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# The Wilms' Tumor Gene *WT1* Can Regulate Genes Involved in Sex Determination and Differentiation: *SRY*, Müllerian-inhibiting Substance, and the Androgen Receptor<sup>1</sup>

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## Abstract

Genital abnormalities associated with Wilms' tumors in the WAGR and Denys-Drash syndromes and the failure of the gonads to develop in Wilms' tumor gene (*wt1*)-homozygous mutant mice suggest that *WT1* may also function in sexual development. To elucidate the mechanism of action of *WT1* in embryonal sexual development, we examined how the four isoforms of *WT1* regulate the transcription of several genes involved in sexual development using cotransfection assays. *SRY* (the sex-determining region of the Y chromosome) promoter was strongly activated by the *WT1* isoforms without the KTS tripeptide, *WT1*(-)/KTS, but was not activated by the *WT1* isoforms with the KTS tripeptide, *WT1*(+)/KTS, in all cells tested. The second alternative splicing site, which inserts the tripeptide KTS, alters the DNA binding capability. The Müllerian-inhibiting substance (*MIS*) promoter was strongly repressed by *WT1*(-)/KTS isoforms and more weakly repressed by the *WT1*(+)/KTS isoforms in Sertoli cells but not in HeLa cells. The androgen receptor (*AR*) promoter was strongly repressed by the *WT1*(-)/KTS isoforms in all cells tested and was more weakly or not repressed by *WT1*(+)/KTS isoforms depending on cell lines. Electrophoretic mobility shift assays showed strong binding by recombinant *WT1*(-)/KTS protein and weaker or no binding by the *WT1*(+)/KTS protein to DNA probes containing *WT1* binding sites from these three promoters. The results of these functional and binding assays suggest that *WT1* has an important role in regulation of genes involved in embryonal sexual development and that *WT1* can function as a transcriptional activator.

## Introduction

Wilms' tumor is one of the most common childhood solid tumors and has an incidence of one in 10,000 (1). GU<sup>3</sup> abnormalities, such as hypospadias and cryptorchidism, are often seen in male Wilms' tumor patients. About 1% of Wilms' tumor patients have the Wilms' tumor, aniridia, GU abnormalities, and mental retardation syndrome (WAGR), which is usually caused by large chromosomal deletions at 11p13. Another 5-7% of WT patients have congenital GU anomalies but not aniridia (2, 3). In another rare *WT1*-associated disease, DDS, which is usually caused by a point mutation in the *WT1* gene, affected 46 XY individuals have severe male pseudohermaphroditism, with streak gonads and ambiguous external genitalia, as well as Wilms' tumor and nephropathy (4, 5). To explain the severity of GU abnormalities in DDS, Pelletier *et al.* (4) hypothesized that the point mutations found in DDS patients function in a dominant negative fashion, and this hypothesis was supported by recent reports (6, 7).

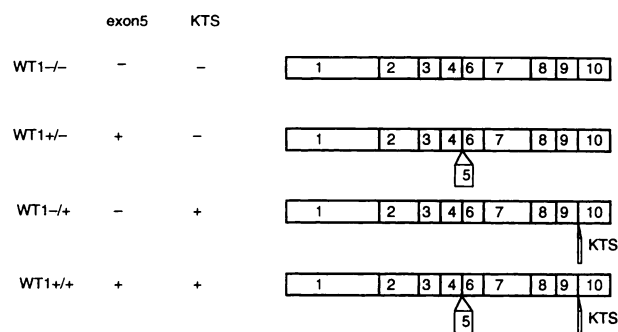
*WT1* cDNA contains an open reading frame of 1347 bp (449 amino acids) encoding a proline-rich domain and four (Cys)<sub>2</sub>-(His)<sub>2</sub>-type zinc fingers, both of which are hallmarks of transcription factors (8, 9). There are four *WT1* isoforms that are generated by use of two alternative splice sites (Fig. 1), the ratios of which are thought to be constant in various tissues and at various developmental stages. Splicing at the first alternative site inserts exon 5, which encodes 17 amino acids, and splicing at the second alternative site inserts 9 bp that encode three amino acids, KTS, at the 3' end of exon 9. This KTS insertion between zinc fingers 3 and 4 changes the DNA binding capability of the zinc-finger domain (10, 11). Although *WT1* isoforms without the KTS tripeptide *WT1*(-)/KTS bind to the EGR-1 transcription factor binding site, as expected by the homology of the zinc finger region in EGR-1 to *WT1* (12), binding sites of *WT1* isoforms with the KTS tripeptide *WT1*(+)/KTS are not well characterized. In human and mouse embryos, *WT1* is expressed in the gonads as well as the kidneys and mesothelial lining (13-15), and the kidneys and gonads of *wt1*-homozygous mutant mice do not develop (16). These experimental data and clinical findings strongly suggest that *WT1* is important in embryonal sexual development.

Recent studies have identified several genes that play a role

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<sup>3</sup> The abbreviations used are: GU, genitourinary; *WT1*(-)/KTS, *WT1* isoform without the KTS tripeptide; *WT1*(+)/KTS, *WT1* isoform with the KTS tripeptide; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; EGR-1, early growth response-1; *SRY*, sex-determining region of the Y chromosome; *MIS*, Müllerian inhibiting substance; *AR*, androgen receptor; EMSA, electrophoretic mobility shift assay; DDS, Denys-Drash syndrome; dpc, day(s) postcoitus; NF, nuclear factor.



**Fig. 1** The four isoforms of WT1. Four splice isoforms were generated by the inclusion or exclusion of exon 5 and the KTS tripeptide. The smallest isoform, WT1<sup>-/-</sup>, lacked both exon 5 and the KTS tripeptide. These four isoforms were cloned in the CMV promoter-based expression vector pCB6+ (29).

in embryonal sexual development of mammals (17). Among them, *SRY*, *MIS*, androgen, and *AR*, as well as *WT1*, have been relatively well studied, but how they interact has not been determined. Embryonal sexual development in mammals can be divided into three stages. First, the bipotential undifferentiated gonad develops from intermediate mesoderm. Second, the bipotential gonad becomes sexually determined to form either a testis or an ovary. Third, the internal and external genitalia develop from the sexual duct system and urogenital sinus. *SRY* is known to play a dominant role in gonadal sex determination, and two testicular hormones, *MIS* and testosterone, play significant roles in sex differentiation (18). The facts that *WT1* is expressed as early as 9 dpc in mice (14, 15) and that undifferentiated gonads do not form in mice with homozygous *WT1* mutations suggest that *WT1* may act at the development of the undifferentiated gonad (16). However, *WT1* may also function in the later stages, because its long-term expression overlaps with that of other embryonal sexual development genes both spatially and temporally.

