunoblotting. Cell hypertrophy was measured by protein/DNA ratio and ^{3}H-leucine incorporation as described in Methods. The role of p21^{WAF1} was assessed by transient transfection of p21^{WAF1} small interfering RNA (p21siRNA). (**A**) p21siRNA, but not scrambled siRNA, attenuated high glucose (30 mM)-induced p21^{WAF1} protein expression. (**B**) and (**C**) p21siRNA, but not scrambled siRNA, attenuated high glucose-induced cell hypertrophy. Data were expressed as the mean±S.E.M. of three independent experiments. $^*P<0.05$, $^{**}P<0.01$ vs. 5.5 mM glucose. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. 30 mM glucose.

the Sp1-3 element) and p93-S mut#3 (mutated at the Sp1-4 element) plasmids were used (Fig. 5A). Thus, there was a 1.54- and 3.67-fold induction of p21^{WAF1} gene transcriptional activity for p93-S mut#2 and p93-S mut#3 by high glucose, respectively (Fig. 5B). Thus, the Sp1-3 (but not Sp1-4) element is required for high glucose-induced p21^{WAF1} gene transcriptional activity.

High Glucose Increased Sp1 and Smad2/3 DNA-Binding Activities in LLC-PK$_1$ Cells

Electrophretic mobility shift assay was used to study the DNA-binding activities of Sp1 and Smad2/3 in LLC-PK$_1$ cells. Thus, high glucose (30 mM) time-dependently (4–8 h) increased Sp1 DNA-binding activity (Fig. 6A). Moreover, high glucose (30 mM) time-dependently (24–72 h) increased Smad2/3 phosphorylation (Fig. 6B). The specificity of Sp1 DNA-binding activity is shown in Figure 6C. Thus, Sp1 antibody (but not control rabbit IgG) detected high glucose-induced Sp1 binding activity. Moreover, Sp1 binding was blocked by 25- and 50-fold excess of the cold (but not mutant) probe.

Role of Smad3 in High Glucose-Induced p21^{WAF1} Expression and Hypertrophy in LLC-PK$_1$ Cells

The role of high glucose-activated Smad3 can be studied by specific inhibitors of Smad3. For

