WT1-Mediated Growth Suppression of Wilms Tumor Cells Expressing a *WT1* Splicing Variant

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A human Wilms tumor cell line (RM1) was developed to test the tumor suppressor activity of WT1, a zinc finger transcription factor that is expressed in the developing human kidney and is mutationally inactivated in a subset of Wilms tumors. Transfection of each of four wild-type *WT1* isoforms suppressed the growth of RM1 cells. The endogenous *WT1* transcript in these cells was devoid of exon 2 sequences, a splicing alteration that was also detected in varying amounts in all Wilms tumors tested but not in normal kidney. Production of this abnormal transcript, which encodes a functionally altered protein, may represent a distinct mechanism for inactivating *WT1* in Wilms tumors.

The ability of a cloned gene to revert transformed properties of tumor cells has been central to the characterization of tumor suppressor genes. Both p53 and the retinoblastoma susceptibility gene product (Rb) demonstrate dramatic growth-suppressing properties when they are reintroduced into cells containing inactivated endogenous genes (*1*). Similar studies in Wilms tumors have been complicated by the existence of multiple genetic loci implicated in different subsets of tumors (*2*) and by the unavailability of appropriate target cell lines. Mutations that inactivate *WT1* have been detected in ~10% of sporadic Wilms tumors and appear to be an early genetic event, present in precursor lesions known as nephrogenic rests (*3*). To obtain an appropriate cell line for studying *WT1* function, we inoculated minced human Wilms tumors subcutaneously into nude mice and then adapted the tumor explants to growth in vitro (*4*). Of 14 cell lines, one (RM1) could be propagated indefinitely in tissue culture without loss of tumorigenic potential. Subcutaneous inoculation of RM1 cells into nude mice yielded tumors with the histologic characteristics of Wilms tumors (Fig. 1).

We used RM1 cells to investigate whether *WT1* could revert their transformed phenotype. The wild-type *WT1* transcript exists as four isoforms, resulting from the presence or absence of two alternatively spliced sequences (*5*). Alternative splice I results from the variable insertion of exon 5, located between the transactivation and DNA binding domains of WT1. Alternative splice II results from the use of a different splice donor site at the 3′ end of exon 9, leading to the insertion of three amino acids (Lys-Thr-Ser or KTS) between WT1 zinc fingers 3 and 4; this change alters the protein's DNA binding properties (*6, 7*). RM1 cells were transfected by lipofection with cytomegalovirus (CMV)-driven expression constructs encoding the four *wt1* murine isoforms (*8*) along with the neomycin resistance (*neo*^{r}) gene. Each *WT1* isoform independently suppressed the emergence of *neo*^{r} colonies, as did a combination of the four isoforms (Table 1). By contrast, *WT1-201*, a point mutant of *WT1* that was identified in a Wilms tumor and whose product has altered transactivational properties (*9*), had no growth-suppressing effect.

Stable cell lines were derived from six RM1 colonies expressing the transfected *WT1* construct. RNA–polymerase chain reaction (PCR) amplification and nucleotide sequencing of *WT1* mRNA with mouse-specific primers did not reveal any mutations in the transfected murine *wt1* gene. However, expression of the transfected constructs was low in these cell lines, which suggests that there was selection against overexpression of wild-type *WT1*. *WT1*-transfected RM1 cells showed reduced colony formation in soft agar (*10*) as well as delayed tumorigenicity in nude mice (*11*). Transfection of *WT1* into a rhabdoid tumor cell line (SM2), derived from a pediatric kidney cancer that is histologically and genetically distinct from Wilms tumor (*4*), did not reduce the number of *neo*^{r} colonies. This observation is consistent with the inability of a fragment of human chromosome 11 containing the *WT1* locus to suppress tumorigenicity of G-401 cells (*12*), which have been used as a model for Wilms tumors but appear to be derived from a rhabdoid tumor (*13*).

