Regulation of taurine transporter gene (*TauT*) by WT1

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Abstract In the present study we have demonstrated that WT1 (Wilms tumor suppressor gene) enhances the expression of *TauT* (taurine transporter gene) in human embryonic kidney 293 cells in a dose-dependent manner. *TauT* promoter activity was increased five-fold by cotransfection of a full-length *TauT* promoter–reporter construct with WT1. Electrophoretic mobility shift assays (EMSAs) using nuclear extracts from WT1-overexpressing 293 cells showed a putative WT1-binding site in the basal promoter region of *TauT*, which bound to WT1 in EMSAs. Mutation of this WT1 consensus sequence abolished binding of WT1. These results demonstrate that *TauT* may represent a downstream target gene of WT1 during renal development.

Key words: WT1; *TauT*; Gene expression; Renal; Development

1. Introduction

Taurine has been shown to be essential for the development and survival of mammalian cells, particularly cells of cerebellum, retina, and kidney [1–3]. Sturman et al. [2] demonstrated that the surviving offspring of taurine-deficient female cats have a large number of neurological defects, including degeneration of retinal pigmented epithelium and ocular tapetum, delayed cerebellar granule cell division and migration, and abnormal cerebral cortical development. Recently, Maar et al. confirmed the observations that taurine depletion severely affects migration of cerebellar granule neurons during early postnatal development by using cultures of dissociated cerebellum of 5- to 6-day-old mice and N2A neuronal cells [4]. They found that taurine depletion by culturing the cells in the presence of a taurine transport inhibitor, guanidoethane sulfonate, resulted in a decreased rate of cell migration, suggesting that normal function of the taurine transporter is required during brain development.

Recent studies demonstrated that taurine and the taurine transporter play an important role in kidney development [1,3]. In the F1 generation of inbred taurine-deficient cats, taurine deficiency results in renal malformation with significantly diminished renal size and progressive kidney damage. Histological examination of these kidneys showed ureteral dilatation, enlarged glomeruli, proximal tubular flattening and abnormal differentiation of renal tubular epithelium, especially in the distal tubule, where taurine has been found to be in highest concentration in immunohistochemical studies [3,5]. Consistent with these findings, Heller-Stilb et al. [1] demonstrated that knockout of *TauT* (taurine transporter gene) resulted in severe and progressive retinal degeneration, a small brain, and shrunken kidneys in a *taut*−/− mouse model. These findings confirm that *TauT* is required for retinal, brain, and kidney development.

We have recently shown that the promoter region of *TauT* contains an overlapping consensus site for WT1 (Wilms tumor suppressor gene)/EGR-1 (early growth response gene 1)/Sp1. It has been demonstrated that WT1 plays a critical role in kidney development [6]. However, the precise mechanism by which WT1 exerts its effects during development awaits identification of actual target gene(s) that it regulates during renal development.

2. Experimental procedures

2.1. Construction of the reporter gene

The promoter region of *TauT* was identified in previous studies [7], and a WT1-binding consensus site was found in the *TauT* promoter sequence, located at −160 to −171. In this study, approximately 1.1 kb of the *TauT* promoter region DNA (GenBank accession number AR151716) was used as the template for PCR and the PCR fragment was cloned into the promoterless luciferase vector pGL3-Basic (Promega, Madison, WI, USA) to generate the plasmid p963 for use in transfections and luciferase assays. The conditions used are 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 58°C, and 1 min of elongation at 72°C. The sense primer (5′-GGGGTACCTTACTGAAGGTCACACAGC-3′) designed for PCR contained a unique site for *Kpn*I, and the antisense primer (5′-AAGATCTTGGCACGGGAGTTCA-3′) contained a unique site for *Bgl*II. PCR products were digested with *Kpn*I and *Bgl*II and re-ligated into *Kpn*I and *Bgl*II sites of pGL3-Basic to generate plasmids containing segments of the *TauT* promoter sequence extending from the +48 nucleotide corresponding to the transcriptional start site. The constructs were verified by DNA sequencing. The 5′-progressive deletions were generated from the p963 plasmid by using sense primers 5′-GGTTCTTTGTGTGTCCGAGCTCCTG-3′ (p265), 5′-GGGGTACCTGTGTGTGGGCGT-3′ (p182), 5′-GGGGTACCAGACCCCGCCCTA-3′ (p-sp1), 5′-GGGGTACCTAGGCCCCGCCCA-3′ (p-sp2), 5′-GGGGTACCAGGCCCGGCCAAG-3′ (p-sp3), and 5′-GGGGTACCAGCAGGATGGGTG-3′ (p-sp4), respectively. The antisense primer used for these constructs was the same as described above. The 3′-progressive deletion was generated from p963 by using the same sense primer for p182 and antisense primers 5′-GGGGCCTGGGAGGTCAGCCACG-3′ (pd-sp1), 5′-GGGGCCTGGGGAGGGGTCTGGGCGGT-3′ (pd-sp2), 5′-CCGGGCCTGGGAGGGGCCTA-3′ (pd-sp3), and 5′-CCGGGCCTGGGCGGGGCCTA-3′ (pd-sp4).

