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WT1 suppresses synthesis of the epidermal growth factor receptor and induces apoptosis

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The Wilms tumor suppressor gene *WT1* encodes a developmentally regulated transcription factor that is mutated in a subset of embryonal tumors. To test its functional properties, we developed osteosarcoma cell lines expressing *WT1* under an inducible tetracycline-regulated promoter. Induction of *WT1* resulted in programmed cell death. This effect, which was differentially mediated by the alternative splicing variants of *WT1*, was independent of *p53*. *WT1*-mediated apoptosis was associated with reduced synthesis of the epidermal growth factor receptor (EGFR), but not of other postulated *WT1*-target genes, and it was abrogated by constitutive expression of *EGFR*. WT1 repressed transcription from the *EGFR* promoter, binding to two TC-rich repeat sequences. In the developing kidney, *EGFR* expression in renal precursor cells declined with the onset of *WT1* expression. Repression of *EGFR* and induction of apoptosis by *WT1* provide a potential mechanism that may contribute to its critical role in normal kidney development and to the immortalization of tumor cells with inactivated *WT1* alleles.

Keywords: apoptosis/epidermal growth factor receptor/ Wilms tumor/WT1

Introduction

WT1 encodes a tumor suppressor gene originally identified by its inactivation in Wilms tumor, a pediatric kidney cancer (Call *et al.*, 1990; Gessler *et al.*, 1990; Haber *et al.*, 1990). The normal expression pattern of *WT1* is consistent with a developmental role in specific tissues, including glomerular precursors of the fetal kidney, stromal cells of the gonads and spleen and mesothelial cells lining the heart, diaphragm and peritoneum (Pritchard-Jones *et al.*, 1990; Pelletier *et al.*, 1991; Armstrong *et al.*, 1992; Park *et al.*, 1993a). Homozygous deletion of *WT1* in the mouse germline results in failure of kidney and gonadal development and in gross abnormalities of the heart and diaphragm (Kreidberg *et al.*, 1993). While tumors have not been observed in mouse models (Glaser *et al.*, 1990; Kreidberg *et al.*, 1993), disruption of *WT1* leads to tumorigenesis in humans, both in kidney and in mesothelial-derived cell types. Mutations inactivating *WT1* have been demonstrated in a subset of cases with genetic susceptibility to Wilms tumor and in ~10% of sporadic tumors (reviewed in Haber and Housman, 1992). Another 10% of Wilms tumors express elevated levels of an aberrant WT1 splicing product (deletion of exon 2), encoding a protein with altered transactivational properties (Haber *et al.*, 1993). *WT1* mutations have also been observed in rare mesotheliomas (Park *et al.*, 1993a), and a chromosomal translocation fusing the putative transactivational domain of the Ewing's sarcoma gene *EWS* to zinc fingers 2–4 of *WT1* has been demonstrated in desmoplastic small round cell tumor, a mesothelial-derived cancer (Ladanyi and Gerald, 1994). Thus, disruption of *WT1* in susceptible target cells leads to tumorigenesis, consistent with its characterization as a tumor suppressor gene. Reintroduction of wild-type *WT1* into a Wilms tumor cell line expressing an aberrantly spliced *WT1* transcript suppressed cell growth, an effect observed with each of four naturally occurring splice variants of *WT1* (Haber *et al.*, 1993).

The mechanism of action of *WT1* is poorly understood. WT1 protein has two recognizable functional domains (Call *et al.*, 1990; Gessler *et al.*, 1990). The C-terminus contains four Cys–His zinc finger domains with extensive homology to those of the *early growth response 1 (EGR1)* gene (also known as *NGFI-A, Zif 268* and *Krox 24). In vitro,* synthesized WT1 binds to the same DNA consensus, 5′-GCGGGGGCG-3′, recognized by the EGR1 gene product, but with 40-fold reduced affinity, and other potential WT1 binding motifs have recently been proposed (Rauscher *et al.*, 1990; Bickmore *et al.*, 1992; Pelletier *et al.*, 1992; Wang *et al.*, 1993b; Nakagama *et al.*, 1995). The N-terminus of WT1 encodes an apparent transcriptional repression domain, suppressing transcription from EGR1-containing promoters in transient transfection assays (Madden *et al.*, 1991). These promoters include those of *EGR1, insulin-like growth factor 2 (IGF2), insulin-like growth factor 1 receptor (IGF1R), platelet-derived growth factor A (PDGF-A), Pax 2, colony stimulating factor 1 (CSF1), transforming growth factor* β *(TGF-*β*)*, among others (Madden *et al.*, 1991; Drummond *et al.*, 1992; Gashler *et al.*, 1992; Wang *et al.*, 1992; Harrington *et al.*, 1993; Werner *et al.*, 1993; Dey *et al.*, 1994). However, *WT1* has not been shown to regulate the expression of any endogenous genes, and the functional significance of these target promoter sequences is unknown. Transcriptional repression by WT1 of an EGR1-containing promoter appears to be modulated by p53, a protein with which WT1 can be co-immunoprecipitated in baby rat kidney cells and Wilms tumor specimens (Maheswaran *et al.*, 1993).

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Wild-type *WT1* transcript contains two alternative splices, resulting in four distinct transcripts (Haber *et al.*, 1991). Alternative splice I comprises exon 5, encoding 17 amino acids that are inserted between the transactivation and DNA binding domains. Alternative splice II results from use of an alternative splice donor sequence between exons 9 and 10. Its insertion leads to three additional amino acids (lysine, threonine, serine or 'KTS') which disrupt the spacing between zinc fingers 3 and 4. WT1 proteins containing the KTS sequence fail to bind the EGR1 DNA consensus sequence *in vitro*, but may bind to other, less well-characterized sequences (Rauscher *et al.*, 1990; Bickmore *et al.*, 1992; Drummond *et al.*, 1994).

To study the growth-inhibiting properties of *WT1* and to determine its effect on any endogenous target genes, we developed an inducible *WT1* expression system. We chose two osteosarcoma cell lines, U2OS and Saos-2 which, unlike Wilms tumor cells, grow well *in vitro* and are highly transfectable. These cell lines have been well-characterized with respect to two other tumor suppressor genes, *p53* and the Retinoblastoma susceptibility gene *Rb*, which are both wild-type in U2OS cells and inactivated in Saos-2 cells (Masuda *et al.*, 1987; Diller *et al.*, 1990). Induction of *WT1* in both U2OS and Saos-2 cells resulted in apoptosis, an effect that was most dramatic in cells expressing *WT1* splice variant B, containing alternative splice I but lacking splice II (KTS). The induction of cell death by *WT1* was associated with reduced synthesis of the epidermal growth factor receptor (EGFR), and it was abrogated by transfection of a cytomegalovirus (CMV)-driven *EGFR* construct. In contrast, induction of *WT1* did not alter detectably the baseline expression of any of its previously postulated target genes. Unlike these EGR1-containing promoters, the WT1 binding site within the *EGFR* promoter consists of two TC-rich motifs, both of which were required for transcriptional repression. In the fetal kidney, both *WT1* and *EGFR* are expressed in the developing nephrogenic zone, with *EGFR* expression preceding *WT1* expression. *EGFR* therefore represents a genuine target gene of *WT1* and its transcriptional repression by this tumor suppressor gene may have implications for its role in both normal development and tumorigenesis.

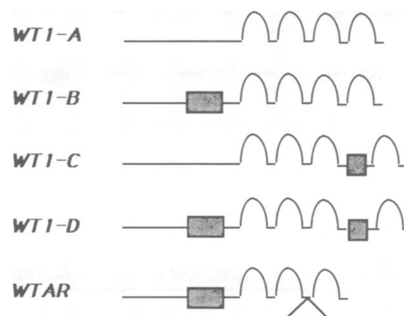
Results

WT1 suppresses growth of osteosarcoma cell lines

Functional studies of *WT1* have been hampered by the absence of appropriate, transfectable cell lines. We have recently demonstrated that transfection of wild-type *WT1* suppresses growth of a Wilms tumor cell line, RM1, expressing an aberrantly spliced *WT1* transcript (Haber *et al.*, 1993). However, RM1 cells are aneuploid, poorly transfectable and contain a truncated endogenous *p53* transcript, complicating interpretation of WT1 function. To establish an appropriate system to study *WT1* function, we therefore chose two well-characterized osteosarcoma cell lines, U2OS and Saos-2, notable respectively for the presence and absence of endogenous wild-type p53. In U2OS cells, expression of endogenous *WT1* was detectable both by Northern and immunoblotting analysis, while *WT1* transcript was detectable in Saos-2 cells by RNA–PCR amplification. In both cell lines, RNA–PCR and nucleotide sequencing demonstrated no mutation in the endogenous *WT1* transcript, and presence of the expected wild-type *WT1* splicing variants (data not shown).

Table I. Suppression of colony formation by *WT1* splice variants

Construct	G418 resistant colonies/dish		
	U2OS	Saos-2	NIH 3T3
Mock	0	2 ± 1	0
Vector	41 ± 3	50 ± 7	34 ± 4
WT1-A	25 ± 6	27 ± 3	24 ± 4
WT1-B	3 ± 2	1 ± 0.2	31 ± 3
WT1-C	64 ± 7	35 ± 4	16 ± 1
WT1-D	35 ± 4	52 ± 5	13 ± 2
WT1 A-D	14 ± 3	42 ± 3	28 ± 3
WTAR	nd	70 ± 4	29 ± 4



U2OS, Saos-2 and NIH 3T3 cells were transfected by calcium phosphate DNA precipitation with CMV-driven constructs encoding the four WT1 isoforms (20 μg), a mixture of all isoforms (5 μg each), or mutant WTAR (20 μg), linked to the Neomycin resistance gene. G418-resistant colonies were stained and counted after 3 weeks. The numbers of colonies/dish shown were derived from a representative experiment (±SD). A schematic representation of the four wild-type WT1 isoforms and of mutant WTAR is shown. Alternative splice I, encoded by exon 5 of WT1, results in the insertion of 17 amino acids between the transactivation and DNA binding domains of WT1. Alternative splice II, derived from use of an alternative splice donor site between exons 9 and 10, consists of three amino acids (KTS) that are inserted between zinc fingers 3 and 4, altering the DNA binding specificity of WT1. The WT1 isoforms are expressed in most cells at a constant ratio of A/B/C/D, 1:2.5:3.8:8.3 (Haber *et al.*, 1991). WTAR is a mutant allele, isolated from a Wilms tumor specimen, with an in-frame deletion of zinc finger 3 and alternative splice II (Haber *et al.*, 1990).

