WT1 Induces Apoptosis through Transcriptional Regulation of the Proapoptotic Bcl-2 Family Member Bak

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Abstract

Wilms’ tumor or nephroblastoma is believed to arise from embryonic nephrogenic rests of multipotent cells that fail to terminally differentiate into epithelium and continue to proliferate. The WT1 tumor suppressor gene, a transcription factor controlling the mesenchymal-epithelial transition in renal development, is mutated in 10% to 15% of Wilms’ tumors. This potentially explains the disordered differentiation and proliferation program of a subset of Wilms’ tumors. To elucidate the role of mutations of WT1 in the etiology of Wilms’ tumor, we used an inducible cellular system for expressing wild-type and tumor-derived missense mutant WT1 proteins. Expression of wild-type WT1, but not mutant proteins, blocked cellular proliferation and DNA synthesis and rapidly induced apoptosis. We showed that wild-type WT1 induced transcription of one of the seven studied proapoptotic genes, Bak. Furthermore, WT1 protein bound to specific DNA-binding sites located in the Bak promoter and Bak was critical to WT1-mediated apoptosis, as overexpression of VDAC2, a specific Bak inhibitor, attenuated WT1-mediated cell death. These data support the hypothesis that Wilms’ tumors arise, in part, because WT1 mutant proteins fail to promote programmed cell death during kidney development.

Introduction

Wilms’ tumor, an embryonic kidney tumor, is one of the most common solid pediatric malignancies and affects ~1 in 10,000 children (1). Wilms’ tumor displays a triphasic histology containing undifferentiated mesenchymal cells, differentiated epithelial cells, and supporting stromal components (reviewed in refs. 2, 3). The Wilms’ tumor susceptibility gene, WT1, was first identified in 1990 (4, 5). Subsequent analysis revealed that WT1 is mutated in 10% to 15% of Wilms’ tumor cases and in >95% of patients with Denys-Drash syndrome (DDS; refs. 6, 7). WT1 is expressed in the condensing mesenchyme, renal vesicle, and glomerular epithelium of the developing kidney. WT1-null mice die by embryonic day 13.5 and exhibit complete agenesis of the kidney as well as abnormal development of the gonads and lungs (8).

The WT1 gene encodes a DNA-binding transcription factor that contains four COOH-terminal C2H2 zinc fingers and a NH2-terminal proline/glutamine-rich transactivation domain. Alternative splicing of WT1 pre-mRNA generates four major isoforms containing or lacking 17 amino acids proximal to the zinc finger region (exon 5) and/or a 3–amino acid insertion (KTS) between the third and the fourth zinc fingers (9). The KTS insertion disrupts the critical spacing between the zinc fingers resulting in a severe reduction of DNA binding to a consensus WT1-binding site (10). However, the WT1 (+KTS) isoforms associate with pre-mRNA splicing machinery and likely play a role in RNA processing (11, 12). The WT1 (−KTS) isoforms bind to GC-rich sites (known as egr1, WRE, and WTE elements) as well as (TCC)n repeat elements (reviewed in refs. 2, 3) and have been shown to either activate or repress transcription. The WT1A isoform, lacking both exon 5 and the KTS insertion, shows the highest specificity for GC-rich consensus DNA-binding sites (9, 13, 14). Genes with promoters containing GC-rich WT1-binding sites have been shown to be bona fide in vivo targets of WT1 and biologically relevant for kidney development and function (15–19).

Wilms’ tumors are believed to arise from nephrogenic rests, lesions composed of undifferentiated blastemal cells, which are present during kidney development but normally disappear after birth (20). Nephrogenic rests that fail to undergo apoptosis are considered premalignant precursors to Wilms’ tumor and their presence in the kidneys of Wilms’ tumor patients highlights a constitutional abnormality of renal differentiation. This suggests that deregulation of apoptosis could be deleterious for kidney development and promote tumorigenesis.