Cell-type–specific growth suppression by *WT1* was consistent with its normally restricted expression pattern (*14*) and implied that endogenous *WT1* function might be disrupted in RM1 cells. Analysis of the endogenous *WT1* transcript in RM1 cells and in the parental Wilms tumor demonstrated a deletion of 123 nucleotides, corresponding to exon 2 of *WT1* (Fig. 2B). The RM1 cells expressed small amounts of WT1 mRNA, consisting primarily of the truncated transcript (*WT1-del2*), as evidenced by RNA-PCR (Fig. 2A) and ribonuclease protection analysis (*15*). Normal kidney tissue surrounding the Wilms tumor showed no evidence of *WT1-del2* mRNA, which indicates that this altered transcript had arisen in tumor cells and did not represent a rare polymorphism. However, we detected no mutations in *WT1* genomic DNA, either in exon 2 or in the flanking intron and exon sequences. Because the deletion did not alter the reading frame, we postulated that the truncated transcript might be produced by alternative splicing, and we looked for its expression in normal tissues. The *WT1-del2* mRNA was undetectable by RNA-PCR in 20-week-old human kidney, which contains the blastemal cells that give rise to Wilms tumor (Fig. 2A). *WT1-del2* mRNA was also absent from 10-day-old mouse kidney, spleen, and uterus—tissues that normally express large amounts of *WT1* mRNA (Fig. 2A). However, all 14 Wilms tumor cell lines and six additional primary Wilms tumor specimens expressed the *WT1-del2* transcript. In most cases, *WT1-del2* represented <10% of the total *WT1* mRNA, although in RM14 cells it comprised ~50% and in RM1 cells vir-

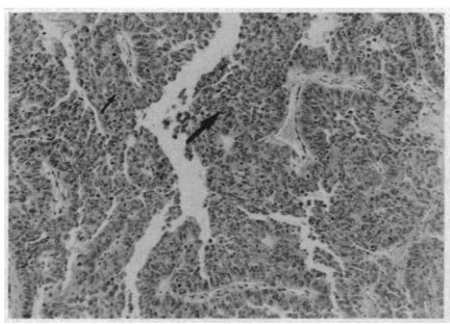

Fig. 1. Histologic characteristics of a subcutaneous tumor derived from the injection of 10^8 RM1 cells into a nude mouse. We established the RM1 cell line by grafting fragments of the parental Wilms tumor subcutaneously in nude mice, passaging the grafts in vivo, and then adapting the tumor graft to culture in vitro. Tumors produced by reinjection of the RM1 cell line into mice exhibited similar histologic features as the primary anaplastic Wilms tumor, with cords of blastemal cells (large arrow) and immature epithelial cells (small arrow) forming tubular or papillary structures. The section is stained with hematoxylin and eosin; magnification is ×80.

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tually 100% of the total *WT1* transcript (Fig. 2A) (*15*). The abundance of the *WT1-del2* mRNA in Wilms tumor specimens was not correlated with the overall level of *WT1* expression, which suggests a distinct mechanism regulating the splicing of *WT1* mRNA.

To determine whether the high proportion of *WT1-del2* expressed in RM1 cells resulted from a specific defect in *WT1* splicing, we analyzed these cells for synthesis of the two other alternatively spliced *WT1* mRNAs. These mRNAs are expressed in a constant proportion in Wilms tumors and in *WT1*-expressing normal tissues, with ~65% containing exon 5 and ~80% containing KTS (*5*). In RM1 cells, the *WT1* transcripts were devoid of not only exon 2, but also exon 5 (Fig. 2A). In contrast, the KTS alternative splice was inserted in the expected proportion of *WT1* mRNAs. Thus, *WT1* exons 2 and 5, which require the use of both alternative donor and acceptor splice sites, were not correctly inserted in RM1 cells, whereas insertion of KTS, which involves a single alternative splice donor site, was unaffected. This defect in alternative splicing appeared to be specific to *WT1*, because *CD44*, a gene that is ubiquitously expressed and has a complex pattern of alternative splicing, was appropriately processed in RM1 cells (*16*). By analogy with the *sex lethal* gene in *Drosophila* (*17*), a specific abnormality in splicing of the *WT1* transcript could involve the disruption of a single regulatory gene. This "in trans" mechanism for disrupting a tumor suppressor gene would be particularly interesting in Wilms tumors, as only a minority of sporadic tumors appear to contain mutations in *WT1* (*2*). Thus, no *WT1* mutations were found in 14 Wilms tumor cell lines, all of which expressed detectable *WT1-del2* mRNAs. We could not determine the effect of the small amounts of *WT1-del2* transcript present in 12 of these cell lines, but the high proportion of *WT1-del2* seen in the RM1 and RM14 cells suggested a functional effect.

