Wilms' Tumor 1 and Signal Transducers and Activators of Transcription 3 Synergistically Promote Cell Proliferation: A Possible Mechanism in Sporadic Wilms' Tumor

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Abstract

Transcription factor Wilms' tumor 1 (WT1) was originally identified as a tumor suppressor for Wilms' tumor, but it is also overexpressed in a variety of cancer cells, suggesting a potential oncogenic function of WT1. It is important to understand molecular mechanisms underlying these dual functions of WT1 in tumorigenesis. In the current study, we report a synergistic role for signal transducers and activators of transcription 3 (STAT3) and WT1 in tumor development, including Wilms' tumor. STAT3 interacts with WT1 through its conserved domains both *in vitro* and *in vivo*. When STAT3 is activated, expression of WT1 enhances STAT3 transcriptional activity. Overexpression of WT1 and STAT3CA in NIH 3T3 increases the expression level of STAT3 target genes, including *cyclin D1* and *Bcl-xL*, which results in an advantage of cell proliferation. Our results suggest that in the presence of activated STAT3, WT1 promotes cell proliferation instead of suppressing cell proliferation. Strikingly, STAT3 translocates to the nucleus and interacts with WT1 in a variety of primary Wilms' tumor cells, raising the hypothesis that WT1 and activated STAT3 in Wilms' tumor accelerate tumorigenesis. (Cancer Res 2006; 66(16): 8049-57)

Introduction

Wilms' tumor 1 (WT1), a transcription factor, was originally identified as a tumor suppressor involved in Wilms' tumor, the most common solid tumor found in children (1, 2). WT1 has been shown to have an important role in kidney, gonad, retina, and spleen development (3–7). WT1 has four major isoforms (WT1A, WT1B, WT1C, and WT1D), which are a result of alternative splicing of exon 5 [−17 amino acids (aa)] and exon 9 (−3 aa, KTS, splice donor site) of the Wilms' tumor gene (*wt1*). The carboxyl terminus of WT1 contains four zinc-finger domains that mediate DNA binding whereas the amino terminus is rich in prolines and glutamines and resembles the transactivation domain of other transcription factors (8–11).

Based on the prototype WT1-responsive promoter, WT1 has been reported to repress expression of growth-inducing genes, such as insulin-like growth factor 2, insulin-like growth factor receptor, platelet-derived growth factor A, epidermal growth factor receptor, transforming growth factor β, bcl-2, c-myc, and others (12–15). The role of WT1 as a tumor suppressor is supported by suppression of colony formation and reduction of tumorigenesis when WT1 is ectopically expressed in several cell lines, including some Wilms' tumor cell lines. Intriguingly, WT1 was shown to be a transcriptional repressor as well as an activator under different conditions (8, 11), suggesting that it plays several roles in cell growth and development.

Although the *wt1* gene was reported to be deleted or point mutated in a subset of sporadic Wilms' tumors (11), the majority of these tumors have the wild-type *wt1* gene. Thus, WT1 seems to lose its tumor suppressor function in these sporadic Wilms' tumors. Moreover, the wild-type (rather than mutated) *wt1* gene is expressed in cells derived from colon cancer and breast cancer (16, 17). WT1 is also overexpressed in primary leukemia (18) and many other cancer cells (19). The wild-type WT1 was thus proposed to play an oncogenic, but not tumor suppressor, role in these WT1-expressing cancers. It is unclear what the possible mechanisms are for WT1 in tumorigenesis and whether other factors are involved in the possible transition of WT1 from a tumor suppressor to an oncoprotein.

STATs are latent transcriptional factors activated by many cytokines and growth factors. Signal transducers and activators of transcription 3 (STAT3) has been found to be overexpressed or constitutively activated in a variety of human malignancies. The constitutive active mutant form of STAT3, STAT3CA, displays cell-transforming activity and tumor formation ability in nude mice (20). Interference of STAT3 activity by overexpressing dominant-negative STAT3β, STAT3Y705F, or by introducing STAT3 small interfering RNA (siRNA) represses tumor cell proliferation and tumor growth, leading to apoptosis in nude mice. It is suggested that STAT3 is involved in tumorigenesis and is an oncoprotein (20–23).