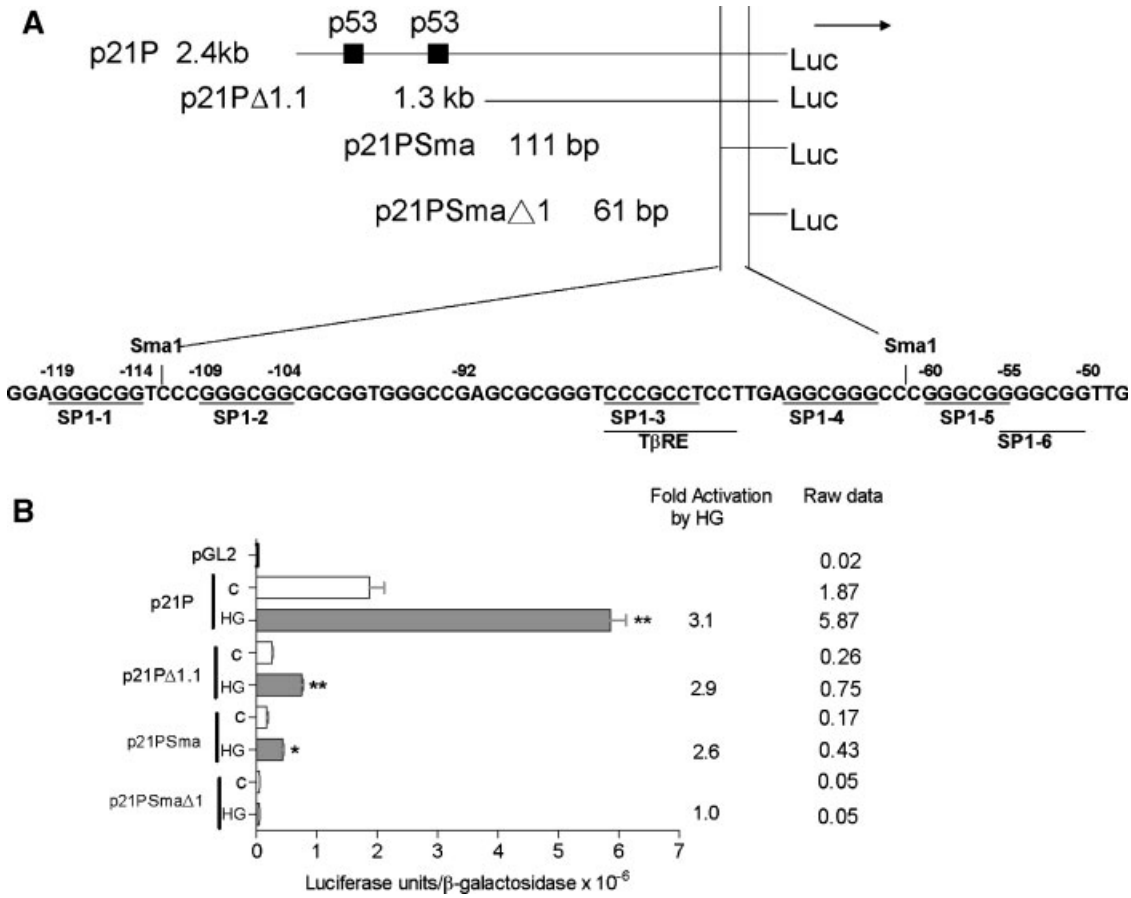

Fig. 4. Identification of DNA elements essential for high glucose-induced p21^{WAF1} gene transcriptional activity in LLC-PK$_1$ cells. Cells were treated with either 5.5 mM glucose (C, empty bars) or 30 mM glucose (HG, gray bars) for 48 h. p21^{WAF1} gene transcriptional activity was studied by luciferase activity assay as described in Methods. Gene transcriptional activity was expressed as luciferase units normalized to β-galactosidase activity. (**A**) A series of 5′-deletion mutants of the 5′ regulatory region of p21^{WAF1} gene constructs. p21P is the full-length 5′ regulatory region of p21^{WAF1} gene. p21P△1.1 is the deletion mutant lacking the p53 binding element. P21PSma is a 111 bp construct containing the Sp1-3 and Sp1-4 binding elements. P21PSma△1 lacks the Sp1-3 and Sp1-4 binding elements. (**B**) High glucose (30 mM)-induced p21^{WAF1} gene transcriptional activity was increased by 3.1-, 2.9-, 2.6- and 1.0-fold for p21P, p21P△1.1, p21PSma and P21PSma△1, respectively. Data were expressed as the mean±S.E.M. of three independent experiments. *$P < 0.05$, **$P < 0.01$ vs. 5.5 mM glucose.

example, we have shown that Smad3 dominant-negative plasmid attenuated β-hydroxybutyrate-inhibited mitogenesis in proximal tubular (HK-2) cells [Guh et al., 2003]. In this study, Smad3 dominant negative plasmid attenuated high glucose-induced p21^{WAF1} protein expression (Fig. 7A) and p21^{WAF1} gene transcriptional activity at 48 h (Fig. 7B). Moreover, Smad3 dominant-negative plasmids attenuated high glucose (30 mM)-induced hypertrophy at 72 h (Fig. 7C).

DISCUSSION

This is the first demonstration that high glucose-induced hypertrophy is dependent on the Sp1-Smad2/3-dependent transcriptional activation of p21^{WAF1} in proximal tubular cells. The elucidation of the molecular mechanism of p21^{WAF1} induction in models of diabetic nephropathy is important owing to the critical role of p21^{WAF1} in the regulation of cell growth [Weinberg and Denning, 2002] and the pathogenesis of diabetic nephropathy [Al-Douahji et al., 1999; Shankland and Wolf, 2000; Wolf, 2000].