The known target genes of *WT1* include growth factor genes, such as platelet-derived growth factor- $\alpha$  chain (19), insulin-like growth factor-II (20), transforming growth factor- $\beta$ 1 (21), and colony-stimulating factor-1 (22), and receptor genes, such as insulin-like growth factor-I receptor (23) and retinoic acid receptor- $\alpha$  (24). Recently, *Pax2* (25), *bcl-2*, and *c-myc* (26) were also identified as other targets of *WT1*. These findings may contribute to understanding the function of *WT1* in kidney development. *WT1* usually represses transcription of reporter constructs containing these gene promoters. However, the role of *WT1* in embryonal sexual development has not yet been established. To understand the mechanism of action of *WT1* in embryonal sexual development, we examined the transcriptional regulation of the *SRY*, *MIS*, and *AR* genes. We found that *WT1* could function as both an activator (of *SRY*), and a repressor (of *MIS* and *AR*). This suggests that *WT1* functions as a sexual development gene through the regulation of *SRY*, *MIS* and *AR*.

## Materials and Methods

**Cell Culture.** HeLa cells, a human cervical carcinoma cell line from American Type Culture Collection (ATCC CCL2;

Rockville, MD), were grown in Eagle's MEM supplemented with 10% FCS. Saos2 cells, a human osteosarcoma cell line (ATCC HTB85), were grown in McCoy's 5a medium supplemented with 10% FCS. T47D cells, a human breast ductal carcinoma cell line (ATCC HTB133), were maintained in RPMI 1640, 0.2 IU/ml bovine insulin, and 10% FCS. Mouse fibroblast Ltk<sup>-</sup> cells were maintained in DMEM with 10% FCS. Mouse Sertoli TM4 cells (ATCC CRL1715) were maintained in a 1:1 mixture of Ham's F12 medium and DMEM plus 4.5 g/liter glucose, and 10% FCS. The cells were transfected while in the exponential growth phase (<75% confluence).

**Cotransfection.** The reporter CAT constructs used were pSRY-CAT1, pSRY-CAT2, and pSRY-CAT5, which contain the human *SRY* promoter (27); pCBMP and pCEMP, which contain the human *MIS* promoter; and pAC<sub>2</sub>, pAR-125, and pAR-40, which contain the human *AR* promoter (28). To construct pCBMP and pCEMP, a 210-bp fragment of the human *MIS* promoter was amplified by PCR of genomic DNA with the primers 5'-CCCAAGCTTCACATCAGGCCAGCTCTATC-ACTGGGGAG-3' and 5'-GCTCTAGACGTGGGTGCTGC-CAGGGGCTGGGCTGCCTG-3'. The 210-bp fragment was subcloned into the *Hind*III and *Xba*I sites of pCATBasic and pCATenhancer (Promega Corp., Madison, WI). The identity of this fragment was confirmed by sequence analysis (29). The *WT1* effector constructs used contained four isoforms of *WT1* cDNA in the CMV promoter-driven expression vector pCB6+ (30). Other CMV promoter-driven effectors (CMV/*WT1* and CMV/*WT1*[R394W]) contain the murine *wtl* cDNA and its<sup>394</sup>Arg to Trp mutation, respectively (24). All DNAs were purified with the Plasmid Maxi kit (Qiagen, Inc., Chatsworth, CA) according to manufacturer's instructions. Cotransfection assays were performed by electroporation as described previously (31). Briefly,  $5 \times 10^6$  cells were electroporated with up to 20  $\mu$ g of plasmid DNA at 250 V and 960  $\mu$ F in 200  $\mu$ l of medium without serum and were cultured for 48–72 h. Two  $\mu$ g of the CMV promoter-driven  $\beta$ -galactosidase plasmid pCMV $\beta$  (Clontech, Palo Alto, CA) were used as an internal control in the transfections, and the empty expression vector (pCB6+) was used as control DNA in each transfection. Cell extracts containing equivalent amounts of  $\beta$ -galactosidase activity were assayed for CAT activity. After TLC, the acetylated [<sup>14</sup>C]chloramphenicol was quantitated by measuring the radioactivity with a Betascope 603 blot analyzer (Betagen Corp., Waltham, MA). The relative activities from three different experiments were averaged and depicted as bar graphs with the standard error of the mean shown as error bars.

**EMSA.** EMSAs were performed in 10- $\mu$ l reaction mixtures containing 15 mM Tris-Cl (pH 7.5), 6.5% glycerol, 90 mM KCl, 0.2 mM DTT, 1 mg/ml BSA, 100  $\mu$ M ZnCl<sub>2</sub>, 1  $\mu$ g of poly(dI-dC), and 50 ng of WT1(-)KTS or WT1(+KTS) recombinant proteins, which were described previously (12). After 5 min on ice, 2.5–5 pmol of competitor oligonucleotide DNA and then 50–100 fmol of <sup>32</sup>P-labeled probe ( $>5 \times 10^4$  cpm) were added, and the reaction mixture was incubated for 15 min at room temperature. The DNA-protein complexes were electrophoresed in 0.5 $\times$  Tris-borate EDTA (45 mM Tris-borate and 1 mM EDTA) 5% polyacrylamide:bis (39:1) gels at 300 V and 4°C, dried, and visualized by autoradiography with Hyperfilm-MP (Amersham Corp., Arlington

Heights, IL). The intensities of the shifted bands were compared by using a Betascope 603 blot analyzer. The oligonucleotides used were EGR-1 (GGCCGGCGGGGGGCGAGGGGCG), SRY1 (TAAACATTGGTGAGTGGGGGGA-CTTTCCTCCC), SRY2 (TTGGCGGGGTTGAGGGGGTGTGAGGGGCG GAG), AR(-)352 (CGTCCTTTTCCTCCCTCCCTCGCCTC-CACCCTG), AR(+ )427 (CCCTTC-TCCCCACCCGCCCGCCCGCCCGTTCG), MIS-175 (GC-TCTATCACTGGGGA GGGAGATAGGCTGCCAG), MIS-117 (CCACTCTGCCTGGAGTGGGGGCGCCGG GCAC-TG), and GATA (CACTTGATAACAGAAAGTGATAACT-CT) and their complements. The double-stranded oligonucleotide probes were labeled with [ $\gamma$ - $^{32}$ P]ATP by using T4 polynucleotide kinase.