STAT3 could participate in oncogenesis through up-regulation of genes encoding apoptosis inhibitors (Mcl-1 and Bcl-xL), cell-cycle regulators (cyclins D1/D2 and c-Myc), and inducers of angiogenesis (24–26). In the present study, we report a novel transcriptional mechanism by which the interaction of WT1 and STAT3 in the nucleus leads to synergistic up-regulation of downstream genes including *cyclin D1* and *Bcl-xL*, resulting in a growth advantage for tumor cells. We present evidence that WT1 helps activated STAT3 stimulate cell proliferation; in this way, WT1 functions as a tumor stimulator rather than as a tumor suppressor.

Materials and Methods

Plasmids and reagents. The mammalian expression vectors including pXJ40-Flag-STAT3, pXJ40-GST, pXJ40-GST-STAT3, a series of

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pXJ40-Flag-STAT3 deletion constructs, and pSGT-c-Src expression plasmid were provided by Dr. Xinmin Cao. pCMV-WT1A, pCMV-WT1B, pCMV-WT1C, and pCMV-WT1D (different isoforms) plasmids were provided by Dr. M.W. Mayo. Luciferase reporter vector pGL3-$(APRE)_4$-luc and pBL2-$(WTE)_3$-luc were provided by Drs. Sachiko Ezoe and Aart J. Jochemsen, respectively. pRc/CMV-Flag-STAT3, Flag-STAT3CA, Flag-STAT3-Y705F, and pRc/CMV-STAT3-S727A were provided by Jame E. Darnell Jr. c-Myc-tagged WT1A (pCMV-Myc-WT1A) was obtained by PCR method from pCMV-WT1. pBS/U6/STAT3-siRNA (for mouse STAT3 siRNA) was constructed according to a previous protocol (27). The target sequence selected is 2,326 to 2,344 bp from the first ATG in the mouse STAT3 mRNA (GenBank accession no. AY299489). Human recombinant interleukin-6 (IL-6) and IL-6 soluble receptor were purchased from Calbiochem (Darmstadt, Germany). Purchased antibodies were anti-Flag M2 monoclonal antibody (Sigma, St. Louis, MO), anti-WT1 rabbit polyclonal antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, CA), anti-STAT3 rabbit polyclonal antibody (C-20, Santa Cruz Biotechnology), anti-p-STAT3 monoclonal antibody (B-7, Santa Cruz Biotechnology), anti–cyclin D1 monoclonal antibody (HD-11, Santa Cruz Biotechnology), anti-βcl-xL monoclonal antibody (H-5, Santa Cruz, Biotechnology), and anti-β-actin monoclonal antibody (C-2, Santa Cruz Biotechnology). The secondary and tertiary antibodies were from Amersham Pharmacia Biotechnology (Piscataway, NJ).

Cell culture, transfection, and generation of stable cells. Human embryonic kidney cell lines 293T and 293, mouse fibroblast cell line NIH 3T3, mouse melanoma cell line B16, and mouse hepatic cell lines $STAT3^{-/-}$ (KO) and $STAT3^{+/+}$ (WT) derived from STAT3 conditional knockout and wild-type mouse were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin. Human hepatoma cell line Hep3B was cultured in MEM complete medium. B16 cells were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The other cell lines were transfected using Vigorous reagent (Vigorous, Beijing, China). For establishing stable cell lines expressing WT1A and/or STAT3CA, expression vector and/or pHygro vector was cotransfected into different cell lines. The positive clones were selected in culture medium containing 1,000 μg/mL G418 and/or 350 μg/mL hygromycin. The resistant cells were then further expanded in 10-cm culture dishes and subsequently confirmed by Western blot.

Glutathione *S*-transferase pulldown assay, coimmunoprecipitation assay, and Western blot. 293T cells in 10-cm dishes were transfected with indicated expression plasmids and were lysed in 1-mL cell lysis buffer [80 mmol/L KCl, 10 mmol/L Na_2HPO_4, 1 mmol/L EDTA (pH 8.0), 0.5% NP40, 10% glycerol, 1 mmol/L DTT, 0.1 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin]. For glutathione *S*-transferase (GST) pulldown assay, 500-μL whole-cell lysate was incubated with 30-μL GST-sepharose beads for 8 hours at 4°C. For the coimmunoprecipitation assay, 500-μL whole-cell lysate was incubated with 2 μg of the indicated antibody and 30 μL of protein G or protein A-sepharose beads. The beads were washed with cell lysis buffer six times and eluted with 2× SDS-PAGE sample buffer and analyzed by Western blot as previously described (27).