The process of apoptosis is under the control of a variety of internal and external signals that activate the mitochondrial pathway or the death receptor pathway, respectively (reviewed in refs. 21–23). Members of the multidomain Bcl-2 gene family play a key regulatory role in the mitochondrial pathway by either suppressing or promoting apoptosis. The antiapoptotic members include Bcl-2, Bcl-XL, Bfl-1, Bcl-W, and Mcl-1, whereas the proapoptotic members include Bax, Bak, and Bik. Activated Bax/Bak induces apoptosis by causing outer mitochondrial membrane permeabilization and release of cytochrome c, leading to cleavage of caspase-9, caspase-3, and eventually poly(ADP-ribose) polymerase (PARP). The activation of Bax/Bak is blocked by Bcl-2/Bcl-XL that function as decoy receptors. Bik indirectly promotes apoptosis by binding and neutralizing Bcl-2/Bcl-XL. Ultimately, it is the net balance between antiapoptotic and proapoptotic proteins in the cell that determines cell fate.

In a previous study with WT1 proteins harboring point mutations in the NH2-terminal regulatory domain, we observed that the transcriptional activation function of WT1 is most critical for its ability to suppress growth (24). The WT1 mutants were defective for transcriptional activation of reporter genes and were unable to suppress growth in colony formation assays. However, the point mutations did not render WT1 completely inactive because the mutants were still competent for transcriptional repression of target genes. To further understand the molecular mechanisms by which WT1 regulates cell growth and to identify
novel target genes of WT1, we were successful in establishing inducible cell lines for wild-type WT1A and two of the previously characterized mutant proteins, WT1A-F112Y and WT1A-P129L. These mutants were isolated from rat kidney tumors caused by administering N-nitroso-N'-methylurea to newborn animals (25). The resultant kidney tumors resembled Wilms’ tumors histopathologically. The nucleotide transversion causing the Phe→Tyr substitution at codon 112 arose in four independent tumors and the Pro→Leu substitution at codon 129 was observed once. Here, we show that WT1A causes growth arrest and promotes apoptosis by up-regulating the expression of proapoptotic genes, particularly Bak, whereas mutant WT1A proteins are defective for these functions.

Materials and Methods

Establishment of Saos-2 WT1 tetracycline-repressible cell lines. Saos-2 tetracycline founder cells (STAS) containing a tetracycline-repressible transactivator (gift of Dr. Daniel A. Haber, Massachusetts General Hospital, Boston, MA; ref. 26) were cultured in DMEM with 10% FCS, 2 mmol/L 1-glutamine, 100 units/mL penicillin, 100 units/mL streptomycin, 0.5 mg/mL G418, and 1 μg/mL tetracycline. Wild-type and mutant Saos-2 Tet-WT1A cell lines were created by transfecting 2 μg pUIHD-WT1A, WT1A-112, and WT1A-129 plasmids along with 200 ng pBabe-puro into STAS cells using the FuGene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN). Positive clones were selected in complete medium containing 0.5 mg/mL G418, 0.2 μg/mL puromycin, and 0.5 μg/mL tetracycline and screened by dot-blot hybridization for WT1 protein.

Immunoblotting. Saos-2 Tet-WT1A cell lines were induced for 24 to 48 hours with medium containing the indicated concentrations of tetracycline. After induction, cells were lysed in SDS buffer [60 mmol/L Tris (pH 6.9), 2% SDS, protease inhibitors (Roche Diagnostics)], subjected to SDS-PAGE, and blotted with the following antibodies: WT1 (C-19) and PARP (Cell Signaling Technology, Beverly, MA); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon International, Temecula, CA). Nonspecific bands were blocked with 5% nonfat dry milk in TBS (0.1% Tween 20) and peroxidase-labeled rabbit IgG or a WT1 antibody (c19) were added and reacted for 1 h at room temperature. Blots were washed and reacted with a chemiluminescent detection reagent (Roche Diagnostics), and bands were visualized and quantified using a Fuji FLA-5000 imaging device. The absorbency was recorded at 490 nm.

[3H]Thymidine incorporation assay. Saos-2 Tet-WT1A cell lines were induced with different concentrations of tetracycline for 24 hours. After induction, cells were washed and incubated with the appropriate medium containing 1 μCi/mL methyl-[3H]thymidine for 5 hours. After labeling, cells were fixed in methanol and lased in 0.25% SDS/0.25 mol/L NaOH. Lysates were neutralized with 40 μL of 1 N HCL and counted by liquid scintillation.

