Gene Expression, Vol. 9, pp. 103–114, 2000
Printed in the USA.

1052-2166/00 $20.00 + .00

The Role of NF-κB in the Regulation of the Expression of Wilms Tumor Suppressor Gene WT1

YIJING CHEN[1] AND BRYAN R. G. WILLIAMS[2]

Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195

The Wilms tumor suppressor gene, WT1, plays an important role in genitourinary development and the etiology of Wilms tumor. WT1 has a spatially and temporally defined expression in the developing genitourinary system and in specific cells of the hematopoietic system, but the regulatory pathways that control WT1 expression are not well understood. Recently, members of the NF-κB family of transcription factors have been proposed as potent activators of the murine WT1 promoter through binding to a NF-κB site. Because the human WT1 promoter contains a conserved NF-κB site, we investigated whether NF-κB also regulates the expression of the human WT1 gene. We activated NF-κB through cytokine stimulation or inhibited NF-κB through expression of a NF-κB "super repressor" in WT1 expressing Wilms tumor, renal carcinoma, and erythroleukemia cultures and examined the level of endogenous WT1 gene expression. Although a transfected NF-κB reporter construct was responsive to these manipulations, we found that altering NF-κB activity had no effect on endogenous WT1 expression in the cell types used in our study. We conclude that despite the presence of conserved NF-κB elements in the murine and human WT1 promoters, NF-κB is not required to regulate the expression of the WT1 gene in its natural context.

Wilms tumor suppressor gene WT1 NF-κB Endogenous gene expression
NF-κB super repressor

DEVELOPMENT is a tightly regulated process that relies upon the accurate interplay of many gene products. Disruption of normal developmental programs as a result of genetic alteration can lead to disease. Wilms tumor or nephroblastoma, the most common solid tumor of childhood (34), is a clear example of disrupted development. Wilms tumors are of embryonic origin and appear to arise from metanephric blastemal tissues of the developing kidney that fail to undergo the normal maturation process (4). The relationship between development and tumorigenesis has been further strengthened by the molecular genetic analysis of WT1, the first Wilms tumor gene to be isolated (25).

The WT1 gene encodes a transcription factor that has four C_2H_2 zinc fingers at the carboxyl-terminus and a glutamine/proline-rich amino-terminus (8,20). Alternative splicing at two sites generates four protein isoforms (22) that appear to have somewhat distinct functions. Cell culture studies have shown that WT1 isoforms can bind to specific DNA sequences and activate or repress transcription from promoter constructs depending upon a variety of different experimental conditions (43) [reviewed in (41)]. A number of genes whose promoters contain WT1 binding sites have been identified, but whether they are bona fide in vivo targets of WT1 remains to be determined.

Mutations in WT1 are associated with the congenital WAGR (Wilms tumor/Aniridia/Genitourinary abnormalities/Retardation) and Denys-Drash syndromes, which both are characterized by genitouri-

Received July 14, 2000; revision accepted August 25, 2000.
[1]Present address: Department of Genetics, University of North Carolina, Chapel Hill, NC 27599.
[2]Address correspondence to Bryan R. G. Williams, Department of Cancer Biology, Lerner Research Institute NB40, The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Tel: (216) 445-9652; Fax: (216) 444-3164; E-mail: williab@ccf.org

nary abnormalities and a predisposition to Wilms tumor. Furthermore, WT1 is inactivated in a subset of nonsyndromic Wilms tumors. These finding both identified WT1 as a tumor suppressor gene and a key regulator of genitourinary development. WT1's function as a tumor suppressor is supported by the finding that wild-type WT1 gene products can suppress the transformed phenotype of both a Wilms tumor cell line (23) and a *ras*-transformed heterologous tumor cell line (32). The requirement for WT1 in normal development is revealed by the phenotypes of mice lacking WT1 function. WT1 null mice die before birth and display abnormal development of the heart, lungs, mesothelium, and complete agenesis of the gonads and kidneys. The genitourinary defects, to some extent, were attributed to failure of renal mesenchyme to respond to normal inductive signals (30).

Consistent with its role in genitourinary development, WT1 is expressed in a temporally and spatially restricted pattern. WT1 expression is limited to precursor cells of the kidneys and gonads and to glomerular podocytes in adults (33,38,40). In addition to these normal tissues, WT1 is highly expressed in Wilms tumors with predominant blastemal components (19,37,51) and in the majority of acute leukemias (6,28,36). Misregulated WT1 expression has been shown to have detrimental consequences. For example, renal cell carcinoma (RCC) is associated with relaxed expression of WT1 in the epithelial cells of the proximal tubule where WT1 is not normally expressed (10). Furthermore, ubiquitous expression of WT1 in mice (35) and precocious expression of WT1 in frogs (48) have been shown to perturb normal development.

Efforts aimed at elucidating the mechanisms that regulate WT1 expression have focused in large part on the identification of *cis* regulatory elements at the WT1 locus and transactivators that function through such elements. Several transcription factors, including Sp1 (11), PAX 2 (12), PAX 8 (13,18), and WT1 itself (45), have been shown to be capable of binding to regions in the WT1 promoter and regulating transcription from the WT1 promoter, at least in cell culture systems. In addition, a 3′ enhancer element containing GATA binding motifs has been shown to function in erythroleukemic K562 cells but not in other cell types (17,50). The fact that master regulatory elements capable of directing appropriate tissue-specific expression of WT1 in transgenic animals are yet to be identified demonstrates the complexity of the regulation of WT1 expression as well as the limitations of the cell culture system, in particular, cells that do not express the endogenous WT1 gene.

Transcription factors of the NF-κB/Rel family regulate the expression of a variety of genes important for immune and inflammatory responses, viral replication, cellular growth, and differentiation [for reviews, see (2,21)]. Mammalian NF-κB/Rel family members include p50, p65, c-Rel, Rel B, and p52, all of which function as homo- or heterodimers. Among them, the prototypic p50/p65 heterodimer is the most potent transactivator (3,44). NF-κB activity is tightly regulated by a family of endogenous inhibitors, IκBs, which complex NF-κB in the cytoplasm in most cells. Exposure of cells to a wide variety of stimuli, including cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), and UV irradiation leads to rapid phosphorylation and degradation of IκB. Upon degradation of IκB, NF-κB is free to translocate into the nucleus where it activates the expression of its target genes (5,7,15,26). Mutation of the inducible phosphorylation sites in IκB blocks its degradation and prevents NF-κB activation (16). Recently, a NF-κB binding site in the murine WT1 promoter was reported to respond to the overexpression of the p50 and p65 subunits of NF-κB in cells transiently cotransfected with murine WT1 promoter constructs (14). Furthermore, nuclear run-on assays revealed that overexpression of p50 and p65 in murine NIH3T3 cells enhanced transcription of the endogenous WT1 gene, which is normally transcriptionally silent in these cells. The observation that this and other putative NF-κB-like sites are conserved between the murine and human promoters led us to investigate the role of NF-κB in regulating WT1 expression in human cells of genitourinary or hematopoietic origin, which normally express WT1. Despite the presence of conserved NF-κB sites in human WT1 promoter, we failed to detect changes in endogenous WT1 expression in these cells when NF-κB activity was demonstrably altered.