To determine the effect of *WT1* overexpression in U2OS and Saos-2 cells, these cells were first transfected with constructs encoding CMV-driven *WT1*, linked to the neomycin resistance gene (Haber *et al.*, 1992). Cells were transfected with each of the wild-type *WT1* isoforms (see Table I), a combination of all four isoforms, or a naturally occurring mutant, *WTAR*, encoding a defective DNA binding domain (Haber *et al.*, 1990). Table I summarizes the results of several independent experiments. In both osteosarcoma cell lines, *WT1-B* significantly reduced the number of drug-resistant colonies. None of the other wild-type *WT1* isoforms had a comparable effect, nor did mutant *WTAR*. Thus, *WT1-B*, a *WT1* isoform that encodes alternative splice I, lacks alternative splice II (KTS) and comprises ~15% of the wild-type *WT1* transcript in most cell types, exerted a potent growth suppressive effect in U2OS and Saos-2 cells. This effect was not observed in all cell types (e.g. NIH 3T3 cells), suggesting that it might result from the disruption of growth pathways in cell lines derived from embryonal tumors.

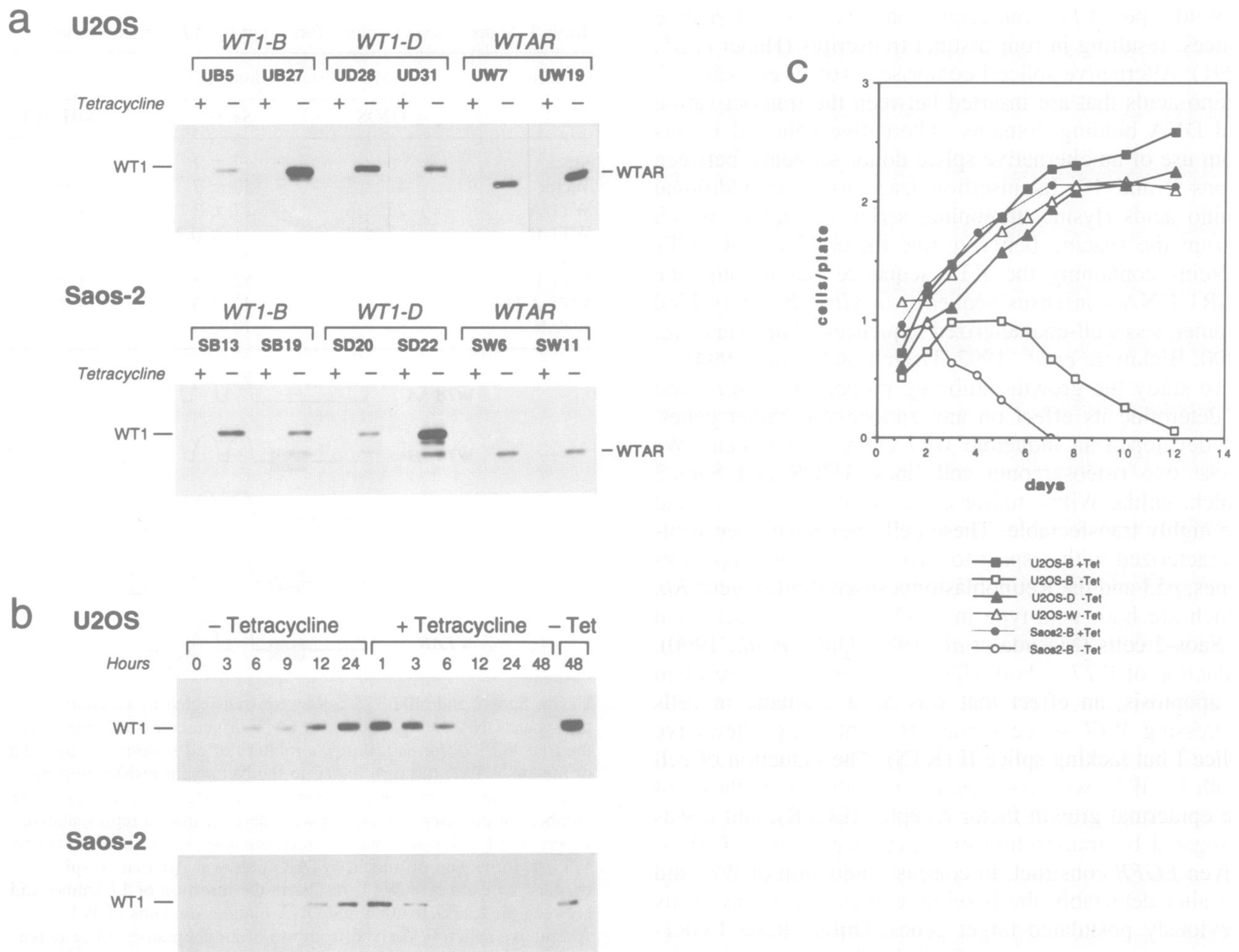


Fig. 1. Inducible WT1 isoforms in U2OS and Saos-2 cells. *WT1*-inducible cell lines were established using the tetracycline-repressible transactivator. In this system, presence of tetracycline in the culture medium suppresses *WT1* expression, while its withdrawal results in induction of *WT1* expression. (**a**) Immunoblot of cellular lysates from U2OS and Saos-2 transfectants grown in the presence or absence of tetracycline for 48 h and probed with anti-WT1 antibody WTc8. Two cell lines are shown for each construct, expressing comparable levels of inducible *WT1-B*, *WT1-D* or mutant *WTAR*. The smaller size of WTAR protein results from a deletion of zinc finger 3. In U2OS cells, low levels of endogenous WT1 are detectable, and in some Saos-2 transfectants a partial degradation product of the induced WT1 can be seen. (**b**) Time-course of WT1 induction. U2OS (clone UB8) and Saos-2 (clone SD20) cells were grown in the absence of tetracycline, and cellular lysates were obtained at timed intervals up to 48 h and analyzed by Western blot with antibody WTc8. To measure WT1 turnover in this system, tetracycline was added back to the culture medium at 24 h, and cellular lysates were prepared at timed intervals. (**c**) Growth curves of U2OS and Saos-2 cells expressing WT1 isoforms. U2OS cells with inducible *WT1-B* (B, clone UB27), *WT1-D* (D, clone UD28) or *WTAR* (W, clone UW19) and Saos-2 cells with inducible *WT1-B* (B, clone SB7) were grown in the presence or absence of tetracycline (±Tet). Cells were seeded in 60 mm dishes at 3×10^4 cells/plate in the presence of tetracycline. Tetracycline was withdrawn after 24 h, cultures were extensively washed with PBS to remove residual drug, and live cells were counted in duplicate plates at daily intervals. The number of cells/plate is plotted as log ($\times 10^4$).

Development of WT1-inducible cell lines

To study the mechanism of growth suppression by *WT1-B*, we established *WT1*-inducible cell lines, using a tetracycline-regulated transactivator (Gossen and Bujard, 1992). In this system, expression of a tetracycline-repressible transactivator allows strict regulation of a promoter containing *tet* operator sequences. Constructs encoding WT1 isoforms under control of this promoter were stably transfected into founder cell lines expressing the transactivator. For these experiments, we chose *WT1* isoforms encoding alternative splice I but varying in their DNA binding domains, either lacking alternative splice II (isoform B), encoding alternative splice II (isoform D), or lacking both zinc finger 3 and alternative splice II (mutant WTAR). For both U2OS and Saos-2 cells, at least three independent cell lines were characterized for each of the three constructs, *WT1-B*, *WT1-D* and *WTAR*. These were named respectively SB, SD and SW (for Saos-2-derived cells) and UB, UD and UW (for U2OS-derived cells). As an additional control, we used cell lines transfected with the non-recombinant plasmid. No expression of the transfected *WT1* gene was observed in the presence of tetracycline by immunoblot analysis (Figure 1a). Withdrawal of tetracycline led to induction of *WT1* expression, detectable within 6 h and peaking at 48 h. Readdition of tetracycline led to a rapid decrease in WT1 levels by 6 h, with no protein detectable after 24 h (Figure 1b). The turnover of WT1 protein following the addition of tetracycline was ~2 h, although this estimate may reflect kinetics of tetracycline transport and turnover of the transactivator, as well as the half-life of WT1 protein itself. The maximal induction of the transfected *WT1*