Fluorescence-activated cell sorting analysis. Saos-2 Tet-WT1A cell lines were induced for 24, 48, or 72 hours at indicated concentrations of tetracycline. At each time point, floating cells were combined with trypsinized cells for analysis by propidium iodide staining or terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay. For propidium iodide staining, cells were fixed in ice-cold 70% ethanol for 10 minutes at 4°C and resuspended in staining solution (100 μg/mL RNase A, 50 μg/mL propidium iodide in PBS). For TUNEL assay, an In situ Cell Death Detection kit (Roche Diagnostics) was used according to the manufacturer’s instructions. Labeled cells were analyzed using a Becton Dickinson (Franklin Lakes, NJ) FACSCalibur flow cytometer and CellQuest software. Saos-2 Tet-WT1A cell lines were infected with an amphotrophic retrovirus harboring the expression vector MSCV-IBRS-green fluorescent protein (GFP) for expression of GFP with VDAC1 or VDAC2 (gift of Dr. Stanley Korsmeyer, Dana-Farber Cancer Institute, Boston, MA; ref. 27). GFP-expressing cells were sorted and treated as above.

**R**nase protection assay. Total RNA from Saos-2 Tet-WT1A cell lines grown with trilzol reagent (Invitrogen, Carlsbad, CA), treated with DNase I, and reverse transcribed by SuperScript II reverse transcriptase according to instructions in Invitrogen. CDNA templates (3 μL; 1:100 dilution) were used with the Quantitect SYBR Green PCR kit (Qiagen, Valencia, CA) and monitored by the DNA Engine Opticon System (MJ Research, Waltham, MA). The PCR conditions were 95°C for 15 minutes, 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds for 40 cycles. Data were calculated as fold induction relative to 1,000 ng/mL tetracycline concentration after normalizing with GAPDH. The primers were GAPDH (5'-CCTAGGGGCAGT-3' and 5'-GAAGTTCGAAAGTGTTCCGT-3'), Bak (5'-TGGAGAATTCCTCTGGTTCAAG-3' and 5'-CTCTCAACGCCTTCAGCCTG-3'), Bik (5'-CCGATTTGGGCTGCGACTG-3' and 5'-GAATCCCTGATGGTCGCT-3'), Bax (5'-TCCAGACTGCTCCACCAAGAAG-3' and 5'-GCAAGTTGAAAGAGCGACACG-3'), and Bcl-2 (5'-GAATCGAGATGGCGAGATGTC-3' and 5'-ACGGCGGAGAAGATGC-3').

Electrophoretic mobility shift assay. Double-stranded oligonucleotide probes corresponding to the two WT1-binding sites within the murine BAK promoter were created by annealing complementary single-stranded oligonucleotides and end-labeling with [α-32P]dCTP and Klenow. The oligonucleotide probes were site1, 5'-CAGAGAGAACCCTGGGGGGGCTG-3' CTCCTTCCCATATT-3' and site2, 5'-GCCGGGGGCGTCCCTTCCCATATT-3'. Synthetic WT1 oligonucleotide and mutant pSp64-WTIA (24, 28) and insertless pSp64 control vector using rabbit reticuloocyte lysate (Promega). DNA-binding reactions with in vitro translated WT1 proteins were done as described previously (16), except that each lysate (2 μL) was preincubated at 4°C for 30 minutes before addition of probe. Excess (50×, 250×, and 500×) cold site1, site2, or mutant nuclear factor-κB (NF-κB; GOCATAGGTCC) oligonucleotides or 1 μg rabbit IgG or a WT1 antibody (c19) were added during the preincubation period as competitors.