MATERIALS AND METHODS

Cell Culture and Transient Transfection

The Wit49A Wilms tumor cell line and the mouse TM3 Leydig cell line were cultured in medium containing equal portions of high-glucose DMEM and F12 nutrient mixture, 10% fetal bovine serum (FBS), and 100 μg/ml of penicillin/streptomycin. The RCC1 renal carcinoma cell line was maintained in RPMI-1640 supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 12.5 mM HEPES, and 100 μg/ml of penicillin/streptomycin. K562 erythroleukemia cells were cultured in RPMI-1640 supplemented with 10% FBS and 100 μg/ml of penicillin/streptomycin. All tissue culture reagents were obtained from GIBCO, BRL. For transient transfection experiments, 4 μg of pGbp2-luc, a firefly luciferase reporter

driven by the NF-κB-inducible promoter of the guanylate-binding protein (Gbp) gene (31), and 0.5 μg of pRLTK, a plasmid expressing the *renilla* luciferase (Promega), were transfected into 2×10^6 Wit49A cells/10-cm dish using lipofectamine plus reagents (GIBCO, BRL) according to the manufacturer's protocol. The luciferase acitvity of the cotransfected pRLTK was used to control for transfection efficiency among samples.

Adenovirus Infection

The recombinant adenoviruses used in this study have been described (29). A multiplicity of infection of 50 was used for all adenovirus infection experiments. Adherent cells were allowed to reach confluency just before infection. The nonadherent K562 cells were plated at a density of 2×10^6/ml. Before infection, cells were washed three times with PBS to deplete serum. The appropriate amount of virus was diluted in the minimal volume of OPTI-MEM needed to cover the tissue culture dish and incubated with the cells for 10–12 h. For adherent Wit49A and RCC1, but not for nonadherent K562 cells, the virus-containing medium was then removed and the cells were cultured in full serum medium for another 12 h before additional treatment and/or analysis.

Luciferase Assay

Transiently transfected Wit49A cells were split into 12-well plates and infected with the appropriate adenovirus 24 h after transfection. Twenty-four hours after viral infection, the cells were treated with or without human TNF-α at 10 ng/ml for 4 h. At the end of the TNF-α treatment, luciferase activity was determined using the Dual-Luciferase Assay System (Promega) following the manufacturer's protocol. The firefly luciferase activity of the pGbp2-luc construct was normalized against the *renilla* luciferase activity of the cotransfected pRLTK construct.

Electrophoretic Mobility Shift Assay

Actively growing or virus-infected Wit49A, RCC1, and K562 cells were treated with 10 ng/ml human TNF-α for 30 min. The nuclear extracts were prepared basically according to the method of Andrews and Faller (1) and Sakamoto et al. (46). At the end of the TNF-α treatment, the cells were washed two times with cold PBS and lysed in modified buffer A [10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.05% NP40, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin] on ice for 15 min. Samples were centrifuged briefly to pellet crude nuclei, which were resuspended in modified buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 700 mM NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin) and incubated on ice for 30 min. The crude nuclear extract was collected and stored at −70°C. For EMSA, annealed oligonucleotides forming the consensus NF-κB site, 5′-GGGGACTTTCCC-3′, were end-labeled with [γ-^{32}P]ATP and purified over a Sephadex G50 column. Crude nuclear extract (5–10 μg of protein) was incubated in the binding buffer (50 mM HEPES-KOH, pH 7.5, 20% glycerol, 5 mM $MgCl_2$, 50 mM KCl, 10 μM $ZnSO_4$, 1 mM DTT) with 1 μg of poly(dI-dC) (Boehringer Mannheim) and 100,000 CPM of end-labeled probe in a total volume of 21 μl for 20 min at room temperature. For supershift experiments, 200 ng of anti-p65 antibody (Santa Cruz Biotechnology, Inc.) was incubated with nuclear extracts for 10 min at room temperature prior to the addition of radiolabeled probes. The protein–DNA complexes were resolved on a 5% nondenaturing polyacrylamide gel in 0.5× TBE buffer at room temperature.

Analysis of WT1 Transcripts and Proteins

Wit49A, RCC1, and K562 cells were treated with human TNF-α (10 ng/ml) for the indicated amount of time or infected with the appropriate adenoviruses. Total RNA was isolated using Trizol reagent (GIBCO, BRL) and reverse transcribed using Superscript II reverse transcriptase (GIBCO, BRL). RT/PCR of WT1 transcripts was performed using the forward primer, 5′-ATCCTCTGCGGAGCCAATAC-3′ and the reverse primer, 5′-ACTGTGCTGCCTGG GACAC-3′. As an internal control for RNA integrity and equal loading, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified in the same tube using 5′-CCATGGAGAAGGCTG GGG-3′ and 5′-CAAAGTTGTCATGGATGACC-3′. Twenty-five cycles of amplification were performed for all samples under the following conditions: denature at 94°C for 45 s, anneal at 60°C for 1 min, and extend at 72°C for 1 min. For Western blot analysis of WT1 proteins, the treated cells were lysed in buffer containing 50 mM Tris-HCl, pH 7.4, 0.5% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 2 μg/ml aprotinin on ice for 30 min. The lysate was cleared of debris and the total protein concentration was measured using the Protein Assay Reagent (BioRad). Equal amounts of total protein were separated on an 8% SDS-PAGE polyacrylamide gel and transferred to PVDF membrane (Millipore).

The anti-WT1 peptide antibody, C19 (Santa Cruz), was used to detect all isoforms of the WT1 protein. The anti-HA antibodies, HA probe (Santa Cruz), was used to detect the expression of the hemagglutinin (HA) epitope-tagged IκBα super repressor protein.

RESULTS

The Conserved Arrangement of Putative NF-κB Binding Sites in the Human and Murine WT1 Promoters

The human and murine WT1 promoters are members of the GC-rich, CCAAT-less and TATA-less class of RNA polymerase II promoters. Several transcription start sites for the human and murine WT1 genes have been mapped to a region that shows extensive sequence homology between these two species (9,17,39). Analysis of the sequence flanking the conserved major transcription start site in the human WT1 promoter revealed four decamers that resemble the consensus NF-κB binding site, 5′-GGGRNNY YCC-3′. Similar sites are present in analogous positions in the murine WT1 promoter (Fig. 1a). Among them, NF-κB sites A and D in the human WT1 promoter are identical to their counterparts in the murine promoter, whereas one or two mismatches are present in sites C and B, respectively (Fig. 1b).