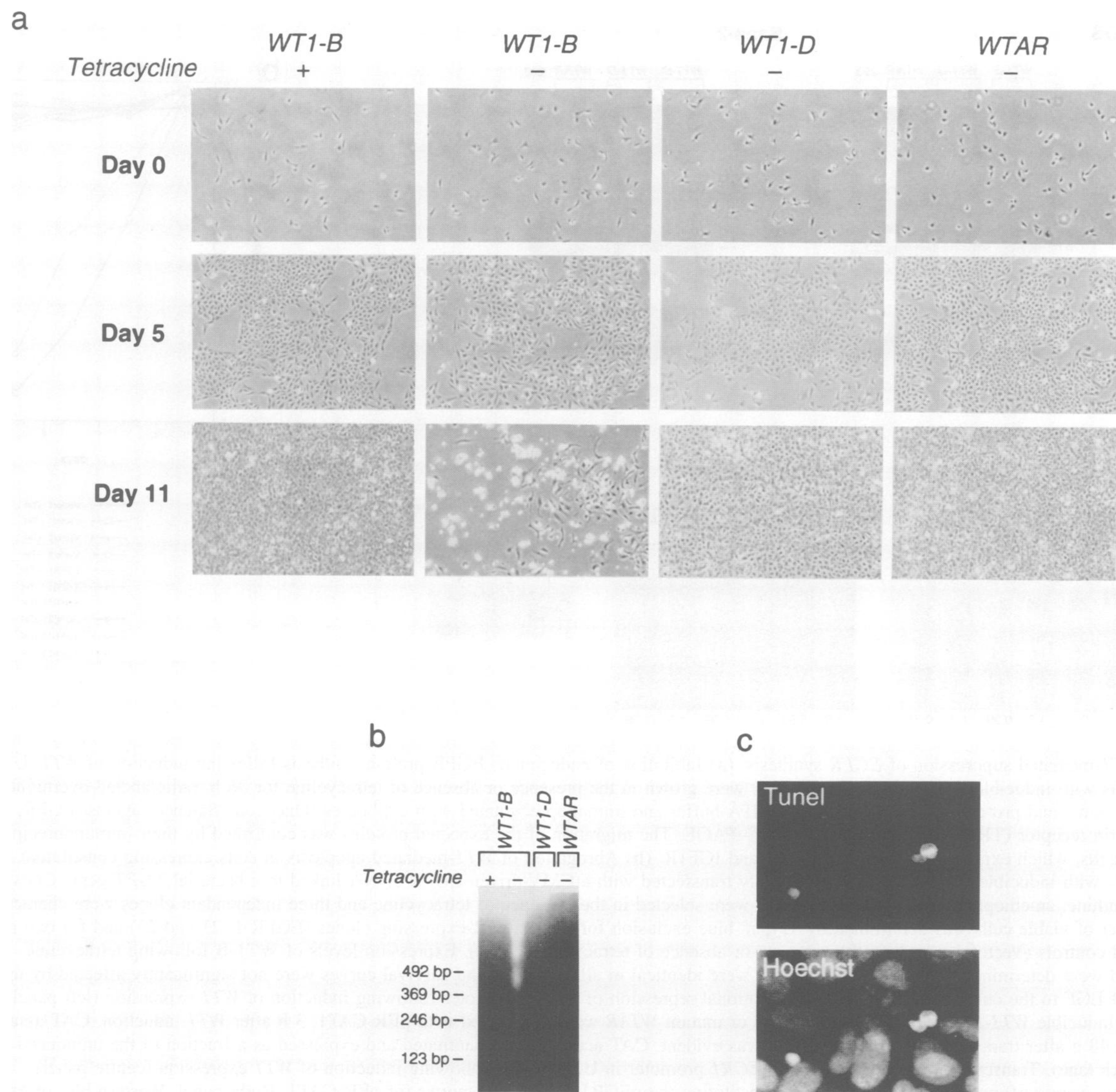


Fig. 2. Induction of apoptosis by WT1. (a) Phase contrast micrographs (×40) of U2OS cells expressing inducible *WT1-B* grown in the presence or absence of tetracycline, and of cells expressing comparable levels of *WT1-D* or *WTAR* grown in the absence of tetracycline. Dying, non-adherent cells are seen as refractile by phase contrast at this magnification. (b) DNA fragmentation induced by *WT1* expression. Poorly adherent cells were harvested from U2OS cells with inducible *WT1-B* (in the presence or absence of tetracycline) or *WT1-D* and *WTAR* (in the absence of tetracycline). Genomic DNA was isolated and electrophoresed on a 2% agarose gel. The isolation of DNA from non-adherent cells resulted in enrichment for apoptotic cells, which would otherwise constitute a small fraction of the population at any given time. Few cells were harvested from non-dying cultures, and the assay was therefore normalized to the starting cell number for each culture. (c) TUNEL staining and nuclear condensation in cells expressing *WT1-B*. U2OS cells with inducible *WT1-B* were grown in the absence of tetracycline for 4 days and double-stained with rhodamine-conjugated Apoptag reagent (Oncor) to detect free DNA 3′-OH-ends (TUNEL), and with Hoechst to demonstrate nuclear condensation and fragmentation. A representative field is shown, through respective filters.

genes was consistent with the 100–200-fold induction observed with a luciferase reporter construct (see Materials and methods). The level of transfected WT1 induced at 24–48 h in the transfectants was comparable with that observed in podocytes of the developing kidney, expressing endogenous *WT1* (data not shown).

WT1 induces apoptosis in U2OS and Saos-2 cells

Induction of *WT1* in U2OS and Saos-2 transfectants confirmed the growth inhibition observed with whole cell populations. Expression of *WT1-B* resulted in a normal growth rate for 3–4 days, followed by rapid cell death (Figure 1c). A correlation was observed between the level of *WT1* expression and the induction of cell death. Thus, *WT1-B* appeared to be the isoform most potent in the induction of cell death, but high levels of *WT1* expression achieved in some cell lines revealed the ability of *WT1-D* to induce cell death (as well as *WT1-A* and *WT1-C;* data not shown). A striking observation in these experiments was the reversibility of growth inhibition upon suppression of *WT1* expression. In both U2OS and Saos-2 cells, addition of tetracycline to the culture medium at any time resulted in renewed growth of any remaining viable cells. Thus, the cellular processes leading to cell death required continuous expression of *WT1,* and no irreversible time point triggering death of the entire cell population could be identified, consistent with a stochastic effect.

Cell death induced by *WT1* bore the consistent hallmarks of apoptosis (Oberhammer *et al.*, 1992; Jacobson *et al.*, 1993; Miura *et al.*, 1993). Phase contrast microscopy of

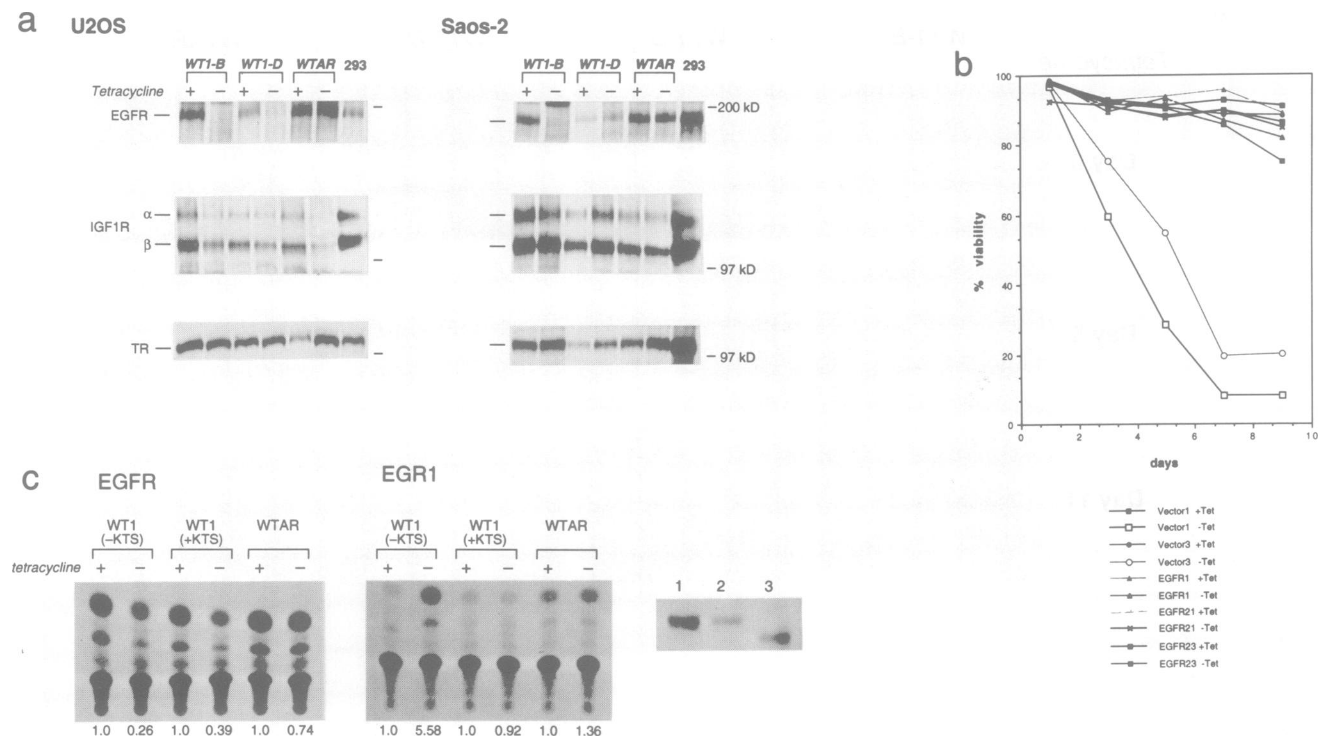


Fig. 3. *WT1*-mediated suppression of *EGFR* synthesis. (**a**) Inhibition of endogenous EGFR protein synthesis following induction of *WT1*. U2OS and Saos-2 cells with inducible *WT1-B, WT1-D* or *WTAR* were grown in the presence or absence of tetracycline for 36 h, radiolabeled overnight with [^{35}S]methionine, and proteins were extracted with RIPA buffer and immunoprecipitated with antibodies (Oncogene Science) against EGFR, IGF1R or transferrin receptor (TR) control, followed by SDS–PAGE. The migration of the expected proteins was confirmed by their immunoprecipitation from 293 cells, which express high levels of EGFR and IGF1R. (**b**) Abrogation of *WT1*-mediated apoptosis in cells expressing constitutive EGFR. U2OS cells with inducible *WT1-B* (UB27) were stably transfected with a CMV-driven *EGFR* cDNA linked to a bacterial *XGPT* gene. Cells resistant to hypoxanthine, amethopterin and thymidine (HAT) were selected in the presence of tetracycline and three independent clones were characterized. The number of viable cells was determined by Trypan blue exclusion for three EGFR-expressing clones (EGFR 1, 21 and 23) and for two mock-transfected controls (Vector 1 and 3) in the presence or absence of tetracycline (±Tet). Expression levels of WT1-B following tetracycline withdrawal were determined by immunoblotting and were identical in all cell lines, and survival curves were not significantly affected by the addition of EGF to the culture medium. (**c**) Transcriptional repression of *EGFR* promoter following induction of *WT1* expression (left panel). U2OS cells with inducible *WT1-B* (-KTS), *WT1-D* (+KTS) or mutant *WTAR* were transfected with pER-CAT1, 3 h after *WT1* induction. CAT assays were performed 48 h after transfection, before cell death was evident. CAT activity was quantitated and expressed as a fraction of the uninduced baseline (below each lane). Transcriptional activation of the *EGR1* promoter in U2OS cells, following induction of *WT1* expression (centre panel). Experiments were performed as described above, substituting the pEGR1-1.2-CAT1 reporter for pER-CAT1. Right panel: Western blot of extracts from U2OS cells used for CAT assays expressing inducible *WT1-B* (lane 1), *WT1-D* (lane 2) or *WTAR* (lane 3), probed with anti-WT1 antibody WTc8.