In this study, we found that high glucose increased p21^{WAF1} protein and p21^{WAF1} mRNA expression in LLC-PK$_1$ cells. Moreover, high glucose-induced hypertrophy was attenuated by p21^{WAF1} siRNA, a versatile method for gene

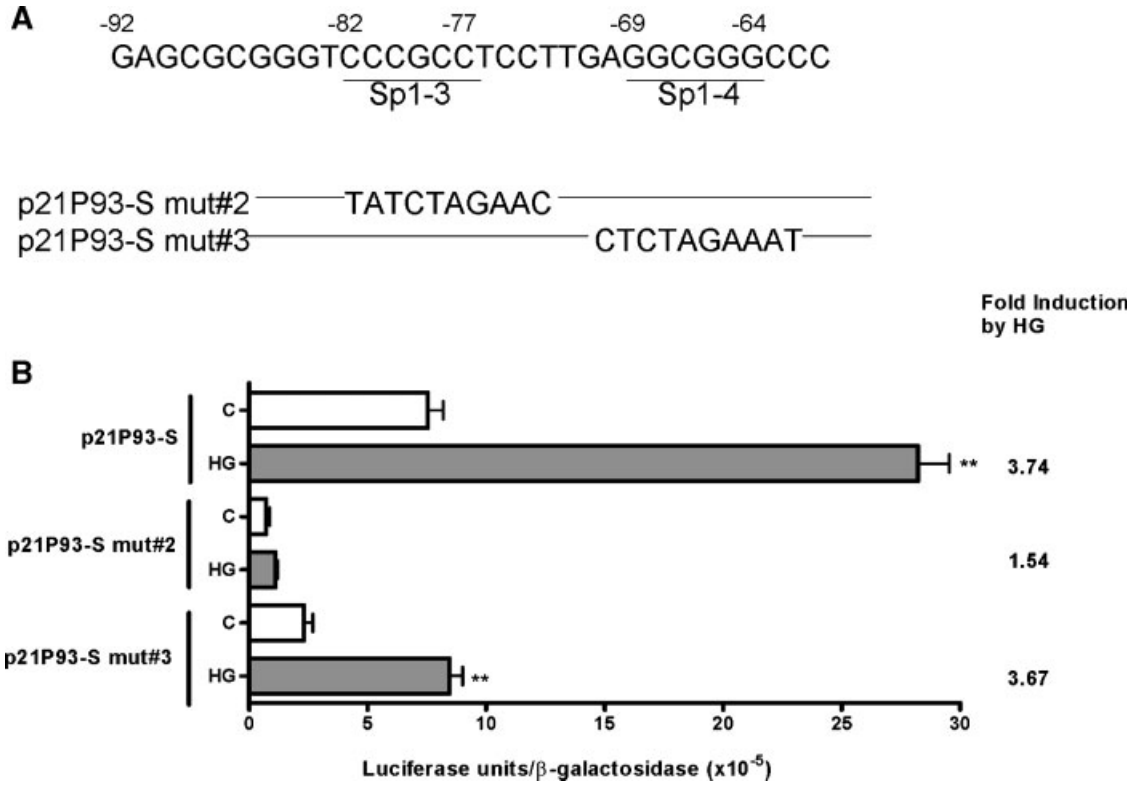

Fig. 5. Identification of DNA elements essential for high glucose-induced p21^{WAF1} gene transcriptional activity in LLC-PK$_1$ cells. Cells were treated with either 5.5 mM glucose (C, empty bars) or 30 mM glucose (HG, gray bars) for 48 h. p21^{WAF1} gene transcriptional activity was studied by luciferase activity assay as described in Methods. Gene transcriptional activity was expressed as luciferase units normalized to β-galactosidase activity. (**A**) p21P93-S mut#2 is the deletion mutant mutated at the Sp1-3 binding element. p21P93-S mut#3 is the deletion mutant mutated at the Sp1-4 binding element. (**B**) High glucose (30 mM)-induced p21^{WAF1} gene transcriptional activity was increased by 1.54- and 3.67-fold for p21P93-S mut#2 and p21P93-S mut#3, respectively. Data were expressed as the mean±S.E.M. of three independent experiments. $^{**}P < 0.01$ vs. 5.5 mM glucose.

knockdown [Sledz and Williams, 2005]. This observation corroborates a previous study showing that p21^{WAF1} overexpression causes hypertrophy in LLC-PK$_1$ cells [Terada et al., 1999]. Interestingly, the lack of p21^{WAF1} gene has been shown to ameliorate progression to chronic renal failure [Megyesi et al., 1999].