To determine whether the *WT1-del2* transcript encodes a protein with altered function, we deleted exon 2 from a CMV-driven murine *wt1* expression construct. In contrast to wild-type *WT1*, *WT1-del2* did not suppress the emergence of *neo*^{r} colonies when transfected into RM1 cells (Table 1). Thus, this alternatively spliced *WT1-del2*

Table 1. Suppression of colony-forming ability by wild-type *WT1* isoforms. Subconfluent RM1 cells (10^6 cells) were transfected by lipofection with 10 μg of a plasmid encoding the *neo* gene driven by the SV40 promoter, linked with a complementary DNA encoding one of the murine *wt1* isoforms (*8*) under control of the CMV promoter. *WT1* isoforms have been defined as follows (*5*): A (−exon 5, −KTS), B (+exon 5, −KTS), C (−exon 5, +KTS), and D (+exon 5, +KTS). When the four *WT1* isoforms were tested in combination (A to D), 2.5 μg of each plasmid was used. *WT1-del2* denotes the aberrantly spliced endogenous *WT1* in RM1 cells, and *WT1-201* encodes a Gly → Asp mutation (codon 201, exon 3) found in a Wilms tumor specimen (*9*). The culture medium was changed after 24 hours, and the cells were trypsinized after 48 hours and seeded onto five 60-mm dishes. Selection with G418 (100 μg/ml) was initiated 72 hours after transfection, and the cells were cultured for 6 weeks before staining. Results are shown as mean number of colonies (±SD) per transfection. ND, not determined.

Transfected construct	Number of *neo*^{r} colonies		
	Experiment 1	Experiment 2	Experiment 3
Mock	0	0.5 ± 0.5	0.4 ± 0.8
neo vector	21.0 ± 3.2	56.3 ± 15.8	64.8 ± 11.1
WT1 (A)	4.2 ± 1.1	7.5 ± 3.4	8.8 ± 3.3
WT1 (B)	5.0 ± 1.2	0.8 ± 0.8	23.2 ± 6.6
WT1 (C)	5.0 ± 2.3	3.8 ± 2.5	17.4 ± 4.4
WT1 (D)	6.5 ± 1.1	1.5 ± 1.5	14.2 ± 3.7
WT1 (A to D)	1.0 ± 0.7	2.0 ± 0.7	14.4 ± 1.4
WT1-del2 (B)	16.3 ± 4.9	ND	ND
WT1-201 mutant (B)	18.8 ± 3.9	ND	ND