U2OS transfectants demonstrated retraction of cellular processes, nuclear condensation and loss of adherence to the tissue culture dish (Figure 2a). While prolonged *WT1* expression was required to trigger death of the entire culture, individual apoptotic cells were observed within 2 days of *WT1* induction. Electrophoretic analysis of DNA from dying cells revealed the fragmentation of chromatin into a nucleosomal ladder characteristic of programmed cell death (Figure 2b). Double staining of individual cell nuclei with Hoechst dye and TUNEL reagent demonstrated nuclear condensation and fragmentation, and presence of free DNA 3′-OH ends within the nuclear fragments, consistent with the apoptotic process involved in cell death (Figure 2c).

WT1 represses synthesis of the EGFR

The induction of apoptosis by *WT1* required an intact DNA binding domain, suggesting that this effect was dependent upon its transactivational properties. A number of potential *WT1* target genes have been reported, based on the presence of an EGR1 consensus sequence in their promoter, and transcriptional repression of reporter constructs in transient transfection assays. Potential target promoters have included those of *EGR1, IGF2, Pax 2, PDGF-A, IGF1R*, among others (Madden *et al.*, 1991; Drummond *et al.*, 1992; Gashler *et al.*, 1992; Wang *et al.*, 1992; Werner *et al.*, 1993). The generation of *WT1*-inducible cell lines therefore made it possible to test the effect of *WT1* expression on endogenous genes. RNA was isolated from multiple U2OS and Saos-2 cell lines 48 h after induction of *WT1-B, WT1-D* or *WTAR*, and analyzed by Northern blotting. None of the previously postulated WT1 target genes was found to be repressed following induction of WT1 in either U2OS or Saos-2 cells (data not shown). Thus, *WT1*-mediated apoptosis presumably involved transcriptional regulation of other, unidentified target genes.

The requirement for prolonged expression of *WT1* to trigger apoptosis suggested that this effect might result from repression of a target gene product with slow turnover, such as a growth factor receptor. We therefore screened receptors thought to play a role in kidney and epithelial cell development for any alteration in their synthetic rate following induction of *WT1*. U2OS and

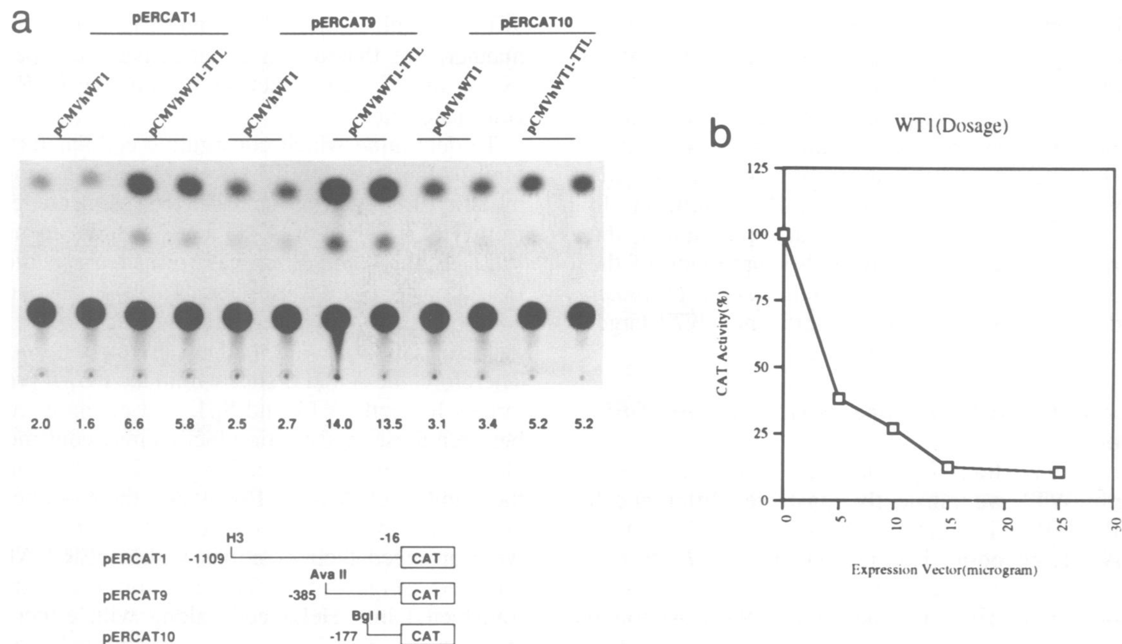


Fig. 4. Localization of WT1-responsive sites in the *EGFR* promoter. (**a**) HeLa cells were transfected with pCMVhWT1 (-KTS) or pCMVhWT1-TTL (truncated mutant) along with the *EGFR* promoter-derived reporter constructs shown below. Since baseline CAT activity was dependent upon the reporter constructs, it is expressed as the percent conversion for each reaction (below each lane). (**b**) Dose-dependent repression of pERCAT9 by WT1. The truncated WT1-responsive reporter was co-transfected with 5, 10, 15, 20 and 25 μg of pCMVhWT1 and a corresponding amount of vector to make up 30 μg of total transfected plasmid. CAT activity is expressed as a percent of the vector-transfected baseline and shown as a graphical representation of the average of two experiments, each performed in duplicate.

Saos-2 cells expressing inducible *WT1-B, WT1-D* or *WTAR* were grown in the absence of tetracycline for 36 h, radiolabeled, and cellular lysates were immunoprecipitated with antibodies against EGFR, IGF1R, platelet-derived growth factor receptor (PDGFR), and a transferrin receptor (TR) control. Synthesis of EGFR was reduced dramatically after *WT1* induction in both Saos-2 and U2OS cells (Figure 3a). This effect was observed primarily with the WT1-B isoform, but it was also evident in cell lines expressing high levels of WT1-D. In contrast, synthesis of neither IGF1R nor TR was affected by *WT1* expression, and PDGFR was not expressed in these cells.

To determine whether suppression of EGFR synthesis was responsible in part for the induction of apoptosis by *WT1*, we transfected U2OS cells expressing inducible *WT1-B* (UB27 cells) with constructs encoding a CMV-driven *EGFR* cDNA. Multiple independent clones were isolated, demonstrating constitutive expression of *EGFR* and unaltered induction of *WT1-B* following withdrawal of tetracycline. Whereas vector-transfected controls underwent apoptosis as expected following *WT1* induction, cells expressing transfected *EGFR* showed protection against *WT1*-mediated cell death (Figure 3b). In the presence of tetracycline, *EGFR* -expressing cells grew at the same rate and to the same density as vector-transfected controls, and addition of EGF to the tissue culture medium did not enhance their proliferation (data not shown), suggesting that constitutive *EGFR* expression did not result in non-specific growth stimulation. Upon prolonged *WT1* induction, constitutive *EGFR* expression did not fully protect against *WT1*-mediated cell death (Figure 3b), suggesting that additional *WT1* target genes may also contribute to this effect.

Transcriptional repression of EGFR promoter by WT1

To determine whether suppression of EGFR synthesis was a direct result of transcriptional repression by WT1, *WT1*-inducible U2OS cells were transiently transfected with a reporter plasmid containing 1.1 kb of the human *EGFR* promoter (Johnson *et al.*, 1988a) upstream of the chloramphenicol acetyl transferase (CAT) gene (pERCAT1). For these experiments, cell lines expressing comparable levels of inducible *WT1-B, WT1-D* or *WTAR* were selected (Figure 3c). CAT activity was determined at 48 h after WT1 induction, before *WT1*-induced apoptosis became evident. Four-fold transcriptional repression of the *EGFR* promoter was observed following induction of *WT1-B*. Mutant WTAR had a minimal effect on the *EGFR* promoter, and WT1-D demonstrated an intermediate level of transcriptional repression, suggesting that insertion of alternative splice II (KTS) reduced binding to the regulatory sequences in the promoter.

To compare the effect of WT1 on the *EGFR* promoter with that on the *EGR1* promoter commonly used as a target for WT1, we performed these transient transfection experiments using the pEGR1-1.2-CAT reporter (Madden *et al.*, 1991) (Figure 3c). WTAR and WT1-D had minimal effects on transcription from the *EGR1* promoter, consistent with observations that deletion of zinc finger 3 (WTAR) decreases recognition of the EGR1 sequence and insertion of alternative splice II (KTS) abolishes it (Rauscher *et al.*, 1990). However, induction of *WT1-B* resulted in 5–6-fold transcriptional activation of the *EGR1* promoter, rather than repression. This result, although somewhat unexpected, was consistent with our prior

observation that the *EGR1* promoter can be either activated or repressed by WT1, depending upon the cellular context (Maheswaran *et al.*, 1993). However, expression of the endogenous *EGR1* transcript in these cells was neither induced nor repressed following induction of WT1 (data not shown), suggesting that any effect of WT1 on the *EGR1* promoter was not physiologically significant. In contrast, *WT1*-mediated transcriptional repression of the *EGFR* promoter was concordant with suppression of the endogenous gene, leading us to conclude that this promoter contained a physiologically significant *WT1*-target sequence, which we characterized.

Identification of WT1-responsive sites in the EGFR promoter

To characterize the transcriptional repression of the *EGFR* promoter by WT1, we transiently transfected different cell lines with pERCAT1 and a CMV-driven *WT1* cDNA (pCMVhWT1, encoding isoform A) or a *WT1* plasmid (pCMVhWT1-TTL) with an in-frame stop codon (Drummond *et al.*, 1992; Gashler *et al.*, 1992). Although less potent in the induction of apoptosis (Table I and data not shown), WT1-A has been the WT1 isoform generally used in comparing the effect of *WT1* on different promoters. Transfection of *WT1-A* into NRK, HeLa and A432 cells resulted in comparable transcriptional repression of the *EGFR* promoter (data not shown), and HeLa cells were used to characterize its WT1-responsive elements further. We first tested deletion constructs containing 370 (pERCAT9, –385 to –16) and 162 (pERCAT10, –177 to –16) nucleotides upstream of the *EGFR* translation initiation site. pERCAT9 showed a similar degree of repression by *WT1* as pERCAT1, but further deletion of the promoter sequence (pERCAT10) reduced transcriptional repression to less than 2-fold (Figure 4a). These results suggested the presence of the WT1-responsive elements between nucleotides –385 to –177. Transcriptional repression of pERCAT9 was also dependent upon the amount of *WT1* plasmid transfected, with CAT activity decreasing progressively to 10% of the control level (Figure 4b).