The expression of p21^{WAF1} is transcriptionally regulated [Wolf and Wenzel, 2004]. For example, we have shown that tamoxifen induces p21^{WAF1} gene transcription via Sp1-binding sites in lung cancer cells [Lee et al., 2000]. Moreover, TGF-β-induced p21^{WAF1} transcriptional activity requires both Sp1 and Smad3 [Pardali et al., 2000].

We also found that high glucose increased the transcriptional activity of p21^{WAF1}. Thus, a series of deletion mutants of the 5′ regulatory region of p21^{WAF1} gene constructs were used to further delineate the molecular mechanism of high glucose-induced p21^{WAF1} gene transcription. We found that the fold-induction of p21^{WAF1} gene transcription by high glucose decreased from 3.1 to 2.6–2.9 for p21PΔ1.1 and p21PSma relative to p21P (the full-length 5′ regulatory region p21^{WAF1} gene construct). Thus, p53 is only minimally required for high glucose-induced p21^{WAF1} gene transcription. There are several other instances of p53-independent p21^{WAF1} gene transcription, including TGF-β [Datto et al., 1995a; Gartel and Tyner, 1999].

In contrast, the induction of p21^{WAF1} gene transcription by high glucose was completely lost for p21PSmaΔ1. Thus, high glucose-induced p21^{WAF1} gene transcription was not mediated by the elements between −61 and 0 bp in the 5′ regulatory region of p21^{WAF1} gene.