Plasmid construction and luciferase assay. A 1.2-kb section of the mouse BAK promoter (Genbank accession no. Y13232) from -1,332 to -138, relative to the start of transcription, was PCR amplified using mouse genomic DNA as template and 5' (G5TACCCACCAGCCCCCTCCCATTTCTGGGCT) and 3' (5'-AGATCTTTTCTGGCTTTGTTGACTG) primers, introducing KpnI and BglII sites. The PCR product was cloned into the promoterless luciferase vector pGL2-Basic (Promega). Internal deletions of WT1-binding sites were created using QuiChange Site-Directed Mutagenesis (Stratagene, Cedar Creek, TX). Construction of WT1A wild-type and mutant expression vectors was described previously (24). For transient cotransfection assays, the mouse Bak promoter were created by annealing complementary single-stranded oligonucleotides and end-labeling with [α-32P]dCTP and Klenow. The oligonucleotide probes were site1, 5'-CAGAGAGAACCCTGGGGGGGCTG-3' CTCCTTCCCATATT-3' and site2, 5'-GCCGGGGGCGTCCCTTCCCATATT-3'. Synthetic WT1 oligonucleotide and mutant pSp64-WTIA (24, 28) and insertless pSp64 control vector using rabbit reticuloocyte lysate (Promega). DNA-binding reactions with in vitro translated WT1 proteins were done as described previously (16), except that each lysate (2 μL) was preincubated at 4°C for 30 minutes before addition of probe. Excess (50×, 250×, and 500×) cold site1, site2, or mutant nuclear factor-κB (NF-κB; GOCATAGGTCC) oligonucleotides or 1 μg rabbit IgG or a WT1 antibody (c19) were added during the preincubation period as competitors.

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**Results**

Generation of mutant WT1A-inducible cell lines. Inducible expression of WT1B and WT1D in osteosarcoma cells (Saos-2) was previously used successfully for identifying endogenous WT1 target genes involved in growth control and differentiation (26). This same tetracycline-repressible system was used to create inducible...
cell lines for WT1A wild-type and point mutants WT1A-F112Y and WT1A-P129L. Tight inducible expression was observed for all clones after withdrawal of tetracycline for 24 hours (Fig. 1A, top). Wild-type and WT1A-129 proteins were expressed at comparable levels, but WT1A-112 protein was consistently expressed at significantly lower levels. The lower protein levels correlated with lower mRNA levels (data not shown). The kinetics of induction was also slightly delayed (data not shown). WT1A-112 was first detected at 24 hours after induction rather than at 6 to 12 hours as seen for wild-type and WT1A-129. The lower RNA expression was not due to chromosome integration site effects because all clones of WT1A-112 had similar low levels of expression. It is possible that the RNA for the mutant WT1A-112 is less stable and more susceptible to degradation. In addition, the WT1A-112 mutant protein appears as a doublet. This may be the result of introduction of a potential new phosphorylation site due to the

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substitution of a tyrosine residue for a phenylalanine in the mutant protein.

To compensate for the disparate protein levels, the expression of the wild-type WT1A was down-regulated by titrating the concentration of tetracycline in the medium (Fig. 1A, middle). Incubation with 6 ng/mL tetracycline in the growth medium was sufficient to achieve expression levels of wild-type WT1A protein comparable with those of the WT1A-112 clones. Expression of wild-type WT1A at this dosage was used in all subsequent comparisons with WT1A-112.

The expression of wild-type WT1A was also comparable with WT1 endogenously expressed in the embryonic kidney (Fig. 1A, bottom). In the developing kidney, WT1 expression peaks at embryonic day 15.5 and is localized to the S-shaped bodies of the condensing metanephric mesenchyme (2, 3). The level of WT1 in the presence of 6 and 0 ng/mL tetracycline was equal to or twice, respectively, the level detected at embryonic day 16. In fact, the amount of WT1 expressed during kidney development was underestimated because the mesenchymal cells that express WT1 were not dissected from the kidney; instead, the whole kidney was used in the lysate.

Missense mutations attenuate the effects of wild-type WT1A on cellular proliferation and DNA synthesis. We determined the effect of wild-type and mutant WT1A proteins on the cellular proliferation rate. As shown in Fig. 1B, tetracycline had no effect on the proliferation rate of control cells. In contrast, a stepwise removal of tetracycline from the growth medium of wild-type WT1A cells led to a dramatic, dose-dependent inhibition of proliferation, whereas a lesser inhibition of proliferation was observed with WT1A-129 cells. Some cell growth was still observed during full induction of WT1A-129 but not with full induction of wild-type WT1A. In contrast, induction of WT1A-112 had no effect on cellular growth.