We used DNase I footprinting to determine the WT1 binding site within the *EGFR* promoter. A bacterially synthesized WT1 zinc finger domain protected two TC-rich direct repeat sequences, defined as A Box: 5′-CTCCCTCCTCCTCGCATTCTCCTCCTCCTC-3′ and B Box: 5′-TCCCTCCTCCGCCGCCTGGTCCCTCCTCC-3′ (Figure 5a). Gel mobility shift assays confirmed binding of the WT1 zinc finger domain to synthetic oligonucleotides containing either the A or B sequence (Figures 5c and d). The specificity of WT1 binding was examined in competition assays with unlabeled oligonucleotides containing the GC-rich EGR1 and Sp1 consensus sequences or the unrelated E2F sequence. Binding of WT1 to the labeled B sequence was reduced by a 10-fold molar excess of the unlabeled B Box and abolished by a 50-fold excess (Figure 5d). Similar competition for WT1 binding was achieved with a 50-fold molar excess of the A sequence, the Sp1 binding site, and the extended EGR1 site derived from the PDGF-A chain promoter. In contrast, no competition was observed with a 50-fold excess of the E2F consensus sequence derived from the *c-myc* promoter. Comparable results were obtained with WT1 binding to the A Box (Figure 5c). These observations suggest that WT1 recognizes the TC-rich motif in a sequence-specific manner, but that it can be effectively competed by an excess of GC-rich sequences such as the EGR1 and Sp1 consensus sites.

To determine which constitutive cellular factors might bind to the A and B Boxes of the *EGFR* promoter, gel mobility shift assays were performed using nuclear extracts from HeLa, NRK or A431 cells, which do not contain WT1. Both A and B probes formed a specific protein–DNA complex that was either abolished or supershifted by addition of antibody directed against Sp1, while antibodies against EGR1 or WT1 had no effect (data not shown). Therefore, the A and B sites within the *EGFR* promoter are targets for both WT1 and Sp1, and potential competition between these transcription factors may contribute to their effects in the relevant cell types. To determine whether the number of A or B Boxes and their orientation were critical for *WT1*-responsiveness of the *EGFR* promoter, we rearranged their positions within pER-CAT9-derived reporters (Figure 5b). These reporter constructs were transfected into HeLa cells along with either wild-type *WT1* or mutant *WT1-TTL*. Transcriptional repression by WT1 required two binding sites (either A and B, A and A, or B and B) in either orientation with respect to the transcriptional start site.

Expression of EGFR precedes that of WT1 in the developing kidney

Transcriptional repression of the *EGFR* promoter by WT1 and suppression of endogenous EGFR synthesis in osteosarcoma cell lines suggested that these genes might be part of a physiological pathway. While the EGF pathway has been shown to be critical for kidney development (Fischer *et al.*, 1989), the expression pattern of *EGFR* has not been characterized. To examine the respective roles of *EGFR* and *WT1* during kidney development, we first compared their developmental time-course of expression in the rat kidney. Expression of *egfr* mRNA was highest at embryonic day 13, and barely detectable thereafter, in contrast to *wt1* expression which peaked at post-natal day 5 (Figure 6a). This developmental sequence was confirmed at the protein level by immunoblot analysis (data not shown). The developmental time course of *wt1* expression in the rodent kidney was consistent with previous reports (Buckler *et al.*, 1991; Sharma *et al.*, 1992; Mundlos *et al.*, 1993).

While analysis of RNA from whole kidneys at different time points indicates the peak expression time for *EGFR* and *WT1*, the developing kidney itself is comprised of co-existing precursor structures at different stages of differentiation. Thus, nephrogenic differentiation is initiated by the condensation of blastemal mesenchyme into an 'S-shaped body', which then gives rise to the podocyte layer of the developing glomerulus, all of which are present within a cross-section of fetal kidney. We therefore examined frozen sections from a 13-week human kidney by immunohistochemistry to identify the developmental structures expressing either EGFR or WT1 (Figure 6b). Both EGFR and WT1 were expressed within the nephrogenic zone of the developing kidney, but in structures at different stages of differentiation. EGFR was expressed in the blastemal mesenchyme and in S-shaped bodies, but was absent when these structures differentiated into

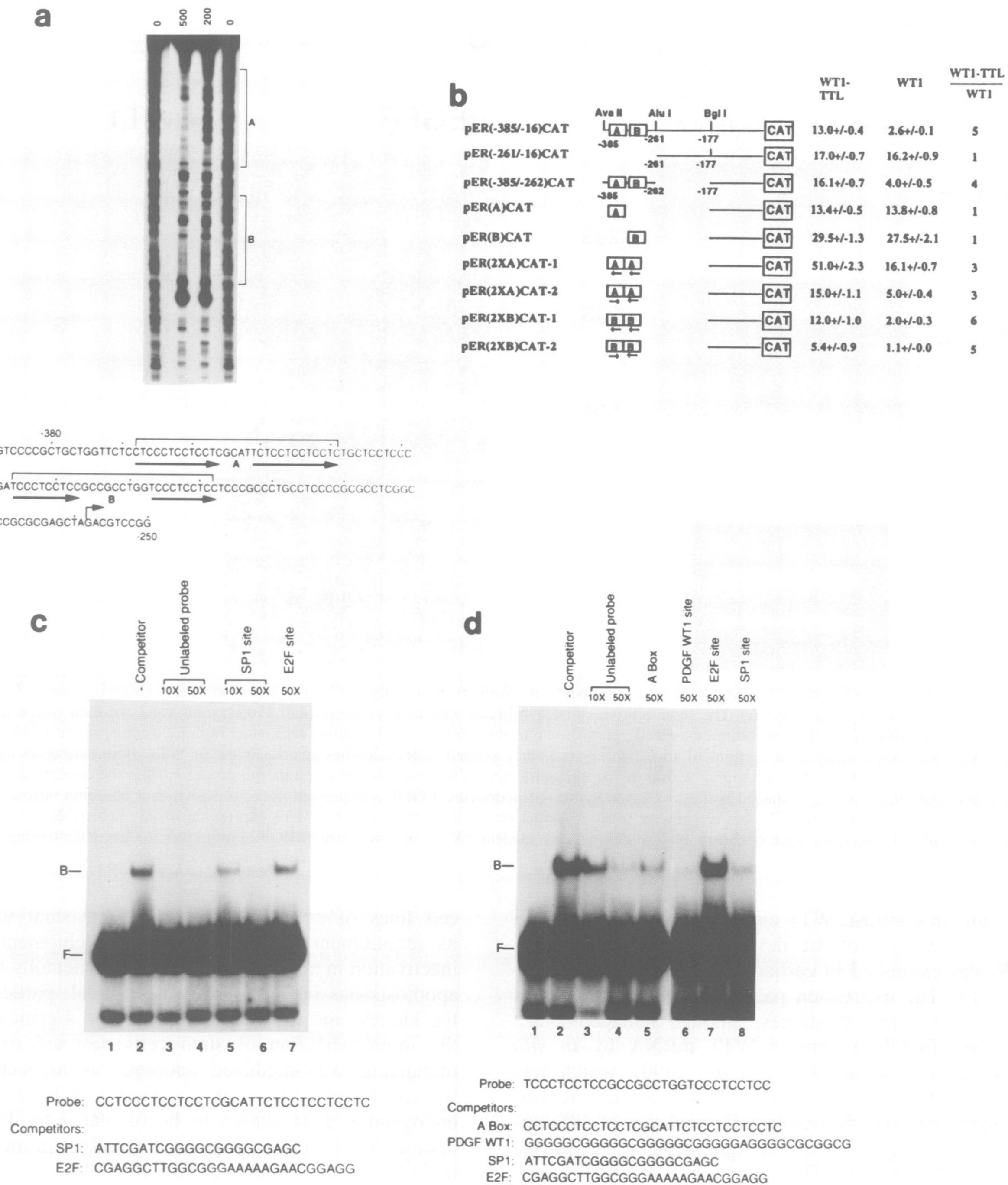


Fig. 5. Identification of novel WT1 binding sites. (**a**) DNase I footprinting of the *EGFR* promoter. Footprinting reactions were performed using end-labeled promoter fragments and purified WT1 zinc finger domain (indicated in nanograms at the top of each lane). The autoradiograph shown is the footprint obtained with the labeled sense strand of the 370 bp (–385 to –16) probe. Two protected sequences are denoted A and B, and their position within the *EGFR* promoter is shown, relative to the transcription start site (depicted by arrow). (**b**) Requirement for two WT1 binding sites for transcriptional repression of the *EGFR* promoter. *EGFR* promoter-derived reporter plasmids were constructed as shown, with various combinations of the A and B boxes (the arrow denotes the 5′ to 3′ orientation). These constructs were transiently transfected into HeLa cells, along with either CMV-driven *WT1* or the truncated mutant *WT1-TTL*. Transcriptional repression is expressed as a ratio of CAT activity of cells transfected with *WT1-TTL*/that of cells transfected with wild-type *WT1*. CAT activities represent the average of at least two experiments performed in duplicate ±SD. (**c**) Gel retardation and competition assays: WT1 binding to the A probe. The annealed, end-labeled A oligonucleotide (lane 1) was mixed with 7 ng of purified protein containing the WT1 zinc finger domain (lane 2). WT1/DNA complexes were competed with unlabeled oligonucleotides (lanes 3–7) in the molar excess indicated above each lane. The protein–DNA mixture was fractionated on a 8% non-denaturing polyacrylamide gel. F denotes the migration of free probe, B that of bound probe. DNA sequences of the probe (sense strand) and competitors are shown below the autoradiograph. Competitors included the unlabeled A Box (probe), Sp1 site and the E2F-responsive site of the *c-myc* promoter. (**d**) WT1 binding to the B probe. Annealed, end-labeled probe B (lane 1) was mixed with purified WT1 zinc finger protein (lane 2) in the presence of unlabeled competitors (lanes 3–8). Competitors included the unlabeled B Box (probe), the A Box from the *EGFR* promoter, PDGF-WT1 from the EGR1-containing WT1-responsive sequence in the *PDGF-A chain* promoter, E2F and Sp1 sites.