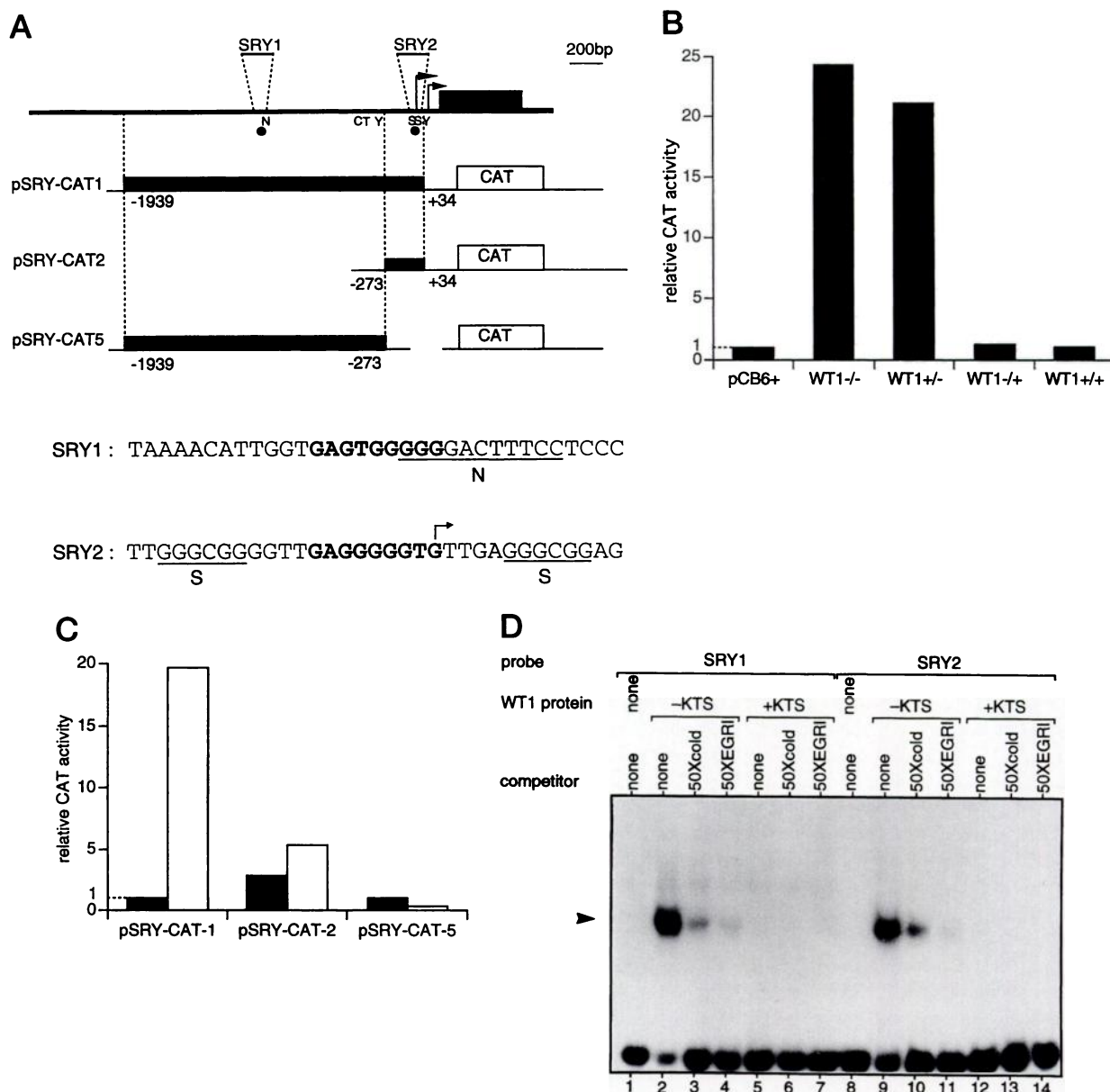
## Results

**Transcriptional Regulation of the SRY Promoter by WT1.** Two transcription start sites have been identified within a 60-bp region in the 1973-bp SRY promoter (27). Two potential WT1 binding motifs with the consensus sequence GNGNGGGNG (32) are located within 2.0 kb of the transcription start sites of the SRY gene (Fig. 2A). One is 860 bp upstream from the first transcription start site and overlaps an NF $\kappa$ B binding site. The second potential WT1 binding site overlaps the first transcription start site. To determine the ability of WT1 to regulate the SRY gene, we cotransfected HeLa cells with expression constructs containing the four WT1 isoforms (Fig. 1) and pSRY-CAT1, which contains the 1973-bp promoter region, including both WT1 binding sites (Fig. 2A). The WT1(-)KTS isoforms strongly transactivated pSRY-CAT1, but the WT1(+ )KTS isoforms had no substantial effect on pSRY-CAT1 (Fig. 2B). The presence of exon 5 did not seem to affect the CAT activity of the pSRY-CAT1 construct, but the presence of the KTS tripeptide prevented transactivation of pSRY-CAT1. The WT1(-)KTS isoform failed to significantly activate transcription of the two deletion constructs pSRY-CAT2 and pSRY-CAT5 (Fig. 2C), which have the proximal and distal WT1 binding sites, respectively, suggesting that both binding sites are required for activation. Similar results were obtained by using Ltk<sup>-</sup> and Saos2 cells in the transient cotransfection assays (data not shown). To verify that the transactivation of the SRY promoter by WT1 is mediated by DNA binding, we determined the ability of a mutant WT1 isoform to transactivate SRY expression. Transactivation by the mouse WT1(-/-) isoform CMV/WT1 was compared to transactivation by the <sup>394</sup>Arg to Trp mutant isoform CMV/WT1 [R394W] (24). This CMV/WT1 [R394W] construct, which contains the mutation most commonly associated with DDS (5), fails to transactivate the RAR $\alpha$  promoter and fails to bind the EGR-1 motif within the hRAR $\alpha$ 1 promoter (24). Similarly, the CMV/WT1 [R394W] construct fails to transactivate the SRY promoter in cotransfection assays in HeLa cells (Table 1). To confirm that normal WT1 protein binds to the SRY promoter, EMSAs were performed with two oligonucleotides derived from the SRY promoter and containing a centrally located WT1 binding site (Fig. 2D). WT1(-)KTS protein strongly bound both oligonucleotides. Formation of these complexes was specifically prevented by competition with an excess of the same unlabeled oligonucleotides or an unlabeled

EGR-1 consensus motif (GCGGGGGCG) but not by the nonspecific oligonucleotide GATA. These EMSA results are consistent with the strong activation of the SRY promoter by the WT1(-)KTS isoform and the lack of transactivation by the WT1(+ )KTS isoform in all cell lines tested.