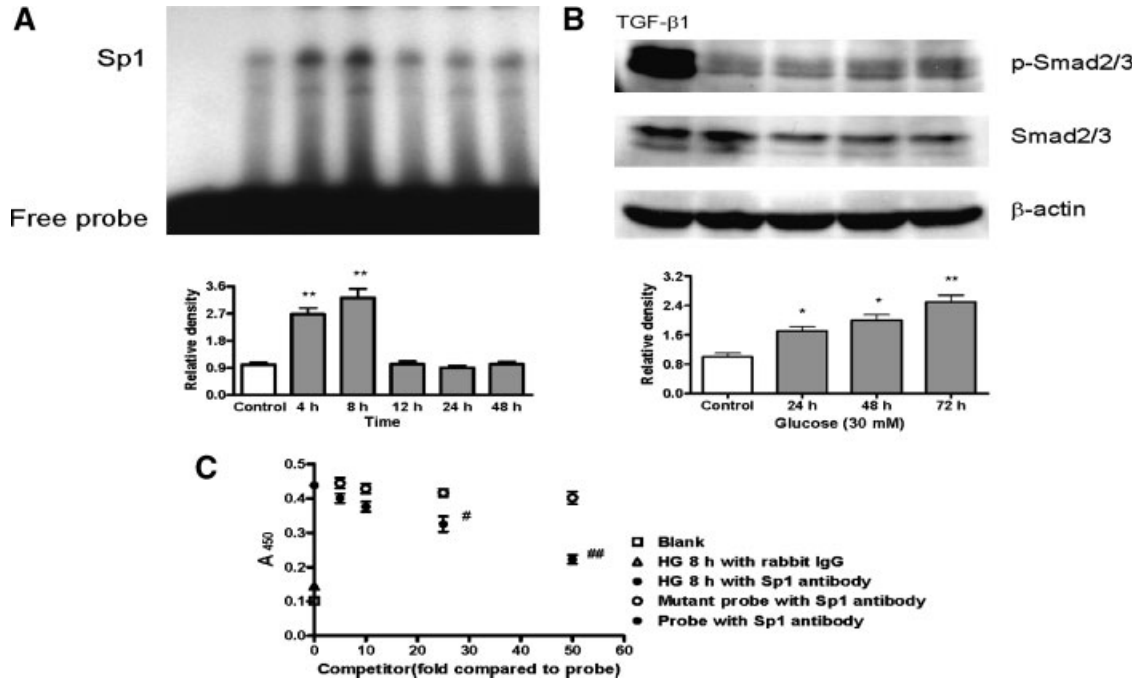

Fig. 6. Effects of high glucose on Sp1 DNA-binding activity and Smad2/3 activity. Cells were treated with either 5.5 mM glucose (empty bars) or 30 mM glucose (gray bars). Sp1 DNA-binding activity was assessed by electrophoretic mobility shift assay using the probe containing the Sp1-3 element. Smad2/3 activity was assessed by immunoblotting phospho-Smad2/3 (p-Smad2/3) and normalized to Smad2/3. The specificity of Sp1 DNA-binding activity was assessed by NoShift transcription factor assay kit as described in Methods. (**A**) High glucose (30 mM) time-dependently (4–8 h) increased Sp1 DNA-binding activity. This is representative of three independent experiments. (**B**) High glucose time-dependently (24–72 h) increased Smad2/3 activity. (**C**) Sp1 antibody (but not control rabbit IgG) detected high glucose-induced Sp1 binding activity. Moreover, Sp1 binding was blocked by 25- and 50-fold excess of the cold (but not mutant) probe. Data were expressed as the mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$ versus 5.5 mM glucose, $^{**}P < 0.01$ versus 5.5 mM glucose. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. HG (high glucose) 8 h with Sp1 antibody.

Additionally, the induction of $p21^{WAF1}$ gene transcription by high glucose was lost in p21P93-S mut#2 (containing the mutated Sp1-3), but not in p21P93-S mut#3 (containing the mutated Sp1-4). Thus, high glucose induces $p21^{WAF1}$ gene transcription via the Sp1-3 response element in the 5′ regulatory region of $p21^{WAF1}$ gene. Interestingly, gene transcriptional activity of p21Psma△1 was only barely above that of the empty vector, indicating that Sp1 is also required for the basal transcription of $p21^{WAF1}$ gene [Datto et al., 1995b].

Indeed, we found that high glucose increased Sp1 DNA-binding activity in LLC-PK_1 cells. Similarly, a previous study found that high glucose increases Sp1 DNA-binding activity in mesangial cells [Chae et al., 2004]. In view of our previous finding that high glucose increases TGF-β and TGF-β receptor expression [Guh et al., 1996] and the fact that Smad2/3 is a downstream target of TGF-β [Eickelberg et al., 2002] in LLC-PK_1 cells, it would be worthwhile to know if high glucose-induced $p21^{WAF1}$ transcription is also mediated by Smad2/3.

Accordingly, we found that high glucose activated Smad2/3. Moreover, Smad3 dominant-negative plasmids attenuated high glucose-induced hypertrophy while attenuating high glucose-induced $p21^{WAF1}$ protein expression and transcriptional activity. These findings corroborate our previous study showing that Smad3 dominant-negative plasmid attenuated β-hydroxybutyrate-induced expression of $p21^{WAF1}$ in HK-2 cells [Guh et al., 2003]. Note that the 5′ regulatory region of $p21^{WAF1}$ gene contains no Smad-binding element [Pardali et al., 2000; Gartel and Radhakrishnan, 2005]. However, Sp1 physically interacts with Smad2/3 [Pardali et al., 2000]. Thus, we found that high glucose increased protein–protein interaction between Sp1 and Smad2/3 in a recent study [Chuang et al., 2007].

The role of Smad2/3 in diabetic nephropathy has been studied by several previous studies.