Luciferase assay. Luciferase assays were done with the indicated plasmid mixtures using 293T cells, Hep3B cells, and NIH 3T3 cells as previously described (27). The reporters used were pGL3-$(APRE)_4$-luc and pBL2-$(WTE)_3$-luc. Data were normalized with an internal control (pRL-TK vector from Promega, Madison, WI). Experiments were done in triplicate and the means with SEs were calculated.

Electrophoretic mobility shift assay. WT1A protein was transiently overexpressed in 293T or COS-1 cells for 36 hours. The nuclear extracts were prepared after the cells were starved for 12 hours and stimulated with 200 ng/mL IL-6 and 200 ng/mL IL-6 soluble receptor (for 293T) or with 100 ng/mL EGF (for COS-1) for 30 minutes. Nuclear extracts containing the same amounts of total protein were subjected to electrophoretic mobility shift assay with ^{32}P-labeled high-affinity SIE/M67 probe (5′-agcttcattttcccgtaaatcccctaaagct-3′; ref. 28). Competitions were done in the presence of unlabeled 100-fold high-affinity SIE oligonucleotide. For the supershift assay, reaction mixtures were preincubated with 1 μg of anti-STAT3 antibody (C-20) or anti–green fluorescent protein antibody (Santa Cruz Biotechnology).

Immunostaining and confocal microscopy. Mouse hepatic $STAT3^{+/+}$ cells were transiently transfected with pCMV-WT1A plasmid and stimulated with IL-6 for 30 minutes. Then the cells were washed with PBS and permeabilized with 0.2% Triton X-100 in PBS for 10 minutes. The cells were blocked with 10% normal goat serum for 45 minutes at the room temperature. The primary antibodies, diluted in PBS with 3% bovine serum albumin, were incubated for 2 hours at room temperature and the bound antibodies were detected with FITC- or TRITC-conjugated goat anti-rabbit or anti-mouse immunoglobulin G (IgG). The staining cells were analyzed using a laser scanning confocal microscopy.

RNA isolation and reverse transcription-PCR amplification. Total RNA was extracted using the Trizol reagent (Invitrogen). Reverse transcription-PCR (RT-PCR) analyses were done with the One-Step RT-PCR kit (Takala, Dalian, Liaoning Province, China). Total RNA (0.5 μg) for each sample was used in an RT-PCR reaction. Fragments of the human WT1 four zinc-finger domains were amplified using the following primers: 5′-gcaccttggttgtgggtgac-3′ and 5′-cgtgctgcttcctggtcc-3′. For detection of STAT3 downstream genes in mouse cell lines, the primers used were c-myc, 5′-tctttccctacccgctcaac-3′and 5′-accctgccactgtccaactt-3′; matrix metalloproteinase 2 (MMP-2), 5′-aacggtcgggaataca-3′ and 5′-tttggcggacagtgac-3′; Mcl-1, 5′-ggcttgaacctgtactgcgg-3′ and 5′-gagtccttggagccggtcgc-3′; and p21, 5′-ctggtgatgtccgaccgacctgtt-3′ and 5′-tgcaagacagcgacaaggcc-3′. PCR was done with 25 reaction cycles. PCR products were resolved on a 2% agarose gel stained with ethidium bromide. β-Actin was amplified as an internal control.

Histologic examination and immunohistochemical analysis. Wilms' tumor specimens were collected and the paraffin-embedded specimens were fixed on a glass slide for immunohistochemical analysis. Rabbit anti-WT1 or anti-STAT3 antibody at a dilution of 1:200 was used and detected with IHC Polymer Detection Kit (Zymed, South San Francisco, CA).

STAT3 siRNA construction, cell proliferation, and cell cycle assays. pBS/U6/STAT3-siRNA (for STAT3 siRNA) was constructed according to a previous protocol (27). B16 cell with STAT3 expression was transiently knocked down by the siRNA construct.