**Transcriptional Regulation of MIS Promoter by WT1.** Shen *et al.* (33) have shown that 180 bp of the MIS 5' flanking region is sufficient for full promoter activity in rat Sertoli cell primary cultures. Inspection of the promoter sequence shows that potential WT1 binding sites are located at -175, -117, and -115 (last two overlap; Ref. 29). We amplified by PCR the region from -200 to +10 of the MIS transcription start site from human genomic DNA and then subcloned the PCR product into two CAT reporter vectors (pCATenhancer, which contains the SV40 enhancer, and pCATBasic, which lacks the SV40 enhancer) to generate pCEMP and pCBMP, respectively (Fig. 3A). We performed cotransfection assays with these CAT constructs as reporters and the four WT1 isoform expression constructs as effectors in TM4 mouse Sertoli and HeLa cells. WT1(-)KTS isoforms strongly repressed and WT1(+ )KTS isoforms relatively weakly repressed transcription of pCEMP in TM4 cells, but in HeLa cells, all isoforms failed to repress (Fig. 3B). Similar results were obtained with the pCBMP construct, which had very low basal activity in TM4 cells (data not shown). The CMV/WT1 expression construct also strongly represses the MIS promoter (Table 1), with CMV/WT1 repression 2-fold greater than the CMV/WT1 [R/W] mutant construct repression (and the latter repression was not significant when compared to the empty vector control pCB6+). To verify that repression by normal WT1 is mediated by DNA binding, EMSAs were performed with the oligonucleotides MIS-175, which has one WT1 binding site, and MIS-117, which has two overlapping binding sites (Fig. 3C). MIS-117 bound 5-fold more strongly than MIS-175 to the WT1(-)KTS isoform, and the WT1(-)KTS isoform binds the MIS-117 site 24-fold stronger than the WT1(+ )KTS isoform. The WT1(+ )KTS bound weakly to both sites. Formation of the WT1(-)KTS complex with the MIS-117 oligonucleotide was specifically prevented by competition with an excess of the same unlabeled oligonucleotide or an unlabeled EGR-1 consensus motif but not by the nonspecific oligonucleotide containing a GATA binding site. The EMSA results are consistent with the repression of the MIS promoter by both the WT1(+ )KTS and WT1(-)KTS isoforms in TM4 cells with strongest repression by the WT1(-)KTS isoform. The weaker DNA binding by the WT1(+ )KTS protein *in vitro* correlates with the relatively weaker repression of the MIS promoter *in vivo* but suggests that additional factors are present in TM4 cells, which may stabilize binding by the WT1(+ )KTS isoform, leading to a modest repression of MIS.

**Transcriptional Regulation of the AR Promoter by WT1.** AR is a member of the steroid and thyroid hormone receptor family. The promoter region of human AR does not have either a TATA box or a CCAAT box but does contain a GC box (34). Within AR -737 to +575, there are 16 potential WT1 binding motifs. Eight of these motifs overlap at three positions (Fig. 4A). We cotransfected expression constructs of the four WT1 isoforms and the reporter construct pAC<sub>2</sub>, which contains AR -737 to +575 and has the 16 AR WT1 binding sites (Fig. 4B), into three different cell lines that do not express



**Fig. 2** Transcriptional regulation of *SRY* promoter by *WT1*. **A**, schematic representation of *SRY* promoter CAT constructs and sequences of oligoprobes SRY1 and SRY2, which were used in EMSAs. *Bent arrows*, transcription start sites; *ORF*, open reading frame; ●, *WT1* binding site; *N*, *NFκB* binding site; *C*, CAATT sequence; *T*, TATA box; *Y*, *SRY* binding site; *S*, *Sp1* binding site (26). SRY-CAT1, the full-length promoter, contains two *WT1* binding sites, whereas the 5' deletion construct, pSRY-CAT2, and the 3' deletion construct, pSRY-CAT5, each contain only one *WT1* binding site. The *WT1* binding sites in each oligo probe are in **boldface**. **B**, relative CAT activity of the full-length *SRY* promoter (10 μg of pSRY-CAT1) cotransfected with 5 μg of each of the four *WT1* isoform expression plasmids in HeLa cells. The CAT activity is depicted relative to the activity of the pCB6 + expression vector alone. **C**, relative CAT activity of 5 μg of the full-length (pSRY-CAT1) and deletion constructs (pSRYCAT2 and pSRY-CAT5) cotransfected with 5 μg of pCB6+ (■) or *WT1*<sup>-/-</sup> construct (□) in HeLa cells. The CAT activity is expressed relative to the activity obtained by cotransfection of pSRY-CAT1 and pCB6+. **D**, EMSA using oligonucleotide probes SRY1 and SRY2 and recombinant *WT1*(-)*KTS* and *WT1*(+)*KTS* proteins. 50× cold, competition by 50-fold molar excess of unlabeled probe; 50× *EGR-1*, competition by a 50-fold molar excess of *EGR-1* oligonucleotide DNA. The complexes are indicated by an *arrowhead*. Note that the *EGR-1* probe contains three *WT1* binding sites.

endogenous *WT1*: HeLa; T47D, a breast cancer cell line that produces *AR*; and Saos2, a p53-negative osteosarcoma cell line in which cotransfected *WT1* has been shown to activate the *EGR-1* promoter (35). *WT1*(-)*KTS* isoforms strongly repressed

the *AR* promoter in all three cell lines (Fig. 4B). *WT1*(+)*KTS* isoforms showed weaker repression in HeLa and Saos2 cells. In contrast, *WT1*(+)*KTS* failed to repress the *AR* promoter in T47D cells (Fig. 4B).