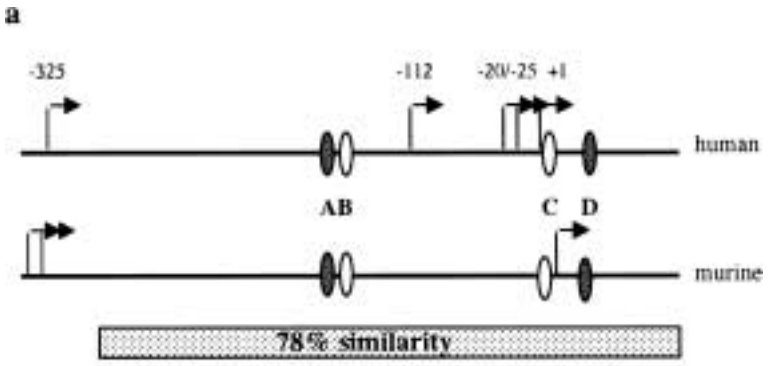
Activation of NF-κB Activity in Wit49A and RCC1 Cells Fails to Augment WT1 Expression In Vivo

Dehbi et al. (14) recently reported that the NF-κB site D present in the murine promoter was able to form a complex with NF-κB and mediate transcriptional activation of the WT1 promoter by overexpressed p50 and p65 subunits of the NF-κB/Rel family of transcription factors. Because the NF-κB site D in the human WT1 promoter sequence is identical to its murine counterpart, we examined the effect of TNF-α, a potent activator of NF-κB, on the expression of the endogenous human WT1 gene in cell lines derived from the genitourinary system where WT1 has a physiological role. The RCC1 cell line was derived from a primary renal cell carcinoma (24). The Wit49A cell line, whose characterization will be described in a separate report, was established from a first-generation xenograft of a Wilms tumor (H. Yeger, unpublished results). Both cell lines express wild-type WT1 transcripts and proteins as revealed by molecular genetic, biochemical, and immunohistochemical analyses [(10); this report].

The Wit49A and RCC1 cells were exposed to human TNF-α and assayed for NF-κB activation and WT1 gene expression. Electrophoretic mobility shift assays (EMSA) using a NF-κB consensus binding site (5′-GGGGACTTTCCC-3′) revealed a basal level of NF-κB binding activity in untreated cells. The basal level of NF-κB binding activity was low in Wit49A cells and much higher in RCC1 cells (Fig. 2a, lanes 1 and 7, respectively). A significant increase in the DNA binding activity to the NF-κB consensus oligo was detected in both cell types treated with TNF-α (Fig. 2a, lanes 4 and 9). The observed binding was specific because it could be competed with excess unlabeled NF-κB oligo (Fig. 2a, lanes 2, 5, 8, and 10). Supershift experiments using antibodies raised against p65 identified that p65 is one of the components in the observed DNA binding activity (Fig. 2a, lanes 3 and 6). These results indicate that NF-κB activity is induced in response to TNF-α in both Wit49A and RCC1 cells. However, examination of WT1 transcript levels by RT/PCR (Fig. 2b) and protein levels by Western analysis (Fig. 2c) revealed no significant changes following an extended period of exposure to TNF-α. These results demonstrate that NF-κB activation by TNF-α is not sufficient to enhance endogenous WT1 expression in these cells.

Inhibition of NF-κB Activity in Wit49A and RCC1 Cells Does Not Affect the Expression of WT1

The detection of constitutive NF-κB DNA binding activity in Wit49A and RCC1 cells prompted us to investigate whether basal NF-κB activity contributes to the constitutive expression of WT1 in these cells. To downregulate the constitutive NF-κB activity, we took advantage of Ad5IκB-SR, a recombinant adenovirus expressing a "super repressor" of NF-κB. The super repressor is a mutated nondegradable IκBα that is resistant to phosphorylation and degradation (29). Both Wit49A and RCC1 cells can be infected by recombinant adenovirus with an efficiency close to 100% as determined by β-galactosidase staining of cells infected with a lacZ-expressing adenovirus, Ad-5laZ (data not shown). The expression of the hemagglutinin (HA)-tagged super repressor protein could be readily detected in cells infected with Ad5IκB-SR (Fig. 4c). To determine if the super repressor was functional in Wit49A and RCC1 cells, infected cells were tested for DNA binding activity to the NF-κB consensus site by EMSA. Remarkably, the super repressor reduced both the constitutive and induced binding to the NF-κB site to undetectable levels in these cells (Fig. 3a, lanes 3, 6, 9, and 12). Furthermore, luciferase assays demonstrated that the super repressor was also able to repress transcription from a NF-κB responsive reporter construct in Wit49A cells



b

site A (-178) and B(-164)

5′GGTGTCTCCGAGAGGGACGCTCC3′ human

5′GGTGTCTCCGAGAtGaACGCTCC3′ murine

site C (+3)

5′GaGGGCAGCC3′ human

5′GGGGGCAGCC3′ murine

site D (+54)

5′GGGGCTCTCC3′ human

5′GGGGCTCTCC3′ murine

NF-κB consensus binding site

5′GGGRNNYYCC3′

Figure 1. Schematic representation of the conserved promoter region of the human and murine WT1 genes. (a) Alignment of the conserved features. The transcriptional start sites are represented by right-angled arrows. Numbers above the arrows indicate the position of the start sites with respect to one of the human major transcription initiation sites, which is designated as +1. Ovals designate the relative positions of the putative NF-κB sites. Filled or open ovals represent sites completely or partially conserved between human and murine sequences, respectively. The stippled bar represents the region within the human and WT1 promoters that is 78% similar at the nucleotide level. (b) Sequences of the four putative NF-κB sites present in the WT1 promoter and of the NF-κB consensus binding site. The putative NF-κB sites are shown in bold and underlined. The mismatches between the human and murine sites are shown in lower case. Numbers in parentheses indicate the position of the first base in the site with respect to the major human transcription initiation site. R, purine; Y, pyrimidine, N, any nucleotide.

(Fig. 3b). The residual luciferase activity observed in cells infected with the super repressor-expressing virus was likely due to basal activation of the reporter construct by transcription factors other than NF-κB. We were unable to assay the activity of the luciferase reporter in RCC1 cells due to the extremely low transfection efficiency in these cells.

When WT1 transcripts and proteins were analyzed by RT/PCR and Western blot analysis, no significant reduction in WT1 expression was detected in cells expressing the NF-κB super repressor protein (Fig. 4a, b, compare lanes 2 and 3; lanes 5 and 6). These results indicate that the constitutive NF-κB activity in Wit49A and RCC1 cells does not significantly contribute to the constitutive expression of WT1 in these cells.