NIH 3T3, B16, $STAT3^{-/-}$, and $STAT3^{+/+}$ cells were used to stably overexpress WT1A and/or STAT3CA. Cellular proliferation was monitored by [^{3}H]thymidine incorporation assay after 24 hours of starvation (without serum). Cells were washed with PBS and followed with 150 μL of 5% trichloroacetic acid for 30 minutes at 4°C. Cells were lysed in 150 μL of 0.5 N NaOH/0.5% SDS, collected into 96-well scintillation plate, and counted. Otherwise, cell numbers were counted or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done after culturing for indicated times. Cell cycle was measured using fluorescence-activated cell sorting.

Chromatin immunoprecipitation assay. 293 cells expressing Flag-STAT3 and Myc-WT1A for 36 hours were used for chromatin immunoprecipitation after 45-minute treatment with leukemia inhibitory factor conditioned medium before sonication. Rabbit IgG antibody, anti-STAT3 antibody (c-20, Santa Cruz Biotechnology), and anti-c-Myc antibody (9E-10, Santa Cruz Biotechnology) were used for precipitation and the precipitants were resolved according to the protocol of the manufacturer (Upstate Bio., New York, NY). The primers for amplifying the precipitated DNA of human *cyclin D1* promoter (GenBank accession no. Z29078) were 5′-aatgaaacttgcacaggggt-3′ (sense) and 5′-agcccaaaagccatccctga-3′ (antisense). PCR was done with 50°C annealing and 33 reaction cycles.

Results

WT1 associates with STAT3 *in vivo*. We identified a partial WT1 fragment containing the transactivation domain (19-228 aa) in a yeast two-hybrid screening using full-length STAT3 as bait (data not shown). To explore possible interaction between STAT3 and WT1 in mammalian cells, we expressed GST-STAT3 and WT1 (isoform A, −17aa-KTS; ref. 10) proteins in 293T cells. GST-STAT3, but not GST alone, pulled down the WT1A protein when they were coexpressed (data not shown). The interaction of WT1 with STAT3

was verified using a Flag-tagged STAT3. In this experiment, expressed STAT3 and WT1 were coimmunoprecipitated reciprocally by antibodies specific to the Flag tag (STAT3) or to WT1 (Fig. 1*A*). We next examined the interaction of WT1 with STAT3 using endogenous proteins. Protein levels were directly measured in different tissues from 7-day neonatal mice with anti-WT1 and anti-STAT3 antibodies. STAT3 protein was expressed ubiquitously in the liver and kidney tissues whereas WT1 was expressed strongly in the kidney. More STAT3 was precipitated with WT1 in cell lysates made from kidney tissue whereas less STAT3/WT1 complex was observed in liver (Fig. 1*B*). These data indicate that the WT1/STAT3 complex exists *in vivo* and correlates with levels of WT1 expression in different tissues.

We next delineated the regions of STAT3 that interact with WT1 using a series of truncated and Flag-tagged STAT3 proteins, which were coexpressed with WT1 in 293T cells and coimmunoprecipitated with anti-WT1 antibody. The DNA binding domain, linker domain, and Src homologue 2 (SH2) domain of STAT3 seemed to be involved in WT1 binding (Fig. 1*C*). Furthermore, the different deletions of WT1 showed that the transactivation domain of WT1 is responsible for the interaction (Fig. 1*D*).

WT1 enhances transcriptional activity of induced STAT3 or STAT3CA. To investigate the functional consequence of the interaction of WT1 with STAT3, we examined transcriptional activities of these proteins independently and when they were coexpressed. We first examined IL-6-induced STAT3 transcriptional activity using a luciferase reporter controlled by a STAT3 response element (APRE). IL-6 treatment induced STAT3-driven luciferase activity (Fig. 2*A, left second column*). In the presence of expressed WT1 (isoform A), luciferase activity was further increased, suggesting that WT1 enhanced the transcriptional activity of STAT3 (Fig. 2*A, left fourth column*). Similarly, cotransfection of STAT3 with c-Src, a STAT3 activator, induced STAT3 transcriptional activity, and coexpression of WT1A also significantly increased this c-Src-induced STAT3 activity (Fig. 2*A, right columns*), indicating that c-Src protein affects STAT3 and WT1 functions. Furthermore, in the presence of constitutively active STAT3CA, WT1 dramatically enhanced the luciferase reporter activity (Fig. 2*B*). In contrast,