To directly assess the effect of WT1 on DNA synthesis, [3H]thymidine incorporation was measured after WT1A induction. Stepwise removal of tetracycline from the medium of WT1A cells resulted in a marked dose-dependent inhibition of [3H]thymidine incorporation, particularly in the wild-type cell line (Fig. 1C). Full and partial induction of wild-type WT1A inhibited [3H]thymidine incorporation by 97% and 74%, respectively, whereas mutant proteins WT1A-129 and WT1A-112 elicited less robust responses of 74% and 50%, respectively. Therefore, induction of wild-type WT1A leads to an almost complete inhibition of cellular proliferation, whereas mutant WT1A proteins have a significantly weaker effect.

Missense mutations impair the ability of WT1A to induce apoptosis. Because expression of WT1A clearly inhibited cellular proliferation and growth, DNA content was measured by fluorescence-activated cell sorting (FACS) analysis after WT1A induction to determine whether this was due to cell cycle arrest or cell death.

After full induction of wild-type WT1A for 24 hours, 20% of the cells had an apoptotic sub-G1 DNA content; in the presence of 6 ng/mL tetracycline, 5% of the cells were apoptotic (data not shown). In contrast, full induction of either WT1A-129 or WT1A-112 for 24 hours did not result in cell death (0.7% and 0.4%, respectively).

After 48 hours, full or limited (0 or 6 ng/mL tetracycline) expression of wild-type WT1A induced apoptosis in 56% of cells, whereas full induction of WT1A-129 and WT1A-112 only led to 11% and 5% induction of apoptosis, respectively (Fig. 1D). By 72 hours of incubation, even the lowest level of expression of wild-type WT1A was able to kill >61% of the cells compared with the 25% and 2% cell death seen with WT1A-129 and WT1A-112, respectively (data not shown).

To confirm the cell cycle analysis data, a TUNEL assay was done on these cells. At 24 hours of induction, little to no significant apoptosis was observed with any of the cell lines (Table 1A). However, by 48 hours of induction, 87.8% of cells expressing wild-type WT1A were apoptotic. Even cultures expressing a low dose of WT1A exhibited significant amounts of apoptosis (33%) compared with WT1A-112-expressing cells (7.7%). Consistent with the cell

| Table 1. TUNEL analysis of wild-type and mutant WT1A cell lines |
|-----------------|----------|----------|
| Cell line       | % Tunnel positive |
| WT1A (6 ng/mL tetracycline) | 8.1      | 5.3      | 33.0   |
| WT1A            | 8.1      | 11.0     | 87.8   |
| WT1A-129        | 5.2      | 7.4      | 45.2   |
| WT1A-112        | 6.0      | 4.2      | 7.7    |

NOTE: Cells expressing wild-type and mutant WT1A were incubated in the presence of 1,000 and 0 ng/mL tetracycline (6 ng/mL tetracycline for WT1A). Where indicated, WT1A-expressing cells were infected with the MIGR1 retroviral vector expressing GFP, GFP + VDAC1, or VDAC2. At the indicated time, floating cells were combined with adherent cells, labeled with fluorescein or TAM red by TUNEL reaction, and processed by FACS analysis.

Figure 1. Differential effects of wild-type and mutant WT1A on growth and apoptosis of Saos-2 cells. A, top, different clones of Saos-2 cells expressing wild-type and mutant WT1A were incubated for 24 hours in either the presence (1 µg/mL) or absence of tetracycline (Tet). Lysates were prepared for immunoblotting with an anti-WT1 antibody. WT1A-112 often appears as a doublet. Middle, wild-type WT1A cells were incubated for 24 hours in 1 ng/mL, 6 ng/mL, 3 ng/mL, or 0 ng/mL tetracycline. Protein expression levels were compared with two WT1A-112 cell lines. The membrane was stripped and rebotted for GAPDH as a loading control. Bottom, expression of wild-type WT1A induced for 24 hours in 6 or 0 ng/mL tetracycline was compared with the level of endogenous WT1 in kidney extracts derived from mice at embryonic day 15.5 and is localized to the S-shaped bodies of the condensing metanephric mesenchyme (2, 3). The level of WT1 in the presence of 6 and 0 ng/mL tetracycline was equal to or twice, respectively, the level detected at embryonic day 16. In fact, the amount of WT1 expressed during kidney development was underestimated because the mesenchymal cells that express WT1 were not dissected from the kidney; instead, the whole kidney was used in the lysate.
cycle analysis, the WT1A-129 mutant had an intermediate effect (45.2%) on apoptosis. Together, these experiments show that expression of wild-type WT1A is able to induce programmed cell death in Saos-2 cells, whereas mutant WT1A-129 was significantly deficient and WT1A-112 was completely defective for this activity.