Altering NF-κB Activity in K562 Cells Does Not Affect the Expression of WT1

In addition to its expression in the genitourinary system, WT1 gene expression has also been detected in specific cells of the hematopoietic system. It is possible that NF-κB does not regulate the expression of WT1 in genitourinary tissues, but is required for WT1 expression in the hematopoietic system. Accordingly, we tested whether NF-κB plays a role in regulating WT1 expression in K562 cells, which are

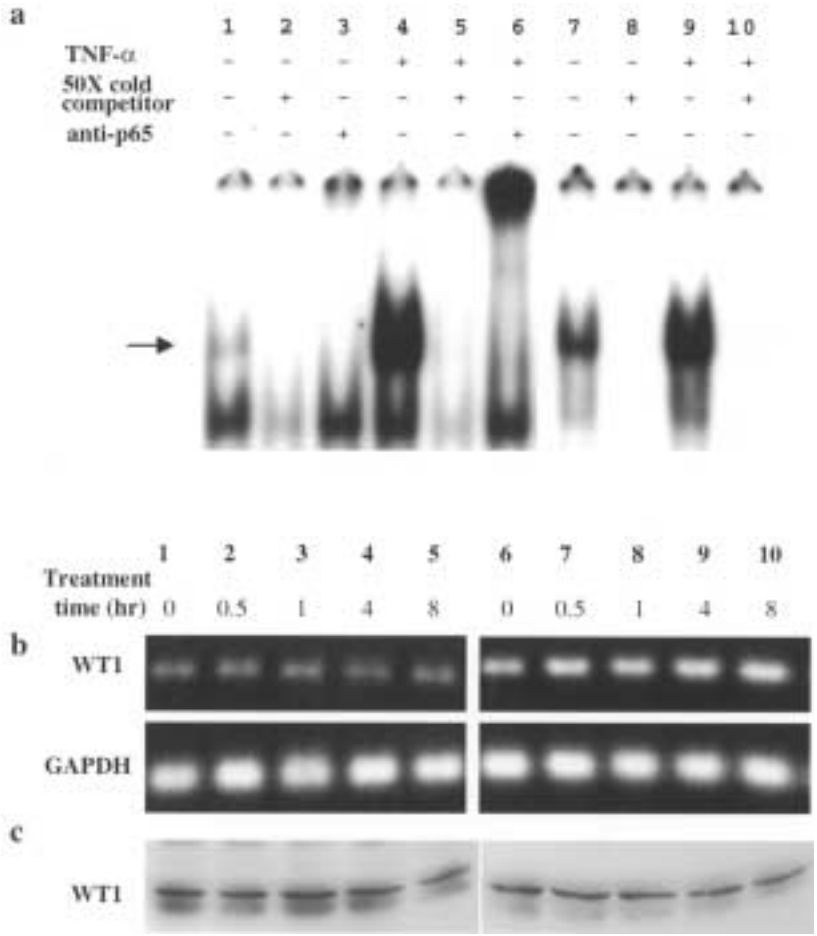


Figure 2. The effect of the activation of NF-κB by TNF-α on WT1 transcript and protein levels in Wit 49A and RCC1 cells. (a) Wit49A cells (lanes 1–6) and RCC1 cells (lanes 7–10) were treated with human TNF-α for 30 min. Crude nuclear extracts were prepared and DNA binding to a consensus NF-κB oligo, 5′-GGGGACTTTCCC-3′, was demonstrated by electrophoretic mobility shift assay (EMSA) as described in Materials and Methods. The NF-κB DNA binding activity is indicated by the arrow. Excess amount of unlabeled probe (50× cold competitor) or 200 ng of anti-p65 antibodies were added as indicated to show the specificity and identity of the DNA binding activity. (b) Wit49A cells (lanes 1–5) and RCC1 cells (lanes 6–10) were treated with human TNF-α for the indicated amount of time. RT/PCR was performed as described in Materials and Methods to determine the relative amount of WT1 transcripts. A portion of the GAPDH transcript was amplified at the same time to control for equal loading. (c) Western blot analysis of the WT1 protein levels in TNF-α-treated samples. Equal amounts of total protein as determined by protein assays were analyzed for each sample. The four WT1 isoforms were resolved as a doublet in most cases under the conditions used for SDS-PAGE.

derived from a chronic myelogenous leukemia in blast crisis and express endogenous WT1. The cells were treated with TNF-α to activate NF-κB activity or infected with Ad5IκB-SR to downregulate NF-κB activity. EMSA revealed that despite constitutive WT1 expression, there was very little, if any, constitutive NF-κB activity in untreated K562 cells (Fig. 5c, lanes 1–3). This suggests that NF-κB activity is not required for initiating WT1 expression in K562 cells. Treatment of the cells with TNF-α resulted in a dramatic increase in the NF-κB DNA binding activity (Fig. 5c, lanes 4 and 5), which could be significantly reduced by expression of the NF-κB super repressor (lane 6). The residual NF-κB DNA binding activity in cells infected with Ad5IκB-SR was most likely due to incomplete infection of the culture (70–80%, β-galactosidase staining not shown). Analysis of WT1 transcripts and proteins by RT/PCR and Western blot revealed no significant change in WT1 expression in response to changes of NF-κB activity (Fig. 5a, b). These results indicate that as in Wit49A and RCC1 cells, NF-κB does not play a significant role in regulating WT1 expression in K562 cells.

DISCUSSION

The 5′ regulatory regions of the human and murine WT1 genes share extensive sequence homology, suggesting that conserved mechanisms may be in-