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Abbreviations: *TauT*, taurine transporter gene; EMSAs, electrophoretic mobility shift assays; WT1, Wilms tumor suppressor gene; EGR-1, early growth response gene 1

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2.2. Cell culture

Human embryonic kidney (293) cells were cultured according to American Type Culture Collection guidelines. Briefly, cells were grown as confluent monolayers in 10 cm diameter tissue culture plates in Dulbecco's modified Eagle's medium/F12 medium with 10% fetal calf serum at 37°C in the presence of 5% CO_2 in a humidified incubator. Cells were plated 18 h before transfection and fed with fresh medium 4 h before transfection.

2.3. Stable expression of WT1 in 293 cells

293 cells were transfected with WT1 and CMV neo Bam plasmid DNA (vector control) by electroporation using a Gene Pulser electroporator (Bio-Rad, Hercules, CA, USA). Geniticin (G418, 1 mg/ml, Clontech, Palo Alto, CA, USA) was used for colony selection. G418-resistant colonies (293/WT1) were screened by Western blot analysis for WT1 expression. In order to test the role of wild-type WT1 in *TauT* expression, WT1-overexpressing 293 cells were used in this study.

2.4. Transient transfection

The reporter gene constructs and WT1 expression vectors (a gift from Dr. John Schuetz at St. Jude Children's Research Hospital, Memphis, TN, USA) were introduced into cultured mammalian cells using cationic liposomes (LipofectAMINE, Life Technologies). Transfection was carried out for 16–18 h, and then cells were washed twice with phosphate-buffered saline and incubated in fresh medium for 24–48 h before harvesting. pGL-control, which contains a luciferase gene driven by the SV40 early region promoter/enhancer, and empty pGL-Basic vectors were used as positive and negative controls, respectively. To standardize the transfection efficiency, 0.1 μg of pRL-CMV vector (pRL Renilla Luciferase control reporter vector, Promega) was cotransfected in all experiments. Cells were harvested 48 h after transfection and lysed in 200 μl of reporter lysis buffer (Promega). A luciferase assay was performed using a dual-luciferase assay kit (Promega), and activity was measured with an Optocomp 1 luminometer (MGM Instruments, Inc., Hamden, CT, USA). Promoter activity (mean ± S.D. of four samples in relative light units) of each construct is represented by relative light output normalized to pRL-CMV control. Graphs represent typical results of three separate experiments. The concentration of protein in the cell extracts was determined using the Bradford method (Bio-Rad, Hercules, CA, USA).

2.5. Northern blot analysis

30 μg of total RNA was separated in an agarose gel and transferred to a nylon membrane by overnight capillary blotting in 10×SSC (sodium chloride/sodium citrate buffer). The Northern blot was hybridized overnight at 42°C with a ^{32}P-labeled riboprobe of the taurine transporter cDNA. The blot was washed successively in standard decreasing concentrations of SSC/0.1% SDS at 65°C for 30 min each and exposed to Kodak film with one intensifying screen at −80°C for 24 h.