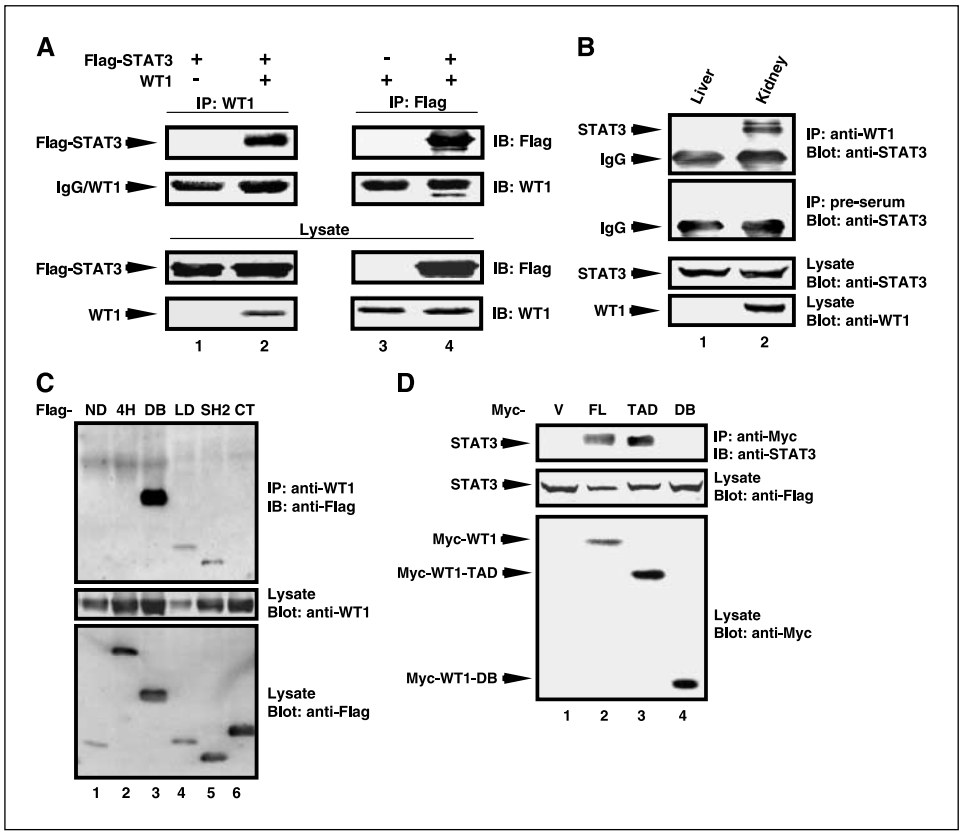

Figure 1. Interaction of WT1 with STAT3. *A,* coimmunoprecipitation of WT1 and STAT3 shows WT1/STAT3 interaction *in vivo*. 293T cells were transfected with plasmids expressing full-size WT1 or Flag-tagged full-length STAT3 as indicated. Whole-cell lysates were used in immunoprecipitation (*IP*) with anti-WT1 antibody (*left*) or anti-Flag M2 antibody (*right*) and blotted with anti-Flag antibody (*top*) or anti-WT1 antibody (*second*), respectively. *Arrows,* levels of Flag-STAT3 in the whole-cell lysates (*third*) and WT1 (*bottom*). *B,* endogenous WT1 and STAT3 interact *in vivo*. Whole-cell lysates from 7-day neonatal mouse liver and kidney were used in immunoprecipitation with anti-WT1 antibody (*top*) or preimmune rabbit serum (*pre-serum*; *second*) and blotted with anti-STAT3 antibody. The levels of endogenous STAT3 (*third*) and WT1 (*bottom*) in the whole-cell lysates of different tissues are indicated separately. *C,* WT1 interacts with the DNA binding domain, linker domain, and SH2 domain of STAT3. 293T cells were transiently transfected with WT1 expression construct and Flag-tagged deletions of STAT3, including NH_2-terminal (*ND*; 1-130 aa), coil-coiled (*4H*; 131-320 aa), DNA binding (*DB*; 321-465 aa), linker (*LD*; 466-585 aa), SH2 (586-688 aa), and transactivation (*CT*; 689-770 aa) domains. Whole-cell lysates were immunoprecipitated with the antibody to c-Myc. After electrophoresis, blots were probed with the mouse monoclonal antibody to Flag (*top*). Expression of WT1 and deletions of Flag-STAT3 were detected by antibody to WT1 (*middle*) or monoclonal antibody to Flag (*bottom*) using whole-cell lysates from transfected cells. *D,* STAT3 interacts with the transactivation domain of WT1. 293T cells were transiently transfected with vector (*V*), full-length Myc-tagged WT1 (*FL*), Myc-tagged WT1 transactivation domain (*TAD*; 1-281 aa), Myc-tagged WT1 DNA binding domain (*DB*; 282-430 aa), and STAT3 expression construct as indicated. Whole-cell lysates were immunoprecipitated with the antibody to c-Myc. After electrophoresis, blots were probed with the mouse monoclonal antibody to Myc (*top*). Expression of STAT3 and deletions of Myc-WT1 was detected by antibody to Flag (*middle*) or to Myc (*bottom*) using whole-cell lysates from transfected cells.