Table 1 Mutation of WT1 diminishes modulation of promoter activity<sup>a</sup>

	Promoter	CB6+/wt1(-/-)	CMV/wt1(-/-)	CMV/mut wt1 (R394W)	% of wild type
Activation by WT1	SRY <sup>b</sup>	12 ± 0.73	31 ± 4.70	0.2 ± 0.9	0.6% <sup>c</sup>
Repression by WT1	AR <sup>d</sup>	0.32 ± 0.04	0.30 ± 0.06	0.75 ± 0.01	40% <sup>e</sup>
	MIS <sup>f</sup>	0.18 ± 0.06	0.22 ± 0.05	0.39 ± 0.11	56% <sup>g</sup>

<sup>a</sup> Mean activity ± SE expressed relative to the empty vector, pCB6+.

<sup>b</sup> Ten µg of pSRY-1 reporter, 5 µg of WT1 expression construct in HeLa cells.

<sup>c</sup> CMV/mut wt1 (R394W) relative to CMV/wt1(-/-).

<sup>d</sup> Five µg of pAC2 reporter, 5 µg of WT1 expression construct in HeLa cells.

<sup>e</sup> CMV/wt1(-/-) relative to CMV/mut wt1 (R394W).

<sup>f</sup> Five µg of pCEMP reporter, 10 µg of WT1 expression construct in TM4 cells.

<sup>g</sup> CMV/wt1(-/-) relative to CMV/mut wt1 (R394W).

Next, we analyzed the effects of cotransfection of 5' deletion constructs of pAC<sub>2</sub> lacking five (pAR-125) or six (pAR-40) potential WT1 binding sites. Both of the constructs retained the 10 WT1 binding sites located 3' of the transcription start site. Although the basal activities of pAR-125 and pAR-40 were very low in HeLa cells (Fig. 4C, ■), these constructs were not repressed by the WT1(-)KTS isoforms (Fig. 4C, □), suggesting that the 5' binding sites are required for repression. To verify that repression of the AR promoter is mediated by DNA binding, we cotransfected it with the mutant CMV/WT1 [R394W] construct. This mutant construct weakly represses the AR promoter construct pAC2 in HeLa cells (Table 1), and this repression is not significant when compared to the empty vector control pCB6+. To examine direct binding of WT1 protein to the AR promoter, we performed an EMSA (Fig. 4D) using two oligonucleotides: one [AR(-)352] from the 5' promoter region that contains two overlapping binding sites, and another [AR(+)-427] from the 3' promoter region with four overlapping binding sites. WT1(-)KTS protein bound more strongly than did WT1(+)-KTS protein, to both oligonucleotides. Complex formation was specifically prevented by competition with an excess amount of unlabeled oligonucleotide or unlabeled EGR-1 oligonucleotide but not by an excess amount of GATA oligonucleotide. The EMSA results are consistent with the strong repression of the AR promoter by WT1(-)KTS isoforms in HeLa and Saos2 cells. The weaker repression by the WT1(+)-KTS isoform is also consistent with the very weak complex formation.