2.6. Western blot analysis

Cells were lysed in 50 μl M-PER mammalian protein extraction reagent (Pierce, Inc., Rockford, IL, USA) supplemented with protease inhibitors cocktail for use with mammalian cell and tissues extracts (Sigma, St. Louis, MO, USA). The lysates were cleared by centrifugation at 14 000×*g* for 2 min, and the supernatants were transferred to clean tubes. Equal amounts of protein (50 μg) were separated by electrophoresis on a 12% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA) using a semi-dry electrophoretic transfer system (Bio-Rad, Hercules, CA, USA). Membranes were incubated in 5% non-fat dry milk in Tris base/sodium chloride (TBS) buffer with 0.2% Tween 20 (TBST) at 4°C overnight. The membranes were incubated with primary antibody for 1 h at room temperature at the following dilutions: WT1 (180) (Santa Cruz, CA, USA) 1:2000, and 1:5000 of an affinity-purified antibody against the C-terminal peptide sequence of the taurine transporter [8]. Blots were washed with TBST and incubated with horseradish peroxidase-linked secondary antibody (Sigma). *TauT* or WT1 protein was detected using a chemiluminescent detection kit (Pierce, Inc.).

2.7. Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides corresponding to the putative WT1-binding site in the *TauT* promoter region were end-labelled with polynucleotide kinase using [γ-^{32}P]ATP. Nuclear extracts were isolated by high salt extraction of nuclei [9]. The binding reactions were performed by using a gel shift assay system following the manufacturer's instructions (Promega). Briefly, the labelled oligonucleotides and nuclear extracts were incubated in gel shift binding buffer [20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris–HCl (pH 7.5), and 0.25 mg/ml poly (dI–dC)] on ice for 30 min, and then the DNA–protein complexes were run on 10% non-denaturing acrylamide TBE gels (Bio-Rad) in 0.5× TBE buffer at 190 V for 45 min. The gel was dried at 70°C for 30 min and exposed to Kodak film with an intensifying screen for 1 h at −80°C.

2.8. Statistics

All experiments were performed in triplicate. Luciferase assays are expressed in units of relative light output. The data represent the mean ± S.E.M. of three experiments. Statistical comparisons were made using one-way ANOVA and Student's *t*-test to determine significant differences in the means.

3. Results

3.1. TauT *is up-regulated by WT1*

To study if WT1 regulates *TauT* expression in 293 cells, various doses of WT1 were transiently transfected into the 293 cells by electroporation. The transfected cells were con-

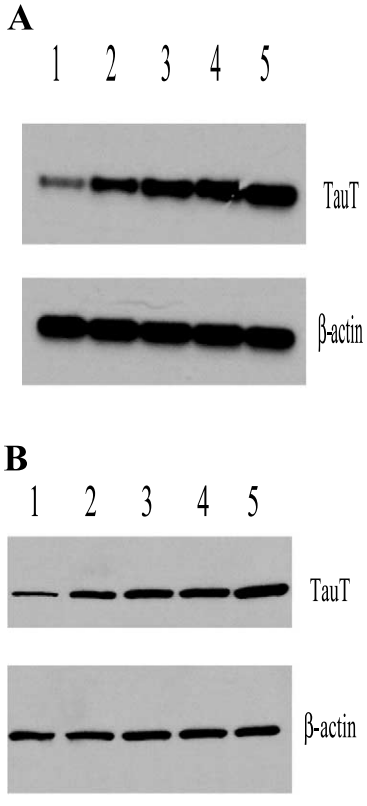

Fig. 1. TauT is up-regulated by WT1. A: Northern blot analysis of *TauT* expression in 293 cells transfected with WT1 for 48 h. Lane 1, control; lane 2, 0.5 μg WT1; lane 3, 1 μg WT1; lane 4, 5 μg WT1; and lane 5, 10 μg WT1. β-Actin was used as an internal control for loading. B: Western blot analysis of *TauT* gene expression in 293 cells transfected with WT1 (0–10 μg) for 48 h. Equal amounts of protein were loaded on an SDS–polyacrylamide gel (12% acrylamide) and the taurine transporter protein was detected as described in Section 2. The housekeeping gene β-actin was used as loading control. The result represented three separate experiments.

tinually cultured for another 48 h. As shown in Fig. 1A, expression of *TauT* was up-regulated by WT1 in a dose-dependent manner. The increase in the amount of WT1 was accompanied by enhanced expression of *TauT* protein (Fig. 1B).