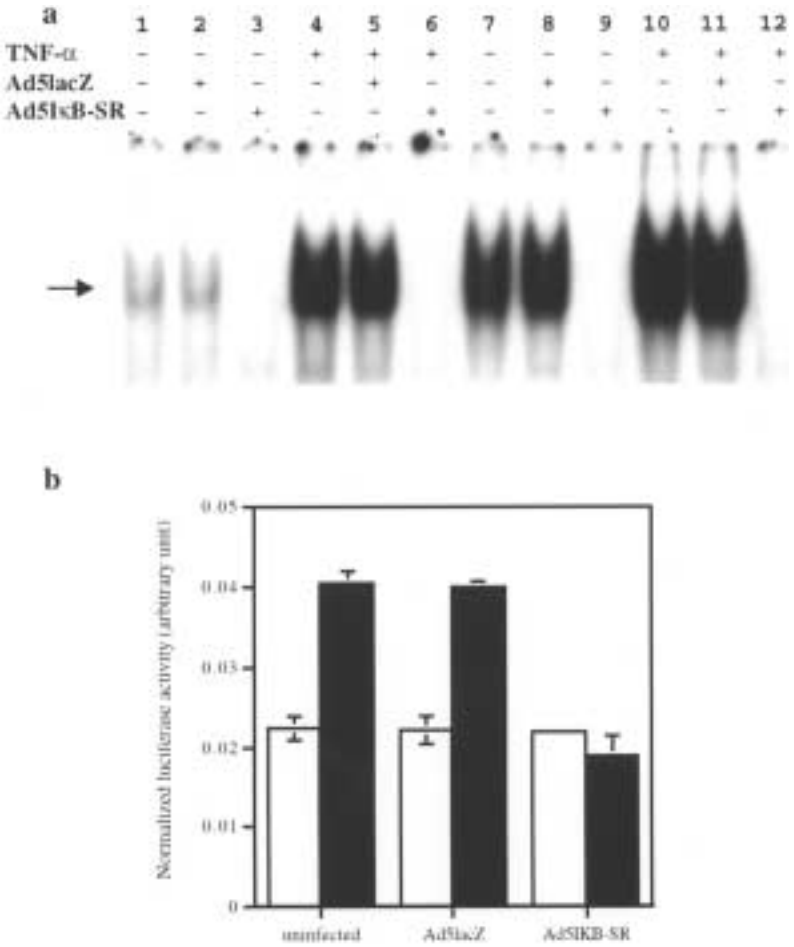


Figure 3. Inhibition of NF-κB activity in Wit49A and RCC1 cells infected by Ad5IκB-SR, an NF-κB super repressor-expressing adenovirus. (a) Wit49A cells (lanes 1–6) and RCC1 cells (lane 7–12) were infected with the indicated adenoviruses for 24 h, after which the cells were treated with human TNF-α (10 ng/ml) or medium alone for 30 min. Crude nuclear extracts were tested for NF-kB site binding by EMSA. The NF-κB DNA binding activity is indicated by the arrow. Ad5lacZ, a lacZ-expressing adenovirus, was used as a control for any nonspecific effects intrinsic to adenoviral infections. (b) Wit49A cells were transiently transfected with pGbp2-luc, a NF-κB inducible luciferase reporter. The cells were then infected with the indicated adenoviruses for 24 h, after which they were either treated with human TNF-α (10 ng/ml) (closed bars) or medium alone (open bars) for 4 h. At the end of the TNF-α treatment, the transcriptional activity of the cells was determined by luciferase assays as described in Materials and Methods. The results represent mean ± SD of duplicate determinations.

volved in the regulation of WT1 gene expression in these two species. The sequence homology includes several putative NF-κB binding sites in the vicinity of the major transcription initiation sites. It has been previously shown that the murine WT1 promoter can be transactivated by the p50 and p65 subunits of NF-κB and that this effect is mediated by a binding site immediately downstream of the major transcription initiation site (14). The fact that this NF-κB binding site (site D in Fig. 1) is conserved in the human WT1 promoter in addition to other NF-κB-like sequences prompted us to investigate whether NF-κB family members play a role in regulating human WT1 expression. We sought to manipulate NF-κB activity in WT1-expressing cells derived from genitourinary or hematopoietic lineage and examine the effect on endogenous WT1 gene expression. We treated cells with TNF-α to globally increase NF-κB activity or infected cells with an adenovirus expressing a mutant IκBα to inhibit NF-κB activity. TNF-α dramatically elevated the NF-κB activity in all cell types used in our study; however, we failed to detect any significant increase in the levels of WT1 transcripts or proteins. Furthermore, when the basal NF-κB activity present in Wit49A and RCC1 cells was completely blocked by adenovirus-mediated expression of the mutant IκBα, we did not observe any effect on the endogenous WT1 gene expression. It has been previously shown that such repression of NF-κB activity by the mutant IκBα is capable of inhibiting the expression of endogenous κB-dependent genes in human intestinal epithelial cells (29). In addition, no in-

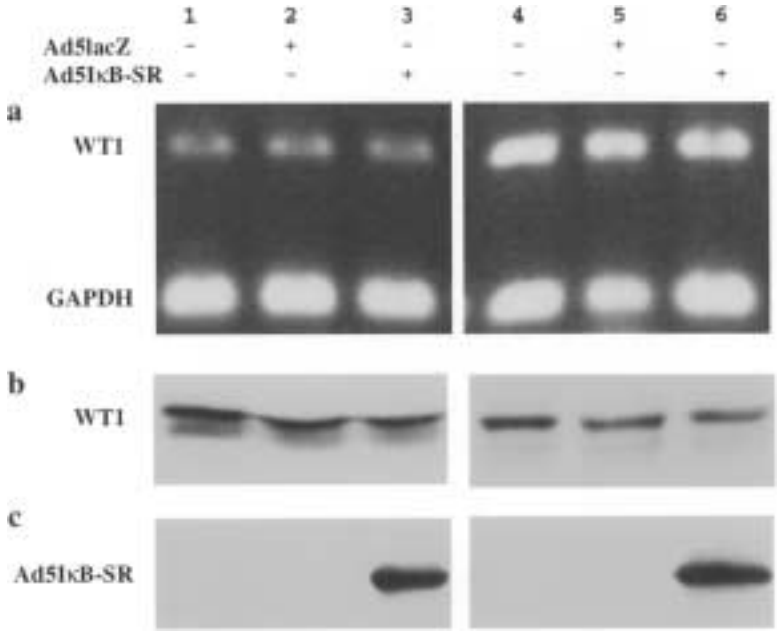


Figure 4. The effect of adenovirus-mediated expression of an NF-κB super repressor on WT1 transcripts and proteins levels in Wit49A and RCC1 cells. Wit49A cells (lanes 1–3) and RCC1 cells (lanes 4–6) were either infected with Ad5IκB-SR, a NF-κB super repressor-expressing adenovirus, or Ad5lacZ, a lacZ-expressing control virus. (a) RT/PCR analysis of WT1 transcript levels. GAPDH was amplified in the same tube at the same time to control for equal loading. (b) Western analysis of WT1 protein levels in uninfected and infected cells. The same amount of total protein as determined by protein assays was used for each sample. (c) Western blot analysis using anti-HA antibodies to detect the expression of the HA epitope-tagged IκBα super repressor protein in Ad5IκB-SR-infected cells.

crease in the endogenous WT1 gene expression was observed when we transiently overexpressed p50 and p65 in Wit49A cells (data not shown). From these results, we conclude that NF-κB does not play a significant role in either initiating or sustaining the expression of WT1 in the human cell lines used in our study.

The discrepancy between our results and the previous finding that NF-κB regulates the mouse WT1 expression might be due to species differences in pathways involved in WT1 gene regulation despite the presence of seemingly conserved *cis* regulatory elements in the human and murine WT1 promoters. Such differences may underlie the molecular basis for differences in phenotype between humans and mice harboring WT1 mutations (30). However, similar to our results in human cells, we were unable to detect changes in endogenous WT1 expression in a murine Leydig cell line, TM3, in response to changes in NF-κB activity (Fig. 6). Therefore, the discrepancy between our results and those of Dehbi et al. (14) cannot be simply accounted for by species differences.