**Wild-type WT1A activates both extrinsic and intrinsic apoptotic pathways.** Because apoptosis can be triggered by external or internal signaling pathways that lead to cleavage and activation of either caspase-8 or caspase-9, respectively (reviewed in ref. 29), Western blots were done to determine which pathway was activated by WT1 (Fig. 2). Upon expression of wild-type WT1A for 24 or 48 hours, there was significant PARP cleavage indicative of apoptosis in general. Caspase-3 cleavage, also common to both intrinsic and extrinsic pathways, was evident at full induction of WT1A at 24 and 48 hours, whereas there was significantly less activation of caspase-3 in WT1A-112 cells. Interestingly, both caspase-8 and caspase-9 were activated by wild-type WT1A. Although activation of caspase-8 is usually associated with death receptor–mediated apoptosis, it also feeds into the mitochondrial caspase-9 pathway through activation of tBid. Hence, it seems that WT1A activates both intrinsic and extrinsic apoptotic pathways.

**Wild-type WT1A activates transcription of the proapoptotic factor Bak.** Next, using a multiplex RPA, we determined whether several known apoptotic-related genes were regulated by WT1A (Fig. 3A). The results were calculated as fold change relative to the 1 µg/mL tetracycline condition and normalized to GAPDH (Fig. 3B). Of the nine genes probed, only Bak was significantly up-regulated by wild-type WT1A. In support of our result, Bak gene expression can be found in the human kidney, particularly in the podocytes where WT1 expression is also seen (30, 31). Induction of wild-type WT1A for 24 hours led to a dose-dependent expression of Bak, ranging from 9- to 23-fold, whereas WT1A-129 and WT1A-112 induced Bak expression 4- and 2-fold, respectively. Consistent with the RNA expression, expression of Bak protein was also induced by wild-type WT1A, to a lesser extent by WT1A-129 and not at all by WT1A-112 (data not shown). Expression of the related mitochondrial death effector, Bax, was not affected by WT1A. The expression of two genes, Bcl-2 and Bfl-1, were below the level of detection by this assay. The RPA results were confirmed by real-time PCR analysis. Bak showed a stepwise induction of 3-fold at 10 hours up to 11-fold by 24 hours (Fig. 3C). The early induction of Bak following WT1A expression strongly suggests that Bak is a direct target of WT1. As in the RPA, WT1A-112 did not induce Bak expression and wild-type WT1A did not induce Bax expression. Microarray analysis of these cell lines was consistent with the RNA protection and real-time PCR results, except that Bik, a BH3-only gene that was not affected in the RPA, was induced up to 8-fold in the real-time PCR and 2.6-fold by microarray analysis.5 Consistent with the RPA, the effect of WT1A on Bcl-2 expression could not be determined because the RNA level was below detectable limits by real-time PCR assay and microarray analysis.