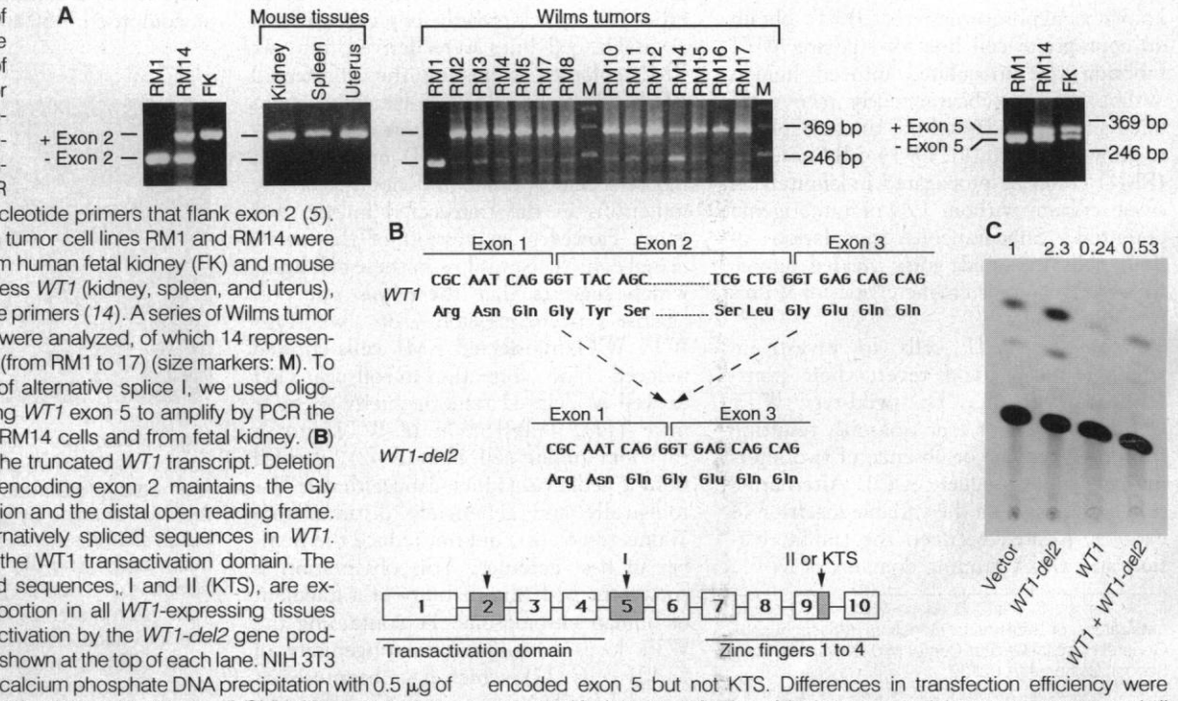

Fig. 2. Characterization of the *WT1-del2* alternative splice. (**A**) Expression of *WT1-del2* in Wilms tumor specimens. RNA was reverse-transcribed with random hexanucleotide primers and subjected to PCR amplification with oligonucleotide primers that flank exon 2 (*5*). RNA samples from Wilms tumor cell lines RM1 and RM14 were analyzed next to RNA from human fetal kidney (FK) and mouse tissues that normally express *WT1* (kidney, spleen, and uterus), with corresponding mouse primers (*14*). A series of Wilms tumor specimens and cell lines were analyzed, of which 14 representative samples are shown (from RM1 to 17) (size markers: **M**). To demonstrate expression of alternative splice I, we used oligonucleotide primers flanking *WT1* exon 5 to amplify by PCR the transcript from RM1 and RM14 cells and from fetal kidney. (**B**) Nucleotide sequence of the truncated *WT1* transcript. Deletion of the 123 nucleotides encoding exon 2 maintains the Gly residue at the splice junction and the distal open reading frame. Arrows indicate the alternatively spliced sequences in *WT1*. Exon 2 encodes part of the WT1 transactivation domain. The other alternatively spliced sequences, I and II (KTS), are expressed in constant proportion in all *WT1*-expressing tissues (*5*). (**C**) Transcriptional activation by the *WT1-del2* gene product; the induction (fold) is shown at the top of each lane. NIH 3T3 cells were transfected by calcium phosphate DNA precipitation with 0.5 μg of the *EGR1-CAT* reporter (*7*), along with 20 μg of CMV-driven constructs encoding *WT1*, *WT1-del2*, or an equal amount of these two constructs (20 μg each). The total amount of transfected CMV promoter sequence (40 μg) was equalized by addition of vector DNA. Both the *WT1* and *WT1-del2* constructs encoded exon 5 but not KTS. Differences in transfection efficiency were corrected for by cotransfection of the human growth hormone reporter, and all experiments were performed at least three times. Transactivational activity was quantitated by cutting spots from the thin-layer chromatography plate and determining their radioactivity in a scintillation counter.

product was similar to *WT1-201*, a Gly → Asp mutation in *WT1* exon 3. To test the transactivational properties of *WT1-del2*, we used transient transfections of NIH 3T3 cells, in which *WT1* has been shown to act as a transcriptional repressor of the early growth response promoter (*EGR1*) (*18*). As expected, transfection of wild-type *WT1* repressed base line *EGR1*-CAT (chloramphenicol acetyltransferase) activity to one-fourth its original level. In contrast, transfection of *WT1-del2* led to twofold greater transcriptional activation (Fig. 2C). WT1 proteins with NH_2-terminal alterations [both synthetic deletion constructs as well as naturally occurring point mutants such as *WT1-201* and *WT1-273* (*9*, *19*)] have also been shown to function as transcriptional activators; these results suggest that distinct domains of WT1 are responsible for transcriptional activation and repression. Cotransfection of equal amounts of wild-type *WT1* and *WT1-del2* resulted in an intermediate level of transactivation, which indicates a codominant effect by the two constructs. The antagonistic effect of wild-type *WT1* and *WT1-del2* suggests that *WT1* function may be disrupted in the fraction of Wilms tumors that express the largest amounts of this aberrant splicing product. Inactivation of gene function by expression of an alternatively spliced isoform has been described for a number of transcription factors (*20*, *21*). For transcription factor E3, the alternatively spliced exon also resides within the transactivation domain (*21*), which suggests a functional antagonism analogous to that of *WT1-del2*. Thus, aberrant splicing of *WT1*, resulting in a protein with altered transactivational properties, represents a distinct and apparently common mechanism of *WT1* inactivation in Wilms tumors. Growth suppression by wild-type *WT1* of a Wilms tumor cell line expressing this altered endogenous transcript is consistent with the characterization of *WT1* as a tumor suppressor gene.