In agreement with our results, Dehbi et al. failed to detect any effect of TNF-α on transcription of the endogenous WT1 promoter in cell lines used in their study (i.e., murine NIH3T3 fibroblasts, human embryonic kidney 293 cells, and human erythroleukemic K562 cells). Given that transcriptional activation of the murine WT1 promoter was most readily observed in transient reporter assays when p50 and p65 were expressed from the strong CMV promoter (14), it is likely that the discrepancy between the in vivo and in vitro situations results from limitations of the experimental setup. For example, differences in the chromatin structure of the endogenous promoter and that of the promoter constructs might explain the poor correlation between NF-κB regulation of the endogenous WT1 promoter and that of the promoter constructs used in transient assays. In fact, our manipulation of the NF-κB activity in Wit49A cells altered the transcriptional activity of a transiently transfected NF-κB reporter construct without changing the endogenous WT1 gene expression. This type of discrepancy has also been described in several studies of the transcriptional regulation of the adenovirus E2 promoter, where transactivation of the promoter was observed when *cis* regulatory sequences were present on a plasmid but not on the viral chromosome (42,47). In addition, cell line differences could be a significant factor in determining experimental outcomes. We used nontransformed cell lines expressing endogenous WT1 that were derived from tissues of genitourinary origin. In contrast, Dehbi et al. used nongenitourinary NIH3T3 cells and adenovirus-transformed 293 cells. We believe that our cell lines provide a more native cellular environment for studying

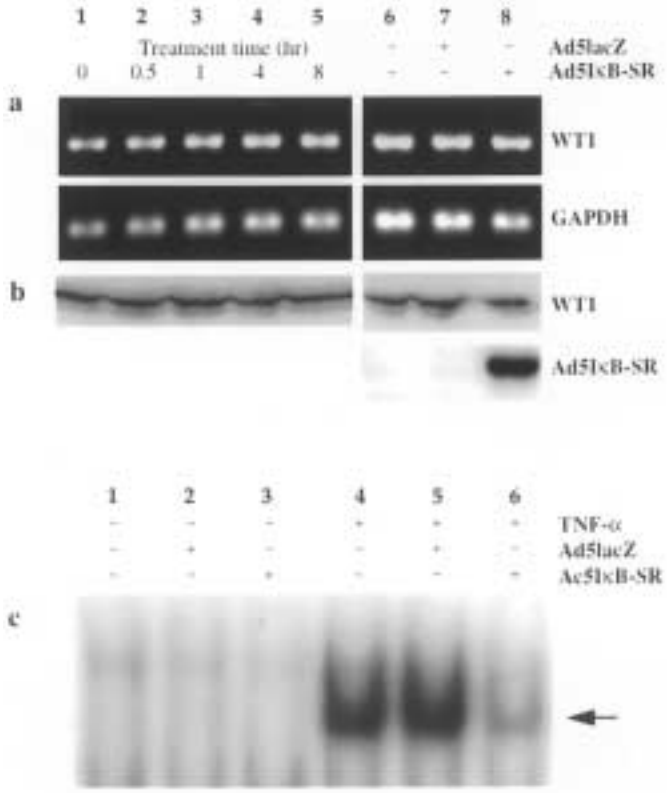


Figure 5. The effect of altering NF-κB activity on WT1 gene expression in erythroleukemia cell line K562. K562 cells were treated with human TNF-α (10 ng/ml) for the indicated amount of time (lanes 1–5) or infected with the indicated adenoviruses (lanes 6–8). (a) RT/PCR analysis of WT1 transcript levels in treated and untreated cells. GAPDH was amplified in the same tube at the same time to control for equal loading. (b) Western analysis of WT1 protein levels in treated and untreated cells (upper panels). The same amount of total protein as determined by protein assays was used for each sample. The lower panel shows the expression of the HA epitope-tagged IκBα super repressor protein in Ad5IκB-SR-infected cells by Western blot analysis using anti-HA antibodies. (c) K562 cells were infected with the indicated adenoviruses for 24 h, after which the cells were treated with human TNF-α (10 ng/ml) or medium alone for 30 min. Crude nuclear extracts were tested for NF-κB DNA binding by EMSA. The NF-κB DNA binding activity is indicated by the arrow.

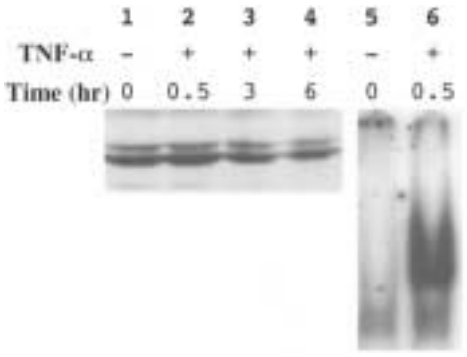


Figure 6. The effect of altering NF-κB activity on WT1 gene expression in TM3 cells. TM3 cells were treated with TNF-α (10 ng/ml) for the indicated amount of time. The level of endogenous WT-1 proteins was detected by Western blot analysis (lanes 1–4). Note that the relative level of the different isoforms of WT-1 protein in TM3 cells is different from what is typically seen in other cell lines used in this study. The induction of the NF-κB DNA binding activity upon TNF-α treatment was shown by EMSA (lanes 5 and 6). The NF-κB DNA binding activity is indicated by the arrow.

the regulation of WT1 expression and therefore are more likely to yield physiologically relevant results.

Our conclusion that NF-κB does not regulate WT1 expression in the genitourinary and hematopoietic cell lines used in this study is substantiated by the very different phenotypes of mice mutant for WT1 and for members of the NF-κB/Rel family. WT1 knock-out mice are embryonic lethal and display severe abnormalities in heart, lung, kidney, and gonad development (30). To date, no defects in mesothelial or genitourinary tissues have been associated with mice lacking NF-κB/Rel family members, including p50, p65, p52, c-Rel, and Rel B [reviewed in (2)]. The fact that some NF-κB/Rel family members are required for the development of blood cells (27,49) and that WT1 is expressed in more than 80% of acute leukemias (6,28,36) raises the possibility that NF-κB regulates WT1 expression primarily in the hematopoietic system. However, this hypothesis was not supported by our finding that the endogenous WT1 gene expression is insensitive to changes in NF-κB

activity in erythroleukemic K562 cells. Taken together, our results show that NF-κB is not a major player in initiating or maintaining WT1 expression. However, our study does not rule out the possibility that NF-κB may regulate WT1 expression under certain circumstances in cooperation with other transcription factors.

The tightly controlled expression pattern of the WT1 gene suggests complex regulation of the locus. To elucidate the mechanisms responsible for WT1 gene regulation, it is necessary to identify regulating factors and the *cis* elements to which these factors bind. However, one needs to be cautious when assessing the physiological importance of such factors and elements identified in transient transfection assays. Our study illustrated the influence of experimental conditions on the sorts of results obtained. It should be emphasized that a more stringent way of studying the regulation of WT1 would be to use physiologically relevant systems, such as Wilms tumor cell lines. Also, more studies focusing on the endogenous gene responses in cell cultures and ultimately, transgenesis experiments studying the regulation of gene expression at the level of a whole animal, will be required to achieve a better understanding of the in vivo factors and signaling pathways involved in controlling WT1 expression.