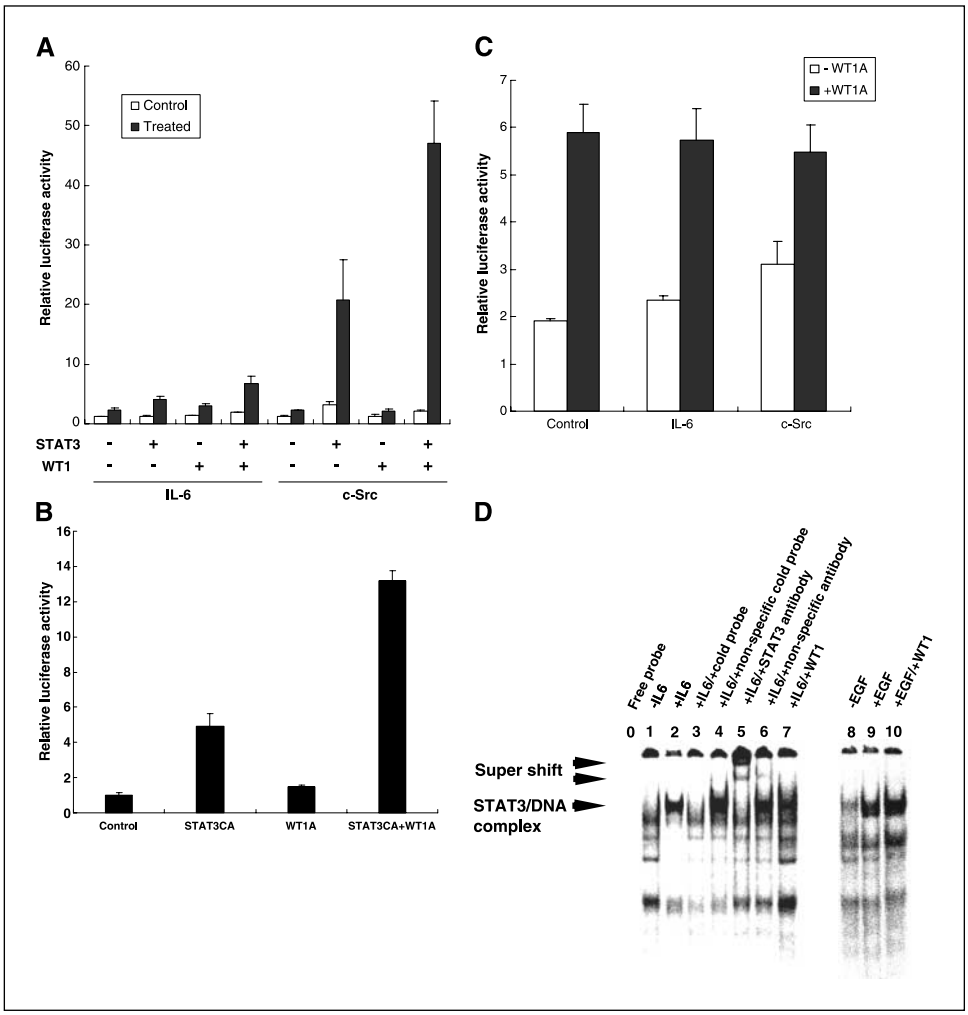

Figure 2. WT1A can affect STAT3 transcriptional activity. *A,* WT1 enhances the transcriptional activities of STAT3. Equal amounts of the indicated plasmids were transiently cotransfected into Hep3B cells treated with IL-6 (*left four columns*) or NIH 3T3 cells cotransfected with c-Src (*right four columns*) together with pGL3-$(APRE)_4$-luc reporter plasmids that could respond to STAT3/3 complex. After 24 hours of transfection, Hep3B cells were starved with serum-free medium for another 24 hours followed by stimulation with cytokines (200 ng/mL IL-6) for 5 hours. All the cells were harvested at 48 hours after transfection. Luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega). Data were normalized by cotransfection with Renilla luciferase vector; *columns,* mean ($n = 3$); *bars,* SD. *B,* WT1 enhances transcriptional activities of constitutively active mutant STAT3CA in 293T cells. The cells were transfected with STAT3CA and WT1 as indicated. The luciferase activity was measured as in (*A*). *C,* induced STAT3 activity has little effect on the transcription activity of WT1. NIH 3T3 cells were transfected with WT1 expression vector and pBL2-$(WTE)_3$-luc reporter plasmid, which responds to WT1 activation. The cells were treated with IL-6 or overexpression of c-Src, respectively. The luciferase activity was measured as in (*A*). *D,* binding of STAT3 to a GAS element is not altered by WT1. Cell extracts were prepared from 293T or COS-1 cells transfected with STAT3 and WT1 and incubated with IL-6/IL-6 soluble receptor or EGF. STAT3 was activated by IL-6/IL-6 soluble receptor or EGF (*lanes 2* and *9*), which could be competed away by STAT3 probe (*lane 3*) but not by nonspecific probe (*lane 4*). The STAT3 complex was recognized by anti-STAT3 antibody to be ''supershifted'' (*lane 5*) but not by nonspecific antibody (rabbit anti–green fluorescent protein antibody; *lane 6*). Expression of WT1 protein had little effect on the STAT3 complex (*lanes 7* and *10*).