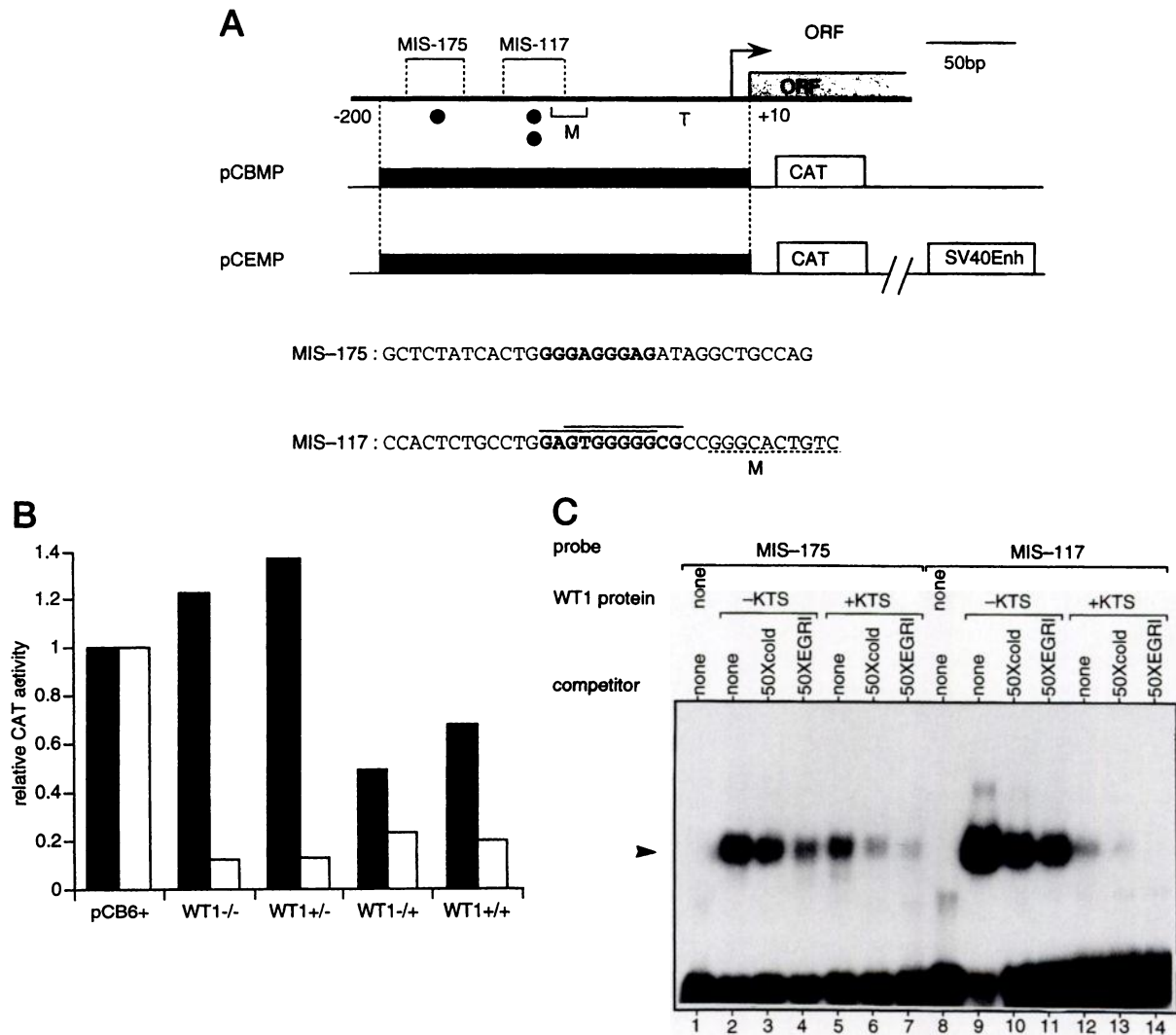
## Discussion

*In situ* hybridization to mouse embryos shows that *WT1* first appears in the urogenital ridge at 9 dpc (15). During the development of the genital organs, *WT1* is expressed strongly in the genital ridge, and expression continues after the genital ridge forms the gonads, especially in the male sex cords, structures that contain the precursors of Sertoli cells. After birth, *WT1* is expressed in the testis, especially in Sertoli cells, but not in Leydig cells or in germ cells (14, 15). Wolffian and Müllerian ducts do not express *WT1*.

*SRY* is a high-mobility group box protein that can function as a DNA binding protein and can commit the undifferentiated gonad to testis (36). Mouse *Sry* is expressed in the nongerm cells that are the precursors of Sertoli cells in the developing testis only during the period from 10.5 to 12.5 dpc (37), which

overlaps with the expression period of *WT1*. Our data indicate that *WT1* protein directly binds to the *SRY* promoter and activates transcription of the *SRY* gene. Because *WT1* expression precedes *SRY* expression, we suggest that transcriptional activation of *SRY* is one of the mechanisms by which *WT1* can function as a sexual development gene (Fig. 5). The absence of *SRY* activation would partly explain how mutations resulting in loss of function of *WT1* lead to genital anomalies as in DDS or in the WAGR constellation of genetic diseases. The finding that *SRY* activation by *WT1* (Table 1) is lost with the *WT1* mutation at R394W, the most common mutation in DDS, is consistent with the clinical findings of DDS (38). DDS primarily involves the feminization of XY (40 of 47 reported DDS patients had XY genotypes including abnormal genital/intersex anomalies and XY pseudohermaphroditism).

*MIS* is an autosomal gene, and its product is a member of the transforming growth factor-β family (29). *MIS* is secreted by Sertoli cells in the developing testis and causes regression of the Müllerian duct, which otherwise would develop into the female genital organs (39). In mouse, *Mis* is expressed from 11.5 dpc in Sertoli cells (40), and *Mis* expression overlaps that of *wl1*. It has been proposed that steroidogenic factor 1 (*SF-1*) is an upstream regulatory gene of *MIS* (33). Although *MIS* plays a role in the differentiation of the sexual duct system, it also influences gonadal differentiation. This effect is demonstrated in the freemartin phenomenon, in which *in utero* exposure of a female twin fetus to the *MIS* in a male twin fetus' blood causes oocyte depletion and the development of seminiferous tube-like structures in the ovaries (41). This phenomenon has been confirmed in XX mice transgenic for human *MIS*, which chronically express *MIS* (42). Additionally, chronic high expression of *MIS* in XY transgenic mice produces feminization of the external genitalia, impairs Wolffian duct development, and leads to undescended testes. The first two abnormalities are thought to be the result of androgen deficiency due to *MIS*-induced inhibition of Leydig cell differentiation and function (42). Our finding that *WT1* can repress *MIS* transcription in Sertoli cells suggests that loss of function of *WT1* could result in high *MIS* levels and so lead to genital anomalies, such as cryptorchidism in XY patients. Although XX DDS patients have normal female external and internal genitalia, they also have gonadal abnormalities, such as dysgenesis and abnormally located ovaries (4). If *WT1* dysfunction also increases the amount of *MIS* in ovarian granulosa cells, which are thought to be derived from the same