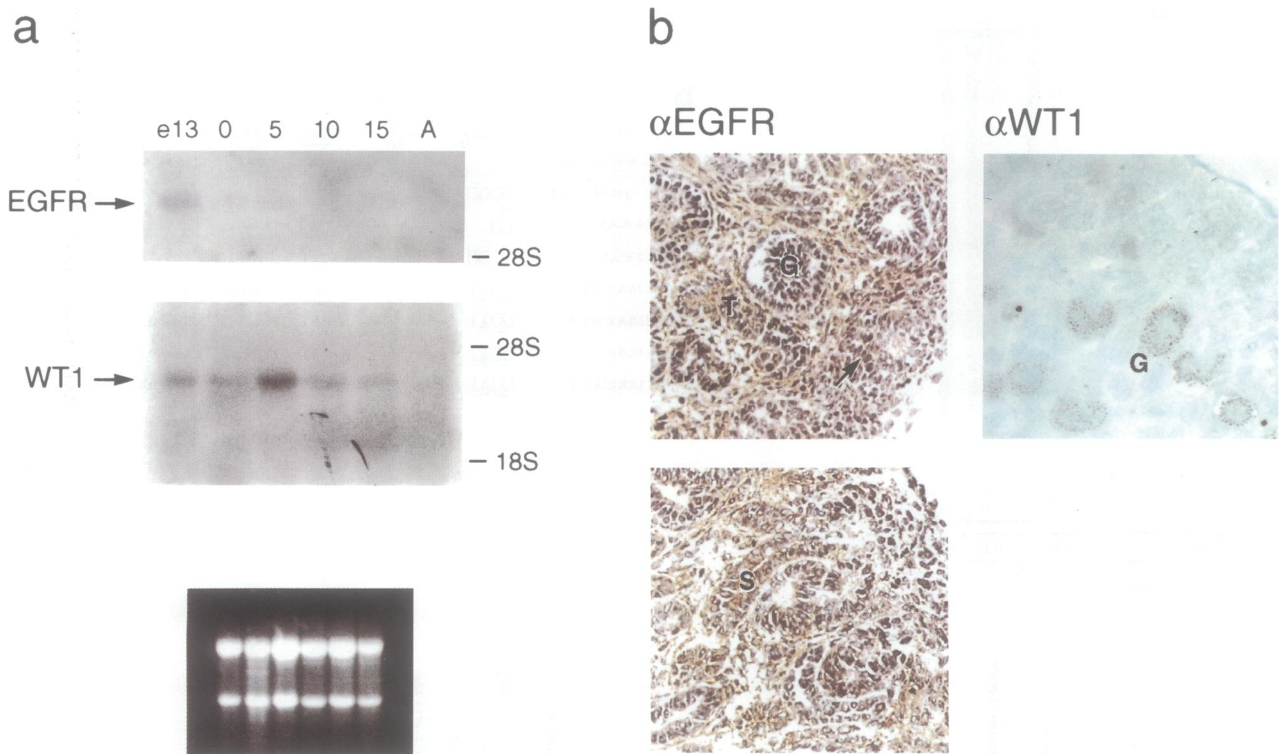


Fig. 6. Expression of *EGFR* and *WT1* during kidney development. (**a**) Northern blot analysis of rat kidneys isolated at embryonic day 13 (e13) and post-natal days 0, 5, 10, 15 and adult (A). The same blot was first hybridized with a rat probe for *egfr* (10 kb transcript) and then with a rat probe for *wt1* (3 kb transcript). The ethidium bromide-stained gel is shown to demonstrate equal loading and intact high molecular weight RNA.
(**b**) Immunohistochemical analysis of sections of 13-week human kidney stained with antibodies against EGFR or WT1. Both antibodies against EGFR and WT1 identify developing structures within the nephrogenic zone. The development of glomeruli proceeds from the condensation of mesenchymal blastema, forming S-shaped bodies, to the formation of podocytes. EGFR is expressed in the mesenchymal blastema (arrow), in S-shaped bodies (S) and in tubules (T), but it is absent from the podocyte layer of glomeruli (G). WT1 expression is only detectable in the podocyte layer of glomeruli (G). Sections were counterstained to demonstrate nuclear (WT1) or cytoplasmic (EGFR) immunohistochemical staining.

glomeruli. In contrast, WT1 expression was restricted to the podocyte layer of the developing glomeruli. Thus, EGFR was expressed in earlier developmental structures than WT1. The expression pattern of WT1 protein was consistent with previous studies, although S-shaped bodies have been found to express WT1 mRNA by *in situ* hybridization (Pritchard-Jones *et al.*, 1990), which was below detection by immunohistochemistry. EGFR was also expressed along the basolateral membrane of differentiating renal tubules, structures that will give rise to the renal collecting system. Thus, expression of EGFR in the kidney is a very early event in the differentiation of both glomerular and tubular renal precursors. Expression of WT1 follows that of EGFR in committed glomerular structures.

Discussion

WT1 has unique properties among tumor suppressor genes, in that its developmental expression pattern appears to mirror its role in tumorigenesis. In the fetal kidney, *WT1* expression is required during a narrow developmental window, and its inactivation within renal precursor cells is responsible for a subset of Wilms tumors. Similarly, absence of *WT1* in mesothelial cells has been linked to developmental malformations of the heart and diaphragm, and its disruption may result in tumors of mesothelial origin. Our observation that *WT1* can induce apoptosis in cell lines of embryonal origin is consistent with both its requirement for normal organ development and its inactivation in malignant cells. Developmentally regulated apoptosis has been shown to be critical, particularly in the kidney, and its suppression may be a critical step in the immortalization of stem cells destined to become malignant. *WT1*-mediated apoptosis is associated with transcriptional repression of *EGFR*, which is the first endogenous gene shown to be regulated by *WT1*. The distinct WT1 binding sites in the *EGFR* promoter may underlie its physiologic regulation by *WT1*.

TC-rich WT1 binding sites within the EGFR promoter

By characterizing the *EGFR* promoter, we have defined a novel WT1-responsive site. Transcriptional repression by WT1 requires the two TC-rich elements in this promoter, based on DNase I footprinting, gel mobility shifts and promoter reconstitution experiments. Another TC-rich sequence has been reported as a potential WT1-target site in the *PDGF-A* chain promoter (Wang *et al.*, 1993a,b). In that study, location of two WT1 target sites, one upstream and the other downstream from the transcription start site, appeared to be critical for transcriptional repression, and transcriptional activation was observed if only one WT1 binding site was present. In contrast, both TC-rich sequences in the *EGFR* promoter were upstream of the transcription start site, and transcriptional activation by

WT1 was not observed if only one site was retained. Since PDGF-A is not expressed in U2OS or Saos-2 cells, the potential physiologic effect of WT1 on expression of the endogenous *PDGF-A* gene could not be determined.

The two WT1 binding motifs in the *EGFR* promoter each contain two repeats of 10–14 pyrimidine residues conforming to the general sequence TCCTCCTCC. Like similar pyrimidine stretches in other promoters, this sequence is associated with an S1 nuclease-sensitive site (Johnson *et al.*, 1988b). These sites, which have been implicated in differential gene expression (Mace *et al.*, 1983; Yu and Manley, 1986), appear to result from changes in secondary and tertiary DNA structure (Liley, 1980; Panayotatos and Wells, 1981; Hentschel, 1982; Htun *et al.*, 1984; McKeon *et al.*, 1984; Pulleyblank *et al.*, 1985; Voloshin *et al.*, 1988). The requirement for two WT1 binding sites for transcriptional repression of the *EGFR* promoter also suggests a potential interaction between binding factors. This possibility is most intriguing because the four repeat elements contained within these two WT1 binding sites are separated from each other by 19, 21 and 23 bp, or by about two full turns of the DNA helix. Homodimerization of WT1 has been reported *in vitro* (Reddy *et al.*, 1995), and we have recently detected co-immunoprecipitation of WT1 isoforms in transfected cell lines (C.Englert *et al.*, submitted). In addition, WT1 could interact with other factors capable of binding to the TC-rich site. We have shown that the general cellular factors that bind to the TC-rich elements in the *EGFR* promoter are Sp1 or antigenically related to Sp1. Further work will be required to determine whether transcriptional repression by WT1 is affected by an interaction with Sp1 or by competition with Sp1 for DNA binding.

Transcriptional repression of WT1-target genes

The potentially complex interactions required for transcriptional repression by WT1 may explain the poor correlation between the regulation of endogenous genes by WT1 and its effect on reporter constructs in transient transfection assays. The development of *WT1*-inducible cell lines enabled us to study the effect of *WT1* expression on its previously postulated target genes. These genes were initially identified by the presence of an EGR1 consensus within their promoter and transcriptional repression of reporter constructs by WT1. Their prototype, the *EGR1* promoter, is transcriptionally repressed by co-transfection of *WT1* in NIH 3T3 and 293 cells (Madden *et al.*, 1991), and transcriptionally activated in Saos-2 cells, in cells expressing temperature-sensitive *p53* in the mutant conformation (Maheswaran *et al.*, 1993) and in U2OS cells with inducible *WT1-B* (Figure 3c). However, no alteration in the baseline level of endogenous *EGR1* mRNA was observed in either Saos-2 or U2OS cells following induction of *WT1-B* (data not shown), indicating that *EGR1* is not a physiologic target of *WT1* in these cells. Similarly, the *IGF1R* promoter is down-regulated by co-transfection of *WT1* in transient transfection assays (Werner *et al.*, 1993), but induction of *WT1* had no effect on the synthesis of IGF1R in U2OS and Saos-2 cells (Figure 3a).

It is possible that *WT1* may repress transcription of different target genes in different tissues, depending upon the baseline activity induced by other transcription factors. However, physiological *WT1* target genes are unlikely to be predicted by *in vitro* binding assays, given the affinity of WT1 zinc fingers for the GC-rich EGR1 and Sp1 consensus sequences as well as to the TC-rich motifs. DNA binding studies of WT1 have relied on the isolated zinc finger domain, since full-length, bacterially synthesized WT1 is insoluble. However, domains outside the zinc fingers may contribute to differential affinity for WT1 binding sites, an observation recently reported for EGR1 (Swirnoff and Milbrandt, 1995). In addition, the DNA-binding specificity of WT1 may be modulated by interactions with other cellular proteins that might not survive the stringent detergents required to recover WT1 from cellular lysates. Thus, the identification of an endogenous gene regulated by WT1 may allow definition of the factors required for transactivation by WT1. The suppression of EGFR synthesis by WT1 is particularly interesting, given that receptor's role in the transduction of growth signals and its overexpression or mutational activation in human cancers (DiFiore *et al.*, 1987; Velu *et al.*, 1987).