To extend this study, the WT1 was stably transfected into 293 cells and expression of *TauT* was determined. As shown in Fig. 2A, overexpression of WT1 increased the transcription of *TauT* mRNA by more than two-fold. Western blot analyses show that the expression of WT1 is undetectable in wild-type 293 cells, whereas a high level of WT1 was found in WT1-expressing cells, in which expression of *TauT* protein was doubled as compared to the control cells transfected with the empty expression vector (Fig. 2B).

3.2. TauT *is transcriptionally activated by WT1*

To determine if up-regulation of the *TauT* gene by WT1 occurs at the transcriptional level, the reporter constructs, including p265 which contains a putative WT1/EGR-1/Sp1-binding site from −160 to −171 and the indicated constructs created by progressive deletion as shown in Fig. 3A, were transiently transfected into 293 cells. Regulation of *TauT* promoter activity by WT1 was examined. As shown in Fig. 3B, reporter gene p265, which contains a TG repeat, shows strong promoter activity that was enhanced five-fold by WT1. Deletion of the TG repeat repressed basal promoter activity of *TauT*, and had no effect on its up-regulation by WT1. Deletion of the WT1/EGR-1/Sp1 site abolished the effect of WT1 on *TauT* promoter function. Consistent with our previous study, we have also demonstrated that an Sp1 site is essential for the basal promoter function of *TauT* in 293 cells.

To determine if the WT1/EGR-1/Sp1 overlapping site is sufficient for the basal promoter function of *TauT* and its regulation by WT1, progressive deletion was carried out from the 3′-sequence of the *TauT* promoter region and the resulting reporter gene constructs were transiently transfected

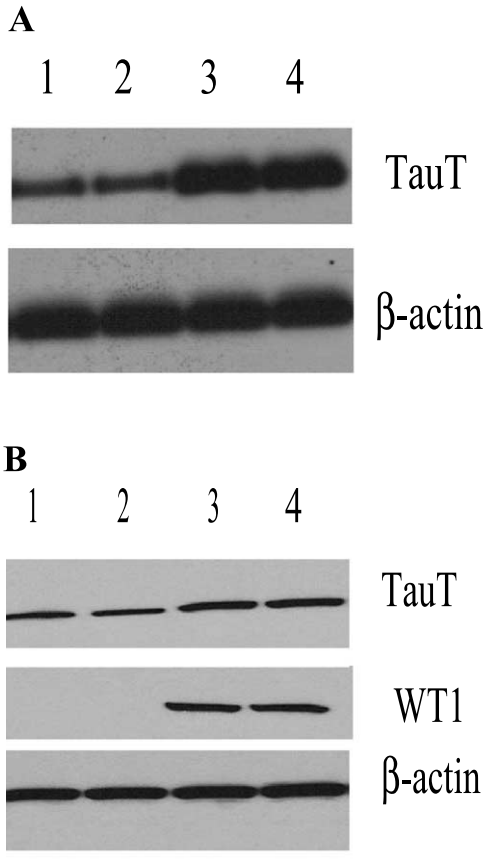

Fig. 2. Up-regulation of *TauT* expression by stable transfection of WT1 in 293 cells. A: Northern blot analysis was carried out using a specific RNA probe generated from human taurine transporter cDNA [12]. Lanes 1 and 2, control 293 cells; lanes 3 and 4, WT1-overexpressing 293 cells. B: Western blot analysis of *TauT* expression was performed using an antibody against the C-terminal peptide sequence of TauT. Expression of WT1 was detected by a WT1 antibody (WT 180, Santa Cruz Biotechnology, CA, USA). Sample loading sequence was the same as described in A.