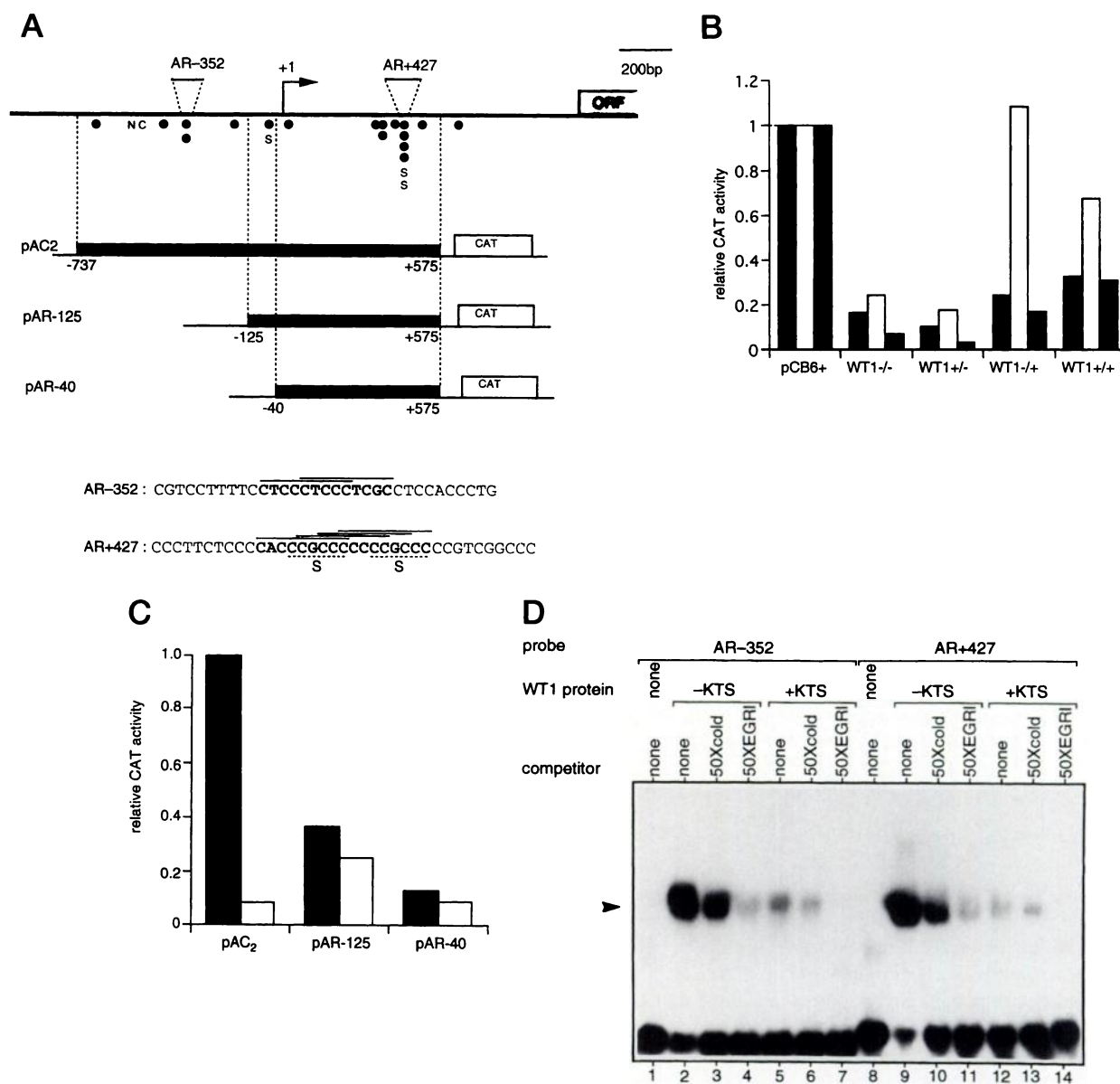
**Fig. 3** Transcriptional regulation of *MIS* promoter by WT1. **A**, schematic representation of *MIS* promoter CAT constructs and sequences of oligoprobes MIS-175 and MIS-117, which were used in EMSAs (**C**). *Bent arrow*, transcription start site; *ORF*, open reading frame; ●, WT1 binding site (28); *M*, conserved element (MIS-RE-1; Ref. 26); *T*, TATA box (26, 28). The full-length *MIS* promoter was subcloned into pCATBasic and pCATEnhancer, which contains the SV40 enhancer, to make pCBMP and pCEMP, respectively. The WT1 binding sites in each oligoprobe are in **boldface**. The lines over the sequences indicate the two overlapping WT1 binding sites. **B**, relative CAT activity of *MIS* promoter (5  $\mu$ g of pCEMP) cotransfected with 10  $\mu$ g of each of the four WT1 isoforms expression plasmids in HeLa cells (■) and in TM4 cells (□). The CAT activity is expressed relative to the activity obtained by cotransfection of pCB6+. **C**, EMSA using oligonucleotide probes MIS-175 and MIS-117 and recombinant WT1(-)KTS and WT1(+KTS) proteins. 50 $\times$  cold, competition by 50-fold molar excess of unlabeled probe; 50 $\times$  EGR-1, competition by 50-fold molar excess of EGR-1 oligonucleotide DNA. The specific complexes are indicated by an *arrowhead*.

progenitors as Sertoli cells in females, it would partly explain the gonadal abnormalities seen in XX DDS patients. The measurement of blood MIS levels in DDS patients might help evaluate this hypothesis.

Another interesting finding in our study is that WT1 repressed the *MIS* promoter in TM4 Sertoli cells but not in HeLa cells. This suggests that there is a cofactor for WT1 in Sertoli cells. Several reports have suggested that WT1 can associate with itself (6, 7, 43) or with other proteins (35). Sertoli cells seem to be a good candidate for the source of the protein that interacts with WT1.

Leydig cells secrete testosterone, which binds to the AR.

Therefore, the AR mediates the function of testosterone in the development of the the sexual duct system and external genitalia (44). AR mutations are associated with a wide spectrum of defects in male sexual development (45), including testicular feminization (46). Developing male reproductive organs, such as efferent ductules, urogenital sinus, Wolffian duct, and their derivatives, express AR (47) but not WT1 (15). This suggests that the absence of WT1 repression of AR during differentiation of the sexual duct system does not explain the genital anomalies in Wilms' tumor patients. Although AR is known to play an important role in differentiation of the sexual duct system, it may also be involved in differentiation of gonads. The high



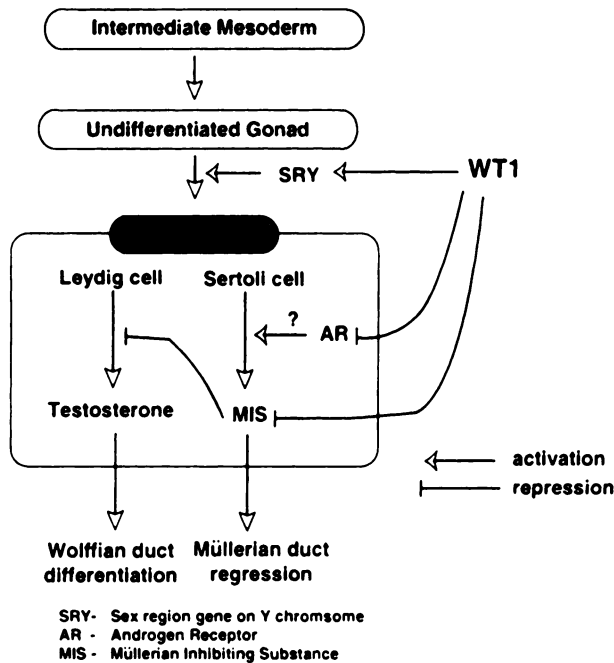
**Fig. 4** Transcriptional regulation of *AR* promoter by WT1. **A**, schematic representation of *AR* promoter CAT constructs and sequences of oligonucleotide probes AR-352 and AR+427, which were used in EMSAs. *Bent arrow*, transcription start site; *ORF*, open reading frame; ●, WT1 binding site; *N*, NF1 binding site; *C*, cAMP response element; *S*, Sp1 binding site (33). The full-length *AR* promoter construct, pAC<sub>2</sub>, contains 16 WT1 binding sites, and the two 5' deletion constructs pAR-125 and pAR-40 contain 11 and 10 binding sites, respectively. In pAR-40, all of the WT1 binding sites are located 3' of the transcription start site. The WT1 binding sites in each oligo probe are in *boldface*, and the *lines* over the sequence indicate the overlapping WT1 binding sites. **B**, relative CAT activity of *AR* promoter (pAC<sub>2</sub>) cotransfected with each of the four WT1 isoforms expression plasmids in HeLa cells (■), T47D cells (□), and Saos2 cells (□). Five, 2.5, and 4 μg of the full-length promoter reporter construct pAC<sub>2</sub> and 10, 5, and 4 μg of expression constructs were transfected into HeLa, T47D, and Saos2 cells, respectively. The CAT activity is expressed relative to the activity obtained by cotransfection of equivalent amounts of the expression construct pCB6+. **C**, relative CAT activity of 5 μg of three *AR* promoter CAT constructs cotransfected with 5 μg of pCB6+ (■) or WT1<sup>-/-</sup> (□), in HeLa cells. The CAT activity is expressed relative to the activity obtained by cotransfection of pAC<sub>2</sub> and pCB6+. **D**, EMSA using oligoprobes AR(-)352 and AR+427 and recombinant WT1(-)KTS and WT1(+)-KTS proteins. 50× *cold*, competitor by a 50-fold molar excess of unlabeled probe; 50× *EGR-1*, competition by a 50-fold molar excess of *EGR-1* oligonucleotide DNA.