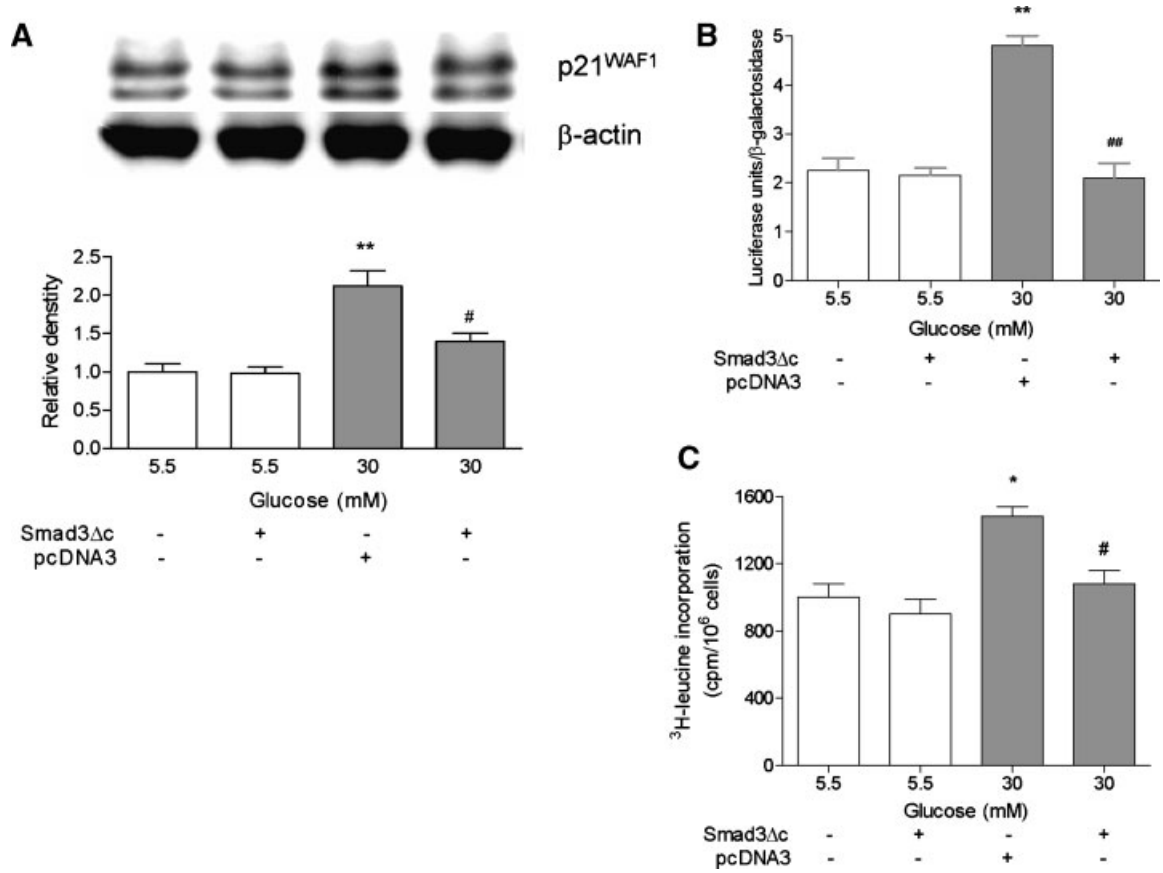

Fig. 7. Role of Smad3 in high glucose-induced p21^{WAF1} protein expression, p21^{WAF1} gene transcriptional activity and hypertrophy in LLC-PK$_1$ cells. Cells were treated with either 5.5 mM glucose (empty bars) or 30 mM glucose (gray bars). p21^{WAF1} protein normalized to β-actin was assessed by immunoblotting, p21^{WAF1} gene transcriptional activity was studied by luciferase activity assay while hypertrophy was assessed by ^{3}H-leucine incorporation as described in Methods. Gene transcriptional activity was expressed as luciferase units normalized to β-galactosidase activity. (**A**) Dominant-negative Smad3 (Smad3Δc) plasmid attenuated high glucose (30 mM)-induced p21^{WAF1} protein expression at 48 h. (**B**) Dominant-negative Smad3 (Smad3Δc) plasmid attenuated high glucose-induced p21^{WAF1} gene transcriptional activity at 48 h. (**C**) Dominant-negative Smad3 (Smad3Δc) plasmid attenuated high glucose-induced hypertrophy at 72 h. Data were expressed as the mean±S.E.M. of 3 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ versus 5.5 mM glucose. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control plasmid (pcDNA3).

Thus, glomerular expression of Smad2/3 was increased in diabetic mice and rats [Hong et al., 2001; Isono et al., 2002; Furuse et al., 2004; Kim et al., 2004]. Similarly, tubular expression of Smad2/3 was increased in diabetic mice and rats [Huang and Preisig, 2000; Hong et al., 2001; Isono et al., 2002]. Moreover, Smad7 attenuated high glucose-induced collagen I synthesis by inhibiting Smad2/3 activation in mesangial and proximal tubular cells [Li et al., 2003]. Finally, Smad3-null mice are protected against streptozotocin-induced diabetic glomerulopathy and albuminuria [Fujimoto et al., 2003].

In conclusion, high glucose-induced hypertrophy is mediated by Sp1-Smad2/3-induced transcriptional activation of p21^{WAF1} in LLC-PK$_1$ cells.

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