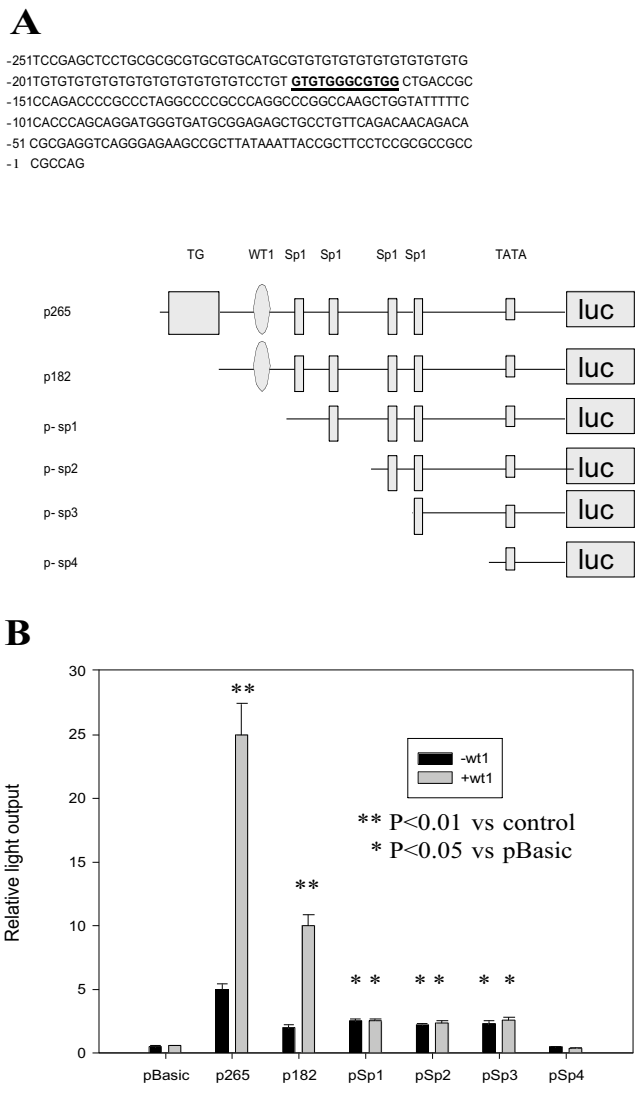

Fig. 3. WT1/EGR-1/Sp1 consensus site is required for WT1 regulation of *TauT* promoter activity. A: Schematic representation of *TauT* promoter–reporter constructs. B: Effect of deletion of the WT1 site on activation of *TauT* promoter by WT1 was carried out in 293 cells. Reporter gene constructs as indicated in A were transiently transfected into 293 cells for 24 h, and then a luciferase assay was performed as described in Section 2. To control for transfection efficiency, cells were cotransfected with pRL-CMV vector (0.1 μg), luciferase activity was measured in cell lysates by the dual-luciferase reporter assay system. The promoter activity (mean ± S.D. of four samples) is represented by relative light output normalized to pRL-CMV control. The graph represents typical results of four separate experiments.