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10. The two RM1 cell lines with the greatest expression of transfected *WT1*, NB2 (isoform B) and ND1 (isoform D), and cells mock-transfected with *neo* were seeded (10^4 cells each) in 0.34% low melting point agarose in culture medium over a cushion of 0.5% agarose in culture medium. After 6 weeks, the colonies were stained with the vital dye *p*-iodonitrotetrazolium violet (Sigma) and counted. The mean number of colonies per plate (±SD) was 1300 (±160) for *neo* cells, 320 (±80) for NB2 cells, and 280 (±80) for ND1 cells.
11. Six nude mice were subcutaneously injected with *neo*, NB2, or ND1 cells (10^8 each). At 21 days, subcutaneous tumors had developed in six of six *neo* mice, five of six NB2 mice, and four of six ND1 mice. The growth rate of the tumors differed between transfected cell lines. At 21 days, the mean maximum tumor diameter was 22 mm for *neo* mice, 16 mm for NB2 mice, and 10 mm for ND1 mice. All tumors were histologically identical and continued to express small amounts of the transfected construct.
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15. A riboprobe spanning *WT1* exons 2 and 3 was annealed with total cellular RNA (10 μg) from different Wilms tumor cell lines and from fetal kidney. Ribonuclease protection analysis demonstrated a truncated transcript, consistent with the absence of exon 2, which accounted for all the detectable *WT1* mRNA in RM1 cells and ~50% of the *WT1* mRNA in RM14 cells. The truncated transcript was absent from normal fetal kidney.
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Peptide Translocation by Variants of the Transporter Associated with Antigen Processing

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Major histocompatibility complex (MHC) class I molecules associate with peptides that are delivered from the cytosol to the lumen of the endoplasmic reticulum by the transporter associated with antigen processing (TAP). Liver microsomes of SHR and Lewis rats, which express different alleles of TAP (*cim*^{b} and *cim*^{a}, respectively), accumulate different sets of peptides. Use of MHC congenic rats assigned this difference to the MHC, independent of the class I products expressed. Both the *cim*^{a} and *cim*^{b} TAP complexes translocate peptides with a hydrophobic carboxyl terminus, but translocation of peptides with a carboxyl-terminal His, Lys, or Arg residue is unique to *cim*^{a}. Thus, the specificity of the TAP peptide translocator restricts the peptides available for antigen presentation.

MHC class I molecules present peptides to antigen-specific receptors on $CD8^+$ T lymphocytes. These peptides are eight to ten residues in length and are acquired by newly synthesized MHC class I molecules in the lumen of the endoplasmic reticulum (ER) (*1*). The source of these peptides is mostly cytosolic (*1*, *2*), and their delivery to the lumen of the ER is mediated by the TAP transporter (*3*). The TAP complex is a member of the adenosine triphosphate (ATP)–binding cassette family of transporters and consists of two subunits, TAP1 and TAP2 (*3*). Both subunits span the membrane six to eight times and have an ATP binding site in the proposed cytosolic domain (*3*). TAP-mediated translocation of peptides across the ER is temperature- and ATP-dependent (*4*–*6*). Little is known about the peptide substrate specificity of this transport process, although a role for the COOH-terminal residue of the peptide has been proposed (*5*, *6*). Allelic variants of TAP1 and TAP2 might have different peptide substrate specificities, a speculation fueled by the location of both TAP genes

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