STAT3 activity induced by IL-6 or c-Src has little effect on transcriptional activity of WT1 as assayed using a luciferase reporter containing a WT1 binding site, WTE (refs. 10, 29; Fig. 2*C*).

Although the DNA binding domain of STAT3 was involved in the protein-protein interaction of WT1 and STAT3 (Fig. 1), we did not observe any obvious effect of WT1 on STAT3 binding to the classic SIE/M67 probe (28) that contained a STAT3 binding site. This finding suggests that WT1 might not affect the DNA binding property of STAT3 (Fig. 2*D*).

STAT3 is activated and interacts with WT1 in primary Wilms' tumor cells. We next examined whether activated STAT3 and WT1 could colocalize in cells. When WT1A was ectopically introduced into cultured normal mouse hepatic cells, it was found diffusely present in the nucleus (Fig. 3*A, top*). IL-6-activated STAT3 translocated into the nucleus and colocalized with WT1 as visualized by confocal microscopy (Fig. 3*A, bottom*).

Because wild-type WT1 was reported to be persistently expressed in the majority of sporadic Wilms' tumors (30), we used primary Wilms' tumor as a disease model to study the possible functional roles of STAT3 and WT1 interaction. First, we examined the locations of STAT3 and WT1 protein in primary Wilms' tumor cells. Normal kidney cells near the tumor were used as controls. In control normal cells near the tumor, STAT3 was mostly in the cytoplasm (Fig. 3*B, i*). However, STAT3 was found mostly located in the nucleus of some primary Wilms' tumor cells including tubular (Fig. 3*B, ii*), stromal (Fig. 3*B, iii*), and blastemal elements (Fig. 3*B, iv*), indicating possible STAT3 activation in these tumor cells. Consistent with the previous reports, WT1 protein was persistently

located in the nucleus of primary Wilms' tumor cells (Fig. 3*C, ii*) but not in normal kidney cells near the tumor except for podocyte (Fig. 3*C, i*