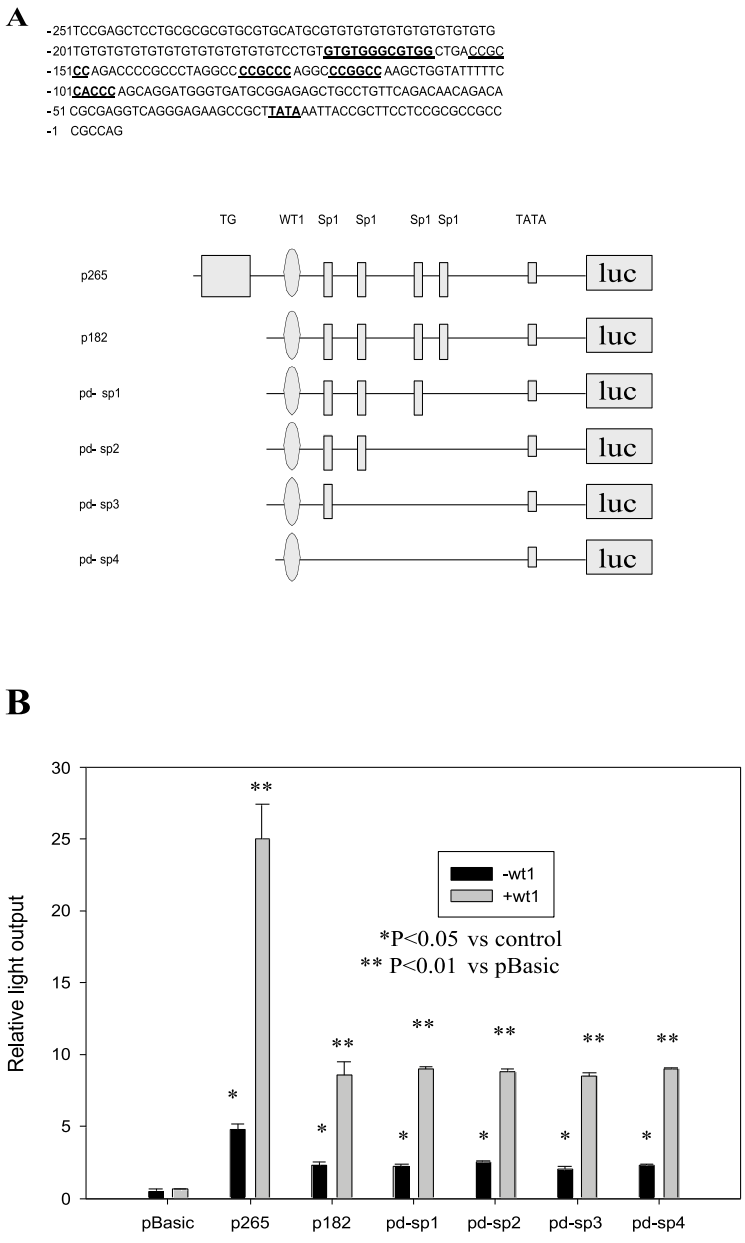

Fig. 4. Sp1 site is not necessary for WT1 regulation of *TauT* promoter. A: Schematic representation of *TauT* promoter–reporter constructs. B: Effect of deletion of the Sp1 sites on activation of *TauT* promoter by WT1 in 293 cells. Reporter gene constructs as indicated in A were transiently transfected into 293 cells for 24 h, and then a luciferase assay was performed as described in the legend to Fig. 3.

into 293 cells. As shown in Fig. 4A,B, deletion of all indicated Sp1 sites (pd–sp4) did not affect *TauT* promoter function and its up-regulation by WT1, as compared to the reporter gene construct p182. These results suggest that theWT1/EGR-1/Sp1 overlapping site is critical for the basal promoter function of *TauT* and its up-regulation by WT1.

3.3. *WT1-binding site is necessary for the transcriptional activation of* TauT *by WT1*

To define whether WT1 binds to this putative WT1/EGR-1/Sp1 overlapping site in the *TauT* promoter, we carried out EMSAs using the synthetic oligonucleotides corresponding to this site (Fig. 5A). As shown in Fig. 5B, nuclear extracts from WT1-overexpressing 293 cells yielded a band (lane 2) that could be competed out by excess unlabelled probe (lane 3). Control 293 cells did not yield a band (lane 1). Mutation of the WT1/EGR-1/Sp1 sequence, in which three thymines (T) were mutated to adenines (A), results in the loss of WT1 binding to this site (lane 5). A polyclonal antibody to WT1 (WT1 180, Santa Cruz, CA, USA) resulted in a super-shifted band (lane 4), suggesting that WT1 specifically binds to the putative WT1/EGR-1/Sp1 overlapping site in the *TauT* promoter region.

4. Discussion

Taurine functions as an osmoregulator and intracellular calcium flux regulator and has been found to play an important role in kidney development [3]. Heller-Stilb and his coworkers have demonstrated that mutation of *TauT* results in retinal degeneration and diminished renal size [1], suggesting that *TauT* per se plays an important role in normal retinal