WT1 and low AR expression in the Sertoli cells of young mice suggests an inverse relationship between WT1 and AR; this is supported by the lower WT1 and higher AR expression in adult mice (48). AR also mediates the function of testosterone in

mature Sertoli cells, which are stimulated by testosterone to maintain spermatogenesis. In contrast, fetal Sertoli cells are insensitive to androgens and fail to support spermatogenesis (44). We speculate that the insensitivity of fetal Sertoli cells to



### Role of *WT1* In Sex Differentiation



**Fig. 5** Model of how *WT1* participates in embryonic male sexual development. *WT1* activates *SRY* and represses *MIS* and *AR*. Loss of *WT1* function is associated with genital abnormalities, possibly because of decreased *SRY* and increased *MIS* expression. Increased *AR* expression due to loss of *WT1* function may contribute to the increase in *MIS* expression.

androgen is due to transcriptional repression of the *AR* gene by *WT1*, which is strongly expressed in fetal Sertoli cells; however, the *AR* expression levels in murine fetal Sertoli cells have not been measured. The mechanism by which defects in *WT1* cause genital anomalies through loss of repression of the *AR* gene in Sertoli cells also must be confirmed.

The results presented here are the first evidence that the CMV promoter-driven *WT1* expression vector can transcriptionally activate a natural promoter. Wang *et al.* (49) proposed that *WT1* could function as an activator when the promoter region of the platelet-derived growth factor (*PDGF*) A-chain was mutated so that there were *WT1* binding sites either 5' or 3' of the transcription start site but not flanking it. This hypothesis is consistent with *WT1* activation of the *SRY* promoter, which has binding sites only on its 5' side, but it is not consistent with *WT1* repression of the *MIS* promoter, which also has binding sites only on its 5' side. Maheswaran *et al.* (35) proposed that *WT1* activates promoters in the absence of p53 expression, but this theory is not supported by our results with the *AR* promoter, which was repressed by *WT1* even in p53-negative Saos2 cells. Recently, Reddy *et al.* (50) reported that when *WT1* is expressed from a CMV promoter-driven expression vector, *WT1* represses *EGR-1* promoter activity, but when *WT1* is expressed from vectors with other promoters, it activates the *EGR-1* promoter. Although the expression vector we used has a CMV promoter, *WT1* activated the *SRY* promoter. Therefore, how

*WT1* switches from a repressor to an activator still remains an enigma.

The functional differences of the four *WT1* isoforms have also not been resolved. Although Reddy *et al.* (43) and Wang *et al.* (51) reported differences between the functions of isoforms with and without exon 5, our data did not show significant differences between these isoforms. On the other hand, there was a substantial difference between isoforms with and without KTS. Substantial activation of *SRY* and repression of *AR* and *MIS* were seen only with the *WT1*(-)KTS isoforms; the *WT1*(+)KTS isoforms showed either weak or no activity. The functional differences between the *WT1*(+)KTS and *WT1*(-)KTS isoforms are probably due to their different binding activities as seen in the EMSAs. The *WT1*(+) isoforms may have *WT1* binding sites other than GNGNGGGNG (10) or may have other functions, such as posttranscriptional RNA processing rather than DNA binding; only *WT1*(-)KTS isoforms may have DNA-binding activity (52).

The function of *WT1* in embryonal sexual development may be stage-specific. At the sex-determination stage, *WT1* induces masculinization through activation of the *SRY* gene, but at the later sex-differentiation stage, *WT1* may induce feminization through repression of *MIS* and *AR* genes. *WT1* only affects *SRY* expression in males, because it is a Y chromosome gene, but *WT1* can affect *MIS* and *AR* expression in both males and females. *MIS* expression must be tightly regulated as both overexpression and no expression result in feminization. There are two interpretations of our repression studies relative to understanding the later stage sex differentiation function of *WT1*. If *WT1* functions the same in males and females, then *WT1* repression of *MIS* in males may just prevent overexpression of *MIS* and thereby prevent feminization. This implies that *WT1* repression of *MIS* may be modest in males and females, and there should be another, female-specific, stronger repressor of *MIS* if *WT1* repression is not sufficient to completely repress *MIS*. The second interpretation assumes that *WT1* functions differently in males and females. This functional difference between males and females can be explained, if there is a difference in *WT1* isoform ratios between males and females. During the sex differentiation stage, granulosa cells may have sufficient *WT1*(-)KTS isoforms to completely repress *MIS*, whereas Sertoli cells may not. To date, *WT1* isoform ratios have been shown to be almost the same between adult testis and ovary (8), but the ratios in embryonal gonads have not yet been determined. One approach to proving this hypothesis is to determine the ratio of *WT1* isoforms in embryonal gonads. If there is a difference in the *WT1* isoform ratios between embryonal male and female gonads, it will be possible to test this theory by developing isoform-specific transgenic mice.

Our data suggest that *WT1* regulates the expression of the sex determination gene *SRY* and the sex differentiation genes *MIS* and *AR*. These findings may explain the genital anomalies associated with Wilms' tumor. However, *WT1* may also regulate the expression of other embryonal sexual development-related genes (Fig. 5). Because embryonal sexual development is thought to involve many more genes, it seems likely that *WT1* may also regulate some of those unknown genes, including the gene(s) responsible for the development of undifferentiated gonad from mesoderm (17), and the relatively early and long-

lasting gene expression of *WT1* during the development of the gonads raises the possibility that its expression overlaps these unknown genes.

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