ACKNOWLEDGMENTS

We thank Drs. Joseph DiDonato and David Brenner for kindly providing the recombinant adenoviruses used in this study, and Drs. Joseph DiDonato and Herman Yeger for helpful discussions. We also thank Drs. Patricia Stanhope-Baker and Sandy Der for their comments on the manuscript. This work was supported by grant DK47101 from the National Institutes of Health.

REFERENCES

1. Andrews, N. C.; Faller, D. V. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res. 19:2499; 1991.
2. Baldwin, A. S., Jr. The NF-kappa B and I kappa B proteins: New discoveries and insights. Annu. Rev. Immunol. 14:649–683; 1996.
3. Ballard, D. W.; Dixon, E. P.; Peffer, N. J.; Bogerd, H.; Doerre, S.; Stein, B.; Greene, W. C. The 65-kDa subunit of human NF-kappa B functions as a potent transcriptional activator and a target for v-Rel-mediated repression. Proc. Natl. Acad. Sci. USA 89:1875–1879; 1992.
4. Beckwith, J. B.; Kiviat, N. B.; Bonadio, J. F. Nephrogenic rests, nephroblastomatosis, and the pathogenesis of Wilms' tumor. Pediatr. Pathol. 10:1–36; 1990.
5. Beg, A. A.; Finco, T. S.; Nantermet, P. V.; Baldwin, A. S., Jr. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: A mechanism for NF-kappa B activation. Mol. Cell. Biol. 13:3301–3310; 1993.
6. Brieger, J.; Weidmann, E.; Fenchel, K.; Mitrou, P. S.; Hoelzer, D.; Bergmann, L. The expression of the Wilms' tumor gene in acute myelocytic leukemias as a possible marker for leukemic blast cells. Leukemia 8:2138–2143; 1994.
7. Brown, K.; Park, S.; Kanno, T.; Franzoso, G.; Siebenlist, U. Mutual regulation of the transcriptional activator NF-kappa B and its inhibitor, I kappa B-alpha. Proc. Natl. Acad. Sci. USA 90:2532–2536; 1993.
8. Call, K. M.; Glaser, T.; Ito, C. Y.; Buckler, A. J.; Pelletier, J.; Haber, D. A.; Rose, E. A.; Kral, A.; Yeger, H.; Lewis, W. H. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell 60:509–520; 1990.
9. Campbell, C. E.; Huang, A.; Gurney, A. L.; Kessler, P. M.; Hewitt, J. A.; Williams, B. R. Antisense transcripts and protein binding motifs within the Wilms tumour (WT1) locus. Oncogene 9:583–595; 1994.
10. Campbell, C. E.; Kuriyan, N. P.; Rackley, R. R.; Caulfield, M. J.; Tubbs, R.; Finke, J.; Williams, B. R. Constitutive expression of the Wilms tumor suppressor gene (WT1) in renal cell carcinoma. Int. J. Cancer 78: 182–188; 1998.
11. Cohen, H. T.; Bossone, S. A.; Zhu, G.; McDonald, G. A.; Sukhatme, V. P. Sp1 is a critical regulator of the Wilms' tumor-1 gene. J. Biol. Chem. 272:2901–2913; 1997.
12. Dehbi, M.; Ghahremani, M.; Lechner, M.; Dressler, G.; Pelletier, J. The paired-box transcription factor, PAX2, positively modulates expression of the Wilms' tumor suppressor gene (WT1). Oncogene 13:447–453; 1996.
13. Dehbi, M.; Pelletier, J. PAX8-mediated activation of the WT1 tumor suppressor gene. EMBO J. 15:4297–4306; 1996.
14. Dehbi, M.; Hiscott, J.; Pelletier, J. Activation of the WT1 Wilms' tumor suppressor gene by NF-kappaB. Oncogene 16:2033–2039; 1998.
15. Devary, Y.; Rosette, C.; DiDonato, J. A.; Karin, M. NF-kappa B activation by ultraviolet light not dependent on a nuclear signal. Science 261:1442–1445; 1993.
16. DiDonato, J.,A.; Mercurio, F.; Rosette, C.; Wu-Li, J.; Suyang, H.; Ghosh, S.; Karin, M. Mapping of the inducible IkappaB phosphorylation sites that signal its ubiquitination and degradation. Mol. Cell. Biol. 16: 1295–1304; 1996.
17. Fraizer, G. C.; Wu, Y. J.; Hewitt, S. M.; Maity, T.; Ton, C. C.; Huff, V.; Saunders, G. F. Transcriptional regulation of the human Wilms' tumor gene (WT1).