Suppression of EGFR by WT1 and induction of apoptosis

Transcriptional repression of the *EGFR* promoter by WT1 and suppression of endogenous EGFR protein synthesis in osteosarcoma cells precedes the induction of apoptosis. Although the induction of apoptosis by *WT1* in embryonal tumor cells may result from its effect on a number of different target genes, the role of *EGFR* suppression is suggested both by its timing and by the rescue from apoptosis of cells constitutively expressing *EGFR*. Transcriptional repression of a growth factor receptor is also compatible with the kinetics of *WT1*-induced apoptosis, including the requirement for continuous *WT1* expression and the renewed growth of cells upon termination of WT1 expression. Following suppression of new EGFR synthesis, remaining EGFR levels would decline at a rate determined by protein turnover and cellular division, with apoptosis triggered at the point where growth factor withdrawal is induced.

Induction of cell death by *WT1* is dependent upon an intact DNA binding domain and is differentially mediated by its alternative splicing variants, correlating well with their ability to repress EGFR synthesis. Although all WT1 isoforms are capable of inducing apoptosis when expressed at high levels, WT1-B, containing alternative splice I and lacking splice II (KTS), appears to be most potent at lower expression levels (Table I). The absence of alternative splice II (KTS) between zinc fingers 3 and 4 may result in enhanced binding to critical target promoters, such as that of *EGFR*. *WT1-D* only induces apoptosis when expressed at high levels, and the presence of the KTS insertion has recently been shown to alter the subnuclear localization of WT1, suggesting that this isoform may have a distinct function in addition to its transactivational properties (Larsson *et al.*, 1995; C.Englert *et al.*, submitted). Insertion of alternative splice I in *WT1* expression constructs results in a small but reproducible enhancement of transactivation (Reddy *et al.*, 1995), an effect that might be physiologically significant. Thus, the potent apoptotic effect of *WT1-B*, which constitutes ~15% of the *WT1* transcript, may be modulated by the expression of other WT1 isoforms in the developing kidney. We have not detected differential expression of the WT1 isoforms in

different tissues (Haber et al., 1991), but these observations were based on whole-tissue extractions and could not exclude differential expression of WT1 isoforms within individual cells undergoing programmed cell death.

The mechanism underlying *WT1*-induced cell death may be analogous to that following cytokine withdrawal in hematopoietic stem cells (Fairbairn et al., 1993), involving the down-regulation of signaling by growth or survival factors that are required by embryonal cancer cells (Baserga, 1994). This cell death pathway differs from that of other transcription factors and tumor suppressor genes implicated in apoptosis. In contrast to *WT1*, induction of apoptosis by *p53* appears to be distinct from its transactivational activity (Yonish-Rouach et al., 1991; Caelles et al., 1994). *WT1*-induced cell death also does not depend upon the presence of 'conflicting signals', such as simultaneous growth stimulation and serum withdrawal, a characteristic of *c-myc*-induced cell death (Evan et al., 1992; Hermeking and Eick, 1994). Unlike *c-myc*-induced apoptosis, *WT1*-mediated cell death is independent of *p53*, occurring both in cells with intact or deleted *p53* genes.

WT1-mediated apoptosis in Wilms tumor and normal kidney development

In the subset of Wilms tumors that contain *WT1* mutations, the timing of these mutations and the proposed apoptotic properties of WT1 may have important functional implications. We have previously shown that *WT1* mutations arise early in sporadic Wilms tumor, within 'nephrogenic rests' that constitute its genetic precursors (Park et al., 1993b). These premalignant lesions consist of persistent primitive blastemal cells that have failed to differentiate (Bove and McAdams, 1976; Beckwith et al., 1990). The high levels of *WT1* expression in these renal stem cells (Pritchard-Jones et al., 1990) suggest a potential role for *WT1* in regulating their fate. Inactivation of *WT1* in nephrogenic rests would therefore result in an expanded population of immortalized stem cells susceptible to additional mutational events. Such a mechanism, analogous to the overexpression of *Bcl2* in lymphoid neoplasms (McDonnell et al., 1989; McDonnell and Korsmeyer, 1991; Strasser et al., 1991), would be consistent with the role of *WT1* mutation as an initial genetic event in Wilms tumorigenesis.

The induction of apoptosis by *WT1* may represent a part of its complex developmental role. Transformed embryonic cancer cells, such as osteosarcoma cell lines, may undergo apoptosis in response to *WT1* expression and suppression of *EGFR*. However, during kidney development, the role of WT1 may be to modulate pathways of terminal differentiation versus programmed cell death. Developmentally regulated apoptosis appears to be particularly important in nephrogenesis, with the kidney being the organ most severely affected in *Bcl2*-null mice (Veis et al., 1993). *WT1*-null mice fail to develop kidneys and the presence of apoptotic cells in their vestigial renal bud has led to the suggestion that *WT1* is required for the survival of blastemal stem cells (Kreidberg et al., 1993). The stage at which renal developmental is arrested in these mice precedes the peak of *WT1* expression associated with the formation of glomeruli. Suppression of EGFR synthesis and induction of apoptosis may therefore be a later event in kidney differentiation.

Renal differentiation has been shown to be dependent upon growth factor signals, and EGF appears to be of particular importance in kidney development (Fischer et al., 1989). Both EGFR and TGF-α, a soluble growth factor that binds to EGFR, are expressed during kidney differentiation and *in vivo* administration of antibodies to TGF-α prevent normal kidney development (Rogers et al., 1992). In the normal developing rat kidney, 3% of cells within nephrogenic areas are apoptotic at any given time, implying large-scale apoptosis during renal development. Intraperitoneal administration of EGF suppresses this developmentally regulated apoptosis, suggesting that it may be mediated by lack of survival growth factors (Coles et al., 1993). Finally, in an *in vitro* model of differentiating metanephric mesenchyme, massive apoptosis results from the failure of induction by co-cultured spinal cord. Addition of EGF to the cultured renal mesenchyme abolishes apoptosis, without itself inducing differentiation (Koseki et al., 1992). Thus, the EGF pathway appears to be critical for the survival of differentiating kidney cells, and its suppression may be a physiologic mechanism by which developmentally regulated apoptosis is induced. The ability of *WT1* to repress *EGFR* transcription, coupled with its temporal and spatial expression pattern, suggest a potentially important developmental pathway.

Materials and methods

Cell culture

Osteosarcoma cell lines (Saos-2 and U2OS), normal rat kidney (NRK), human cervical carcinoma (HeLa), human epidermoid carcinoma (A431) and NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Cells were transfected by calcium phosphate DNA precipitation method (Ausubel et al., 1989). Drug-resistant colonies were selected by growth in G418 (0.5 mg/ml) or puromycin (1 μg/ml for U2OS cells, 0.2 μg/ml for Saos-2 cells). For maintenance of stable cell lines containing a tetracycline-regulated construct, the medium was supplemented with tetracycline (1 μg/ml). To generate growth curves, cells were seeded in 60 mm dishes at 3×10^4 cells/plate in the presence of tetracycline. Tetracycline was withdrawn after 24 h, cultures were washed extensively with PBS to remove residual drug, and duplicate plates counted. To determine viability, both attached cells and those in the supernatant were collected and stained with the vital dye Trypan blue (Sigma).

Development of cell lines with inducible WT1 constructs

Founder cell lines were generated by co-transfecting Saos-2 and U2OS cells with 10 μg of pUHD15-1, a plasmid encoding a tetracycline-repressible transactivator (Gossen and Bujard, 1992) and 1 μg of pCMVneo (Baker et al., 1990). Individual G418-resistant colonies were isolated and characterized by transient transfection with the luciferase reporter plasmid pUHC13-3, whose promoter is induced by the transactivator (Gossen and Bujard, 1992). Luciferase activity in the presence and absence of tetracycline was measured to identify cells with maximal promoter inducibility. Highest induction was 270-fold for Saos-2 cells (clone STA 5) and 180-fold for U2OS cells (clone UTA 6). These two founder cell lines were used to establish cells with *WT1*-inducible constructs. Constructs encoding full-length murine *WT1* (Haber et al., 1992) were cloned into vector pUHD10-3 (Gossen and Bujard, 1992), under control of a promoter containing both CMV and *tet* operator sequences. Thus, presence of tetracycline in the culture medium would suppress WT1 expression, while its withdrawal would result in induction of WT1 expression. Wild-type *WT1* constructs contained either alternative splice I alone (isoform WT1-B) or alternative splices I and II (isoform WT1-D) (Haber et al., 1991). A mutant *WT1* allele was constructed, encoding a naturally occurring mutation (WTAR), with an in-frame deletion of zinc finger 3 and alternative splice II (Haber et al., 1990). Both STA5 and UTA6 founder cells were transfected with 10 μg of each

construct and 1 μg of pBabe puro, conferring resistance to puromycin (Templeton *et al.*, 1991). Puromycin-resistant colonies were isolated in the presence of tetracycline, and screened for *WT1* expression by immunoblotting upon withdrawal of tetracycline. For each *WT1* construct in Saos-2 and U2OS cells, three clones demonstrating tightly regulated induction of the transfected gene were selected for further study.

Generation of anti-WT1 antibodies and immunological methods

To prepare monoclonal antibodies against WT1, a bacterial expression plasmid containing histidine-tagged WT1 was constructed by cloning a DNA fragment encoding amino acids 4–318 (lacking alternative splice I) of WT1 into the vector pET-KH (provided by R.Bernards). Protein was expressed and purified using nickel-chelate affinity chromatography under denaturing conditions as recommended by the manufacturer (Qiagen) and used to immunize mice as described by Harlow and Lane (1988). Monoclonal antibodies were generated by fusing splenocytes to Sp2 myeloma cells, 3 days after the final boost. Positive tissue culture supernatants were identified by ELISA and further characterized by immunoblotting and immunoprecipitation using extracts from cell lines harboring inducible *WT1* constructs. Five different cell lines producing monoclonal antibodies against WT1 were generated by single-cell cloning. The IgG1 monoclonal antibody mWT12 was used for immunocytochemistry experiments. The polyclonal antiserum against WT1 was generated by immunizing rabbits according to standard procedures with the antigen described above. Antisera were characterized extensively, with WTc8 yielding the best results by immunoblotting, immunofluorescence and immunoprecipitation.