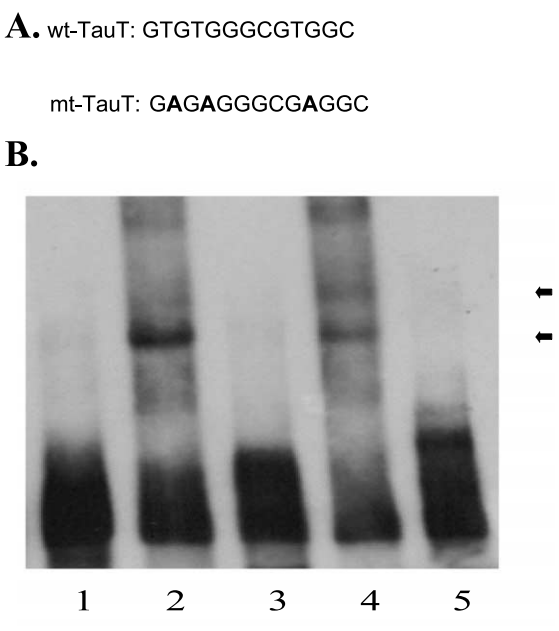

Fig. 5. EMSA using the putative WT1-binding site sequence of the *TauT* promoter. A: Sequences of oligonucleotides corresponding to nucleotide positions −160 to −171 relative to the transcription start site in *TauT* (wt-TauT). The sequence of mutant WT1 probe is shown as mt-TauT. B: EMSAs were done using radiolabelled *TauT* oligonucleotides with nuclear extracts from 293 cells overexpressing WT1. Lane 1, wt-TauT probe alone; lane 2, wt-TauT probe plus nuclear extracts from WT1-overexpressing cells; lane 3, wt-TauT probe plus nuclear extracts and unlabelled probe at 1:100; lane 4, wt-TauT probe with nuclear extracts from WT1-overexpressing 293 cells plus WT1 antibody (WT1 180); lane 5, mt-TauT probe with nuclear extracts from WT1-overexpressing 293 cells.

and kidney development. We have recently shown that *TauT* is a target gene of p53, and that it is negatively regulated by p53 in renal cells. These findings strongly support the role of *TauT* in renal development, since overexpression of p53 in p53 transgenic mice results in severe renal damage, which is similar to the observations made in the taurine-deficient cats [3,10].

In the present study, we show that *TauT* is likely a target gene of WT1, which plays a critical role in kidney development [6]. First, we demonstrated that expression of *TauT* was up-regulated by WT1 in human embryonic 293 renal cells in a dose-dependent manner. This result was confirmed by stable transfection of WT1 into 293 cells. Secondly, our results showed that regulation of *TauT* by WT1 occurred at the transcriptional level, as demonstrated by using a luciferase reporter gene driven by the *TauT* promoter. We have also demonstrated that the WT1/EGR-1/Sp1 overlapping site located at −160 to −171 is essential for WT1 regulation. It appears that the TG repeat, which is located immediately upstream of the WT1/EGR-1/Sp1 site, is important for the maximum effect of WT1 on *TauT* promoter function. Deletion of the TG repeat represses the basal promoter activity of *TauT* by 60%. Thirdly, we showed that WT1 increased *TauT* transcription by directly binding to the DNA sequence between −160 and −171 of the *TauT* promoter. Mutation of this consensus site abolished WT1 binding to the *TauT* promoter, and abrogated the effect of WT1 on *TauT* promoter function.

It has been demonstrated that WT1 plays a critical role in kidney development. The WT1 gene, located on chromosome 11, band p13, encodes a 50 kDa nuclear transcription factor that contains four zinc-finger DNA-binding domains [13]. Unique WT1 mutations have been demonstrated in individuals with Denys-Drash syndrome, which includes intersexual disorders, nephropathy (mesangial sclerosis), and Wilms tumor. Mutations in the zinc finger domains of WT1-gene were found in Frasier syndrome, which is defined by the association of focal and segmental glomerulosclerosis, male pseudohermaphroditism, and gonadoblastoma [14]. Expression of the WT1 gene is restricted during development to mesenchymal tissues, occurring in specific cells of the collecting system within the kidney, non-germ cell components of the gonads, uterus, spleen, and mesothelium [15]. Knockout of the WT1 gene results in embryonic lethality in the homozygous state, secondary to the failure of kidney and gonad development [16]. However, the precise mechanism by which WT1 exerts its effects during normal development and prevents tumor formation in the kidney awaits identification of actual target gene(s) that it regulates during kidney development.

Several lines of evidence suggest that the interaction of p53 and WT1 plays an important role in normal development and in tumorigenesis [17]. Using in vitro immunoprecipitation and Western blot analyses, p53 and WT1 proteins were shown to physically interact in BRK (baby rat kidney) cells [18]. WT1 protein was shown to stabilize p53, modulate its transactivational properties, and inhibit its ability to induce apoptosis (programmed cell death) [19].

Taken altogether, our results suggest that *TauT* is a target gene of WT1. Regarding the action of p53 on *TauT* regulation and the role of the taurine transporter in renal development [11], we concluded that *TauT* plays an important role in normal nephrogenesis. The function of *TauT* is regulated by WT1 and p53, which control a fine balance between cell proliferation and apoptosis during renal development.

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