Cell type-specific enhancer and promiscuous promoter. J. Biol. Chem. 269:8892–8900; 1994.
18. Fraizer, G. C.; Shimamura, R.; Zhang, X.; Saunders, G. F. PAX 8 regulates human WT1 transcription through a novel DNA binding site. J. Biol. Chem. 272: 30678–30687; 1997.
19. Gerald, W. L.; Gramling, T. S.; Sens, D. A.; Garvin, A. J. Expression of the 11p13 Wilms' tumor gene, WT1, correlates with histologic category of Wilms' tumor. Am. J. Pathol. 140:1031–1037; 1992.
20. Gessler, M.; Poustka, A.; Cavenee, W.; Neve, R. L.; Orkin, S. H.; Bruns, G. A. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. Nature 343:774–8; 1990.
21. Ghosh, S.; May, M. J.; Kopp, E. B. NF-kappa B and Rel proteins: Evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 16:225–260; 1998.
22. Haber, D. A.; Sohn, R. L.; Buckler, A. J.; Pelletier, J.; Call, K. M.; Housman, D. E. Alternative splicing and genomic structure of the Wilms tumor gene WT1. Proc. Natl. Acad. Sci. USA 88:9618–9622; 1991.
23. Haber, D. A.; Park, S.; Maheswaran, S.; Englert, C.; Re, G. G.; Hazen-Martin, D. J.; Sens, D. A.; Garvin, A. J. WT1-mediated growth suppression of Wilms tumor cells expressing a WT1 splicing variant. Science 262:2057–2059; 1993.
24. Hashimura, T.; Tubbs, R. R.; Connelly, R.; Caulfield, M. J.; Trindade, C. S.; McMahon, J. T.; Galetti, T. P.; Edinger, M.; Sandberg, A. A.; Dal Cin, P.; Sait, S. J.; Pontes, J. E. Characterization of two cell lines with distinct phenotypes and genotypes established from a patient with renal cell carcinoma. Cancer Res. 49: 7064–7071; 1989.
25. Hastie, N. D. The genetics of Wilms' tumor—a case of disrupted development. Annu. Rev. Genet. 28:523–558; 1994.
26. Henkel, T.; Machleidt, T.; Alkalay, I.; Kronke, M.; Ben-Neriah, Y.; Baeuerle, P. A. Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. Nature 365:182–185; 1993.
27. Horwitz, B. H.; Scott, M. L.; Cherry, S. R.; Bronson, R. T.; Baltimore, D. Failure of lymphopoiesis after adoptive transfer of NF-kappaB-deficient fetal liver cells. Immunity 6:765–772; 1997.
28. Inoue, K.; Ogawa, H.; Sonoda, Y.; Kimura, T.; Sakabe, H.; Oka, Y.; Miyake, S.; Tamaki, H.; Oji, Y.; Yamagami, T.; Tatekawa, T.; Soma, T.; Kishimoto, T.; Sugiyama, H. Aberrant overexpression of the Wilms tumor gene (WT1) in human leukemia. Blood 89:1405–1412; 1997.
29. Jobin, C.; Panja, A.; Hellerbrand, C.; Iimuro, Y.; Didonato, J.; Brenner, D. A.; Sartor, R. B. Inhibition of proinflammatory molecule production by adenovirus-mediated expression of a nuclear factor kappaB super-repressor in human intestinal epithelial cells. J. Immunol. 160:410–418; 1998.
30. Kreidberg, J. A.; Sariola, H.; Loring, J. M.; Maeda, M.; Pelletier, J.; Housman, D.; Jaenisch, R. WT-1 is required for early kidney development. Cell 74:679–691; 1993.
31. Kumar, A.; Yang, Y. L.; Flati, V.; Der, S.; Kadereit, S.; Deb, A.; Haque, J.; Reis, L.; Weissmann, C.; Williams, B. R. Deficient cytokine signaling in mouse embryo fibroblasts with a targeted deletion in the PKR gene: Role of IRF-1 and NF-kappaB. EMBO J. 16: 406–416; 1997.
32. Luo, X. N.; Reddy, J. C.; Yeyati, P. L.; Idris, A. H.; Hosono, S.; Haber, D. A.; Licht, J. D.; Atweh, G. F. The tumor suppressor gene WT1 inhibits ras-mediated transformation. Oncogene 11:743–750; 1995.
33. Madden, S. L.; Cook, D. M.; Rauscher, F. J. d. A structure-function analysis of transcriptional repression mediated by the WT1, Wilms' tumor suppressor protein. Oncogene 8:1713–1720; 1993.
34. Matsunaga, E. Genetics of Wilms' tumor. Hum. Genet. 57:231–246; 1981.
35. Menke, A.; McInnes, L.; Hastie, N. D.; Schedl, A. The Wilms' tumor suppressor WT1: Approaches to gene function. Kidney Int. 53:1512–1518; 1998.
36. Menssen, H. D.; Renkl, H. J.; Rodeck, U.; Maurer, J.; Notter, M.; Schwartz, S.; Reinhardt, R.; Thiel, E. Presence of Wilms' tumor gene (WT1) transcripts and the WT1 nuclear protein in the majority of human acute leukemias. Leukemia 9:1060–1067; 1995.
37. Miwa, H.; Tomlinson, G. E.; Timmons, C. F.; Huff, V.; Cohn, S. L.; Strong, L. C.; Saunders, G. F. RNA expression of the WT1 gene in Wilms' tumors in relation to histology. J. Natl. Cancer Inst. 84:181–187; 1992.
38. Mundlos, S.; Pelletier, J.; Darveau, A.; Bachmann, M.; Winterpacht, A.; Zabel, B. Nuclear localization of the protein encoded by the Wilms' tumor gene WT1 in embryonic and adult tissues. Development 119:1329–1341; 1993.
39. Pelletier, J.; Schalling, M.; Buckler, A. J.; Rogers, A.; Haber, D. A.; Housman, D. Expression of the Wilms' tumor gene WT1 in the murine urogenital system. Genes Dev. 5:1345–1356; 1991.
40. Pritchard-Jones, K.; Fleming, S.; Davidson, D.; Bickmore, W.; Porteous, D.; Gosden, C.; Bard, J.; Buckler, A.; Pelletier, J.; Housman, D.; Heyningen, V.; Hastie, N. The candidate Wilms' tumour gene is involved in genitourinary development. Nature 346:194–197; 1990.
41. Pritchard-Jones, K. Molecular genetic pathways to Wilms tumor. Crit. Rev. Oncog. 8:1–27; 1997.
42. Rajan, P.; Dhamankar, V.; Rundell, K.; Thimmapaya, B. Simian virus 40 small-t does not transactivate RNA polymerase II promoters in virus infections. J. Virol. 65:6553–6561; 1991.
43. Reddy, J. C.; Licht, J. D. The WT1 Wilms' tumor suppressor gene: How much do we really know? Biochim. Biophys. Acta 1287:1–28; 1996.
44. Ruben, S. M.; Narayanan, R.; Klement, J. F.; Chen, C. H.; Rosen, C. A. Functional characterization of the NF-kappa B p65 transcriptional activator and an alternatively spliced derivative. Mol. Cell. Biol. 12:444–454; 1992.
45. Rupprecht, H. D.; Drummond, I. A.; Madden, S. L.; Rauscher, F. J. r.; Sukhatme, V. P. The Wilms' tumor suppressor gene WT1 is negatively autoregulated. J. Biol. Chem. 269:6198–6206; 1994.

46. Sakamoto, Y.; Yoshida, M.; Semba, K.; Hunter, T. Inhibition of the DNA-binding and transcriptional repression activity of the Wilms' tumor gene product, WT1, by cAMP-dependent protein kinase-mediated phosphorylation of Ser-365 and Ser-393 in the zinc finger domain. Oncogene 15:2001–2012; 1997.
47. Swaminathan, S.; Thimmapaya, B. Transactivation of adenovirus E2-early promoter by E1A and E4 6/7 in the context of viral chromosome. J. Mol. Biol. 258: 736–746; 1996.
48. Wallingford, J. B.; Carroll, T. J.; Vize, P. D. Precocious expression of the Wilms' tumor gene xWT1 inhibits embryonic kidney development in *Xenopus laevis.* Dev. Biol. 202:103–112; 1998.
49. Weih, F.; Carrasco, D.; Durham, S. K.; Barton, D. S.; Rizzo, C. A.; Ryseck, R. P.; Lira, S. A.; Bravo, R. Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kappa B/Rel family. Cell 80:331–340; 1995.
50. Wu, Y.; Fraizer, G. C.; Saunders, G. F. GATA-1 transactivates the WT1 hematopoietic specific enhancer. J. Biol. Chem. 270:5944–5949; 1995.
51. Yeger, H.; Cullinane, C.; Flenniken, A.; Chilton-MacNeil, S.; Campbell, C.; Huang, A.; Bonetta, L.; Coppes, M. J.; Thorner, P.; Williams, B. R. Coordinate expression of Wilms' tumor genes correlates with Wilms' tumor phenotypes. Cell Growth Differ. 3:855–864; 1992.