Cell lysates for immunoblotting were prepared from subconfluent cultures by extraction with RIPA buffer. 20–30 μg of protein were analyzed by SDS–PAGE and transferred onto nitrocellulose membranes using standard procedures. Antibody WTc8 was used at a 1/1000 dilution, followed by goat anti-rabbit antibody (Biorad; 1/10 000 dilution) and detection by enhanced chemiluminescence (ECL) system (Amersham). For immunoprecipitation experiments, cultures were radiolabeled with [^{35}S]methionine overnight, followed by incubation with antibodies against EGFR, IGF1R, TR (Oncogene Science) bound to Protein A–Sepharose, and analysis by SDS–PAGE.

DNA fragmentation and TUNEL analysis

Equal numbers of cells were seeded into 150 mm dishes and tetracycline was withdrawn after cells had attached to the dish. After 5–8 days, when cells reached ~80% confluence, cells with reduced adherence were harvested. DNA was isolated and electrophoresed on a 2% agarose gel as described by Smith *et al.* (1989). The isolation of DNA from non-adherent cells resulted in enrichment for apoptotic cells, which would otherwise constitute a small fraction of the population of attached cells at any given time. Few cells were harvested from non-dying cultures, and the assay was therefore normalized to the starting cell number for each culture. Presence of free DNA 3′-OH ends, characteristic of apoptotic cell death was quantitated by the TdT-labeling technique. Cells were grown on coverslips in the presence or absence of tetracycline, fixed in 1% paraformaldehyde in PBS for 20 min at room temperature. Cells were then washed in PBS and apoptotic cells were labeled with digoxigenin-conjugated dUTP using terminal deoxynucleotidyl transferase (Oncor). dUTP incorporation was measured using fluorescein-conjugated anti-digoxigenin antibody and visualized by fluorescent microscopy. To demonstrate nuclear fragmentation, cells were co-stained with 8 μM Hoechst 33258 for 3 min at room temperature before the final washes.

Construction of reporter plasmids

For transient transfection experiments, a full-length human WT1 cDNA driven by the CMV promoter and lacking both alternative splices (pCMVhWT1) was used (Drummond *et al.*, 1992). As a control, a construct was used, containing a synthetic oligonucleotide with stop codons in all three reading frames inserted at a unique *Bam*HI site (amino acid 179) (Gashler *et al.*, 1992). Construction of reporter plasmids pERCAT1, pERCAT9 and pERCAT10, containing fragments of the EGFR promoter, have been described (Johnson *et al.*, 1988a). pERCAT10-1 was constructed as follows: a 162 bp (–177 to –16) *Hind*III fragment of the EGFR promoter was isolated from pERCAT10, digested with *Bam*HI and *Xho*I, and the shorter *Bam*HI–*Xho*I fragment was subcloned into the pBLCAT3 vector (Luckow and Schütz, 1987). All EGFR promoter CAT clones were made by subcloning blunt-ended EGFR promoter fragments into a blunted *Bam*HI site of pERCAT10-1. pER(–385/–16)CAT was created from the 208 bp *Ava*II–*Bgl*I fragment, pER(–261/–16)CAT from the 85 bp *Alu*I–*Bgl*I fragment, and pER(–385/–262)CAT from the 124 bp *Ava*II–*Alu*I. To test the WT1 binding sites, oligonucleotides containing the A sequence 5′-CCTCCCTCCTCCTCGCATTTCTCCTCTCCTC-3′ and B sequence 5′-TCCCTCCTCCGCCGCCTGGTCCCTCCTCC-3′ were synthesized and annealed with their respective complementary strands. The pER(A)CAT was made by ligating the annealed oligonucleotides for the A Box into the blunted *Bam*HI site of pERCAT10-1 and pER(B)CAT was made from the corresponding B Box oligonucleotides. pER(2×A)CAT-1 and pER(2×A)CAT-2 were made by ligating annealed A Box oligonucleotides, subcloning a fragment containing two copies into the blunted *Bam*HI site of pERCAT10-1 and confirming their orientation by direct sequencing. pER(2×B)CAT-1 and pER(2×B)CAT-2 were made with annealed B Box oligonucleotides using a similar approach.

CAT assays

Cells in mid-log phase were transiently transfected either by calcium phosphate or lipofectin (Life Technologies Inc.). Unless stated otherwise, 20 μg of CMV-driven expression constructs and 4 μg of reporter plasmids were used, together with 2 μg of a construct encoding human growth hormone (HGH; Nichols Institute) or 4 μg of a construct encoding β-galactosidase (Norton and Coffin, 1985). For CAT assays using *WT1*-inducible cell lines, tetracycline was withdrawn 3 h before transfection. The total amount of transfected CMV promoter sequence was equalized by addition of vector, and extracts were standardized with respect to an internal control for transfection efficiency (HGH or β-gal). Experiments were performed in duplicate and repeated three times. CAT activity was measured by the method of Gorman (1985), and resolved by thin-layer chromatography (TLC). The TLC plate was either scanned or cut and counted by scintillography. CAT activity is expressed either as a fraction of baseline activity or as the percent conversion value (see legends). In each case, a representative experiment is shown.

DNase I footprinting assays

Asymmetrically radiolabeled DNA fragments were obtained by either using [α-^{32}P]dATP and the large fragment of *Escherichia coli* DNA polymerase or [γ-^{32}P]ATP and T4 polynucleotide kinase (Sambrook *et al.*, 1989), followed by restriction enzyme digestion. The expected probes were isolated by polyacrylamide gel electrophoresis. Binding reactions were prepared by combining radiolabeled probe, polydI/polydC and 2×DFB (DNase I Footprinting Buffer: 20 mM HEPES, pH 7.9, 50 mM KCl, 2 mM DTT, 2 mM $MgCl_2$, 20% glycerol), with or without WT1 protein. Reactions were incubated for 20 min at room temperature, in a volume of 45 μl. DNase I (1 mg/ml in 10 mM Tris–Cl, pH 8.0) was diluted 1:25, and 1 μl was added to binding reactions. After incubation for 30 s at room temperature, the reaction was terminated by the addition of 45 μl stop buffer (100 mM Tris–Cl, pH 7.5, 2% SDS, 20 mM EDTA, 400 μg/ml proteinase K) and incubation for 15 min at 37°C. The reaction mixture was extracted twice with phenol/chloroform, once with chloroform, and precipitated with 2 vol ethanol in the presence of carrier transfer RNA. After a re-precipitation step, the products were resuspended in 2.5 μl 90% formamide, 10 mM EDTA, pH 8.0, 1 mg/ml xylene cyanol, 1 mg/ml bromophenol blue (formamide buffer) and separated in a polyacrylamide sequencing gel. Maxam–Gilbert cleavage at As and Gs was performed as described (Sambrook *et al.*, 1989).

Mobility retardation assays

HeLa cell crude nuclear extracts were prepared as described (Fried and Crothers, 1981). The purified Zinc-finger domain of WT1 was a kind gift from Drs Zhao-Yi Wang and Tom Deuel (Jewish Hospital at Washington University Medical Center, St Louis, MO), and antibodies for Sp1, WT1 and EGR1 were obtained from Santa Cruz Biotechnology, Inc. Synthetic DNA oligomers used for DNA binding analysis were as follows: EGFR A Box: 5′-CCTCCCTCCTCCTCGCATTCTCCTCC-TCCTC-3′; EGFR B Box: 5′-TCCCTCCTCCGCCGCCTGGTCCCTCC-TCC-3′, extended EGR1 consensus from the PDGF-A chain promoter: 5′-GGGGGCGGGGGCGGGGGCGGGGGAGGGGCGCGGCG-3′, Sp1 consensus sequence: 5′-ATTCGATCGGGGCGGGGCGAGC-3′, and E2F consensus sequence from the *c-Myc* promoter: 5′-CGAGGC-TTGGCGGGAAAAAGAACGGAGG-3′. These oligonucleotides were annealed with their complementary strands for DNA binding studies. Electrophoretic mobility-shift experiments were performed as described by Fried and Crothers (1981). The probes were end-labeled with either [α-^{32}P]dATP and the large fragment of *E.coli* DNA polymerase or with [γ-^{32}P]ATP and T4 polynucleotide kinase and radiolabeled fragments were isolated by polyacrylamide gel electrophoresis. Binding reactions were prepared by combining WT1 protein or 10 μg of nuclear extract

and 4 µg of poly[d(I-C)] (Boehringer Mannheim) in 5 mM HEPES, pH 7.9, 10% glycerol, 25 mM KCl, 0.05 mM EDTA, 0.125 mM PMSF. The reaction mixture was incubated on ice for 10 min, after which 30 000 c.p.m. of radiolabeled probe and, where indicated, 100-fold molar excess of unlabeled competitor DNA, was added. The final reaction volume was 20 µl. After 20 min incubation on ice, the DNA–protein complex was resolved from free probe by electrophoresis through a 5% non-denaturing polyacrylamide gel in 0.25× TBE buffer (Sambrook *et al.*, 1989) at 200 V and 4°C.

Northern Blot analysis and staining of tissue sections

Kidneys were dissected from pregnant rats (embryonic day 13) or from neonatal rats (days 0, 5, 10, 15 and adult), and total cellular RNA was isolated using the LiCl/urea method (Auffray and Rougeon, 1980), electrophoresed in 0.8% agarose/formaldehyde gels, and transferred to Genescreen Plus (NEN). Northern blots were probed with probes derived from the rat *wt1* and *egfr* sequences (Petch *et al.*, 1990; Sharma *et al.*, 1992). For immunocytochemistry, frozen sections of 13-week human kidney were fixed with acetone, hydrated with 10% goat serum in PBS and incubated with the primary monoclonal antibody (10 µg/ml), followed by PBS washes and incubation with the secondary goat anti-mouse antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch; 25 µg/ml). Slides were incubated with 3 mg DAB and 3 µl of 30% H_2O_2 in 10 ml PBS, treated with Gill's Hematoxylin #1 for nuclear counterstaining (EGFR) or Light Green for cytoplasmic counterstaining (WT1), and treated with 1% OsO_4 in H_2O, ethanol, xylene and permount. Best results were obtained with the 528 anti-EGFR monoclonal antibody (Oncogene Science) and the anti-WT1 monoclonal antibody mWT12, described above.

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