**WT1 can bind to the murine Bak promoter.** The mouse Bak gene, including promoter sequences, was cloned previously located in the human kidney, particularly in the podocytes where WT1 expression is also seen (30, 31). Induction of wild-type WT1A for 24 hours led to a dose-dependent expression of Bak, ranging from 9- to 23-fold, whereas WT1A-129 and WT1A-112 induced Bak expression 4- and 2-fold, respectively. Consistent with the RNA expression, expression of Bak protein was also induced by wild-type WT1A, to a lesser extent by WT1A-129 and not at all by WT1A-112 (data not shown). Expression of the related mitochondrial death effector, Bax, was not affected by WT1A. The expression of two genes, Bcl-2 and Bfl-1, were below the level of detection by this assay. The RPA results were confirmed by real-time PCR analysis. Bak showed a stepwise induction of 3-fold at 10 hours up to 11-fold by 24 hours (Fig. 3C). The early induction of Bak following WT1A expression strongly suggests that Bak is a direct target of WT1. As in the RPA, WT1A-112 did not induce Bak expression and wild-type WT1A did not induce Bax expression. Microarray analysis of these cell lines was consistent with the RNA protection and real-time PCR results, except that Bik, a BH3-only gene that was not affected in the RPA, was induced up to 8-fold in the real-time PCR and 2.6-fold by microarray analysis.5 Consistent with the RPA, the effect of WT1A on Bcl-2 expression could not be determined because the RNA level was below detectable limits by real-time PCR assay and microarray analysis.

**WT1A activates the Bak promoter.** To confirm that WT1 activates the transcription of Bak, the regulatory regions of the Bak gene, encompassing both WT1-binding sites, were cloned into a promoterless luciferase reporter construct. The Bak promoter reporter plasmid was transfected into 293T cells with expression vectors for wild-type or mutant WT1A. WT1A activated the promoter 10-fold compared with empty vector control (Fig. 5A). Comparable with data from the inducible cell lines, WT1A-129 activated the promoter weakly (1.6-fold), whereas WT1A-112 did not activate the Bak promoter at all. An internal deletion of site1 from the promoter had a minimal effect on transcription; however, deletion of site2 or deletion of both sites severely impaired the ability of WT1A to activate the promoter (Fig. 5B). This result supports the idea that WT1A binds strongly to the WTE site on the Bak promoter and activates transcription and is consistent with our previous observations that mutant WT1A proteins are defective for transcriptional activation (24).

**A specific Bak inhibitor attenuates WT1A-induced apoptosis.** The mitochondrial, voltage-dependent anion channel protein, VDAC2, specifically complexes with the inactive form of Bak (27) and selectively blocks Bak-dependent but not Bax-dependent apoptosis. The related protein VDAC1 does not bind to

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Bak and has no effect on Bak-dependent apoptosis. We used VDAC2 as a specific inhibitor of Bak in Saos-2 cells to determine if Bak is a component of the WT1-induced apoptotic pathway. WT1A wild-type and mutant cells were infected with retrovirus expressing either GFP alone or GFP plus VDAC1 or VDAC2. Following induction of wild-type or mutant WT1A, a TUNEL assay was done to measure the level of apoptosis of infected cells. In the presence of VDAC2, there was a 30% decrease in apoptosis (37% versus 25%) induced by wild-type WT1A, whereas VDAC1 had no effect on cell viability (Table 1B). As expected, WT1A-112 did not induce apoptosis nor were these cells affected by the expression of VDAC1 or VDAC2.

Discussion

WT1 induces apoptosis through up-regulation of the Bak pathway. Wild-type WT1 blocks cell growth and proliferation of Saos-2 cells and induces apoptosis, whereas tumor-derived mutants were attenuated for this effect. Consistent with this, WT1 activates the transcription of the endogenous proapoptotic gene Bak. Transient transfections and EMSAs show that WT1 stimulates the Bak promoter through a direct interaction. WT1 binds to both low- and high-affinity sites within the Bak promoter. Our results are consistent with Nakagama et al. (32) who identified the WT1 high-affinity binding site WTE (5'-GCGTGGGAGT-3') by an in vitro selection assay. This site has been found in several other promoters that are up-regulated by WT1 in vivo (15, 33). Although tumor-derived WT1 mutants bind to high- and low-affinity sites within the Bak promoter, the mutant WT1 proteins only marginally activate the promoter in vitro and weakly induce the expression of the gene in vivo. In addition, the proapoptotic BH3-only gene, Bik, is induced by WT1. Bak-induced apoptosis is a caspase-dependent event (34) and we show that induction of apoptosis in our system activates caspase cleavage. Moreover, we showed that blockade of activated Bak by the specific inhibitor VDAC2 reduced the ability of WT1 to induce apoptosis.

Together, these data suggest that one mechanism by which WT1 induces apoptosis and thereby limits cell growth is by resetting the effective ratio between proapoptotic and antiapoptotic Bcl-2 family members. Changes in apoptotic set points may occur by down-regulation of antiapoptotic proteins or up-regulation of proapoptotic multidomain Bcl-2 proteins and/or BH3-only proteins. WT1 expression in our system is associated with specific increases in the multidomain Bak protein and BH3-only protein Bik. In this regard,
WT1 shares some similarity with the tumor suppressor gene p53. p53 clearly stimulates apoptosis through transcriptional activation of target genes, including Bcl-2 family members, such as the multidomain Bax gene and the BH3-only genes Noxa, PUMA, and Bid (35–39). This leads to stimulation of the intrinsic mitochondrial apoptosis pathway and cleavage of caspase-9. Intriguingly, expression of WT1 in Saos-2 cells was also associated with caspase-8 cleavage as is p53 expression. The mechanism by which WT1 stimulates the extrinsic apoptotic pathway remains to be discovered.

**WT1 as a tumor suppressor gene.** In humans, disruption of WT1 leads to tumorigenesis in kidneys and mesothelial-derived cell types. Expression of WT1 suppresses growth of Wilms’ tumors as well as other malignant cell types (40–42). In addition, reintroduction of wild-type WT1 into a Wilms’ tumor cell line expressing an aberrantly spliced WT1 transcript suppresses cell growth (40). Although WT1 is not mutated in the majority of sporadic tumors, the similar phenotypes of wild-type and mutant WT1 tumors suggests that pathways normally modulated by WT1 might be defective in sporadic Wilms’ tumors. The absence of WT1 in a Wilms’ tumor suggests that it may not have a normal apoptotic set point. In tumors where WT1 is present, it would be reasonable to search for defects in members of the intrinsic apoptotic pathway. Again, this resembles the case of p53. Tumor cells harboring mutant p53 may not undergo certain apoptotic responses and disruption of a downstream p53 target gene, such as PUMA, prevents all p53-mediated apoptosis (43).

There are contradictory reports as to whether WT1 induces or represses apoptosis by regulating Bcl-2 expression. Mayo et al. showed a correlation between Bcl-2 and WT1 expression in sporadic

![Figure 5](image-url)

**Figure 5.** Wild-type WT1A activates the mouse Bak promoter in transfected cells. A, the Bak reporter plasmid was transiently cotransfected into 293T cells along with an expression vector for WT1A, WT1A-112, or WT1A-129 and a Renilla internal control vector. At 48 hours after transfection, a dual luciferase assay was done. B, WT1-binding sites within the Bak promoter were deleted either singly (Del1 and Del2) or in combination (dblDel). The mutated Bak promoters were cotransfected into 293T cells with either empty expression vector or one expressing WT1A. For both (A) and (B), the luciferase data are normalized to total protein concentration and representative of three experiments each done in triplicate. Bars, SD.
WT1 Regulates Proapoptotic Bak

WT1 in kidney development and tumor formation. Nephrogenic rests are clusters of embryonal cells that abnormally persist in the postnatal kidney. A fraction of nephrogenic rests may become neoplastic and cause Wilms’ tumors and other neoplasms. Intralobar nephrogenic rests are thought to occur early in development and are highly prevalent in kidneys of WAGR and DDS patients, two syndromes that are linked to low or abnormal expression of WT1 (50, 51). It is possible that the role of WT1 during development is to determine cell fate, a choice between differentiation and apoptosis. The failure of tumor-derived WT1 mutants to activate the proapoptotic pathway may contribute to the development of Wilms’ tumors. During normal renal development, WT1 is highly up-regulated in the condensing metanephric mesenchyme and plays a controlling role in the mesenchymal-epithelial transition. During this transition, apoptosis occurs concomitantly with differentiation. High WT1 levels, by altering expression of Bcl-2 family members, may allow for the loss of excess renal cells during the normal development of the kidney. In this context, loss of WT1 function could lead to both disordered differentiation and a deficit of normal cell death contributing to the development of Wilms’ tumor and the kidney anomalies associated with WT1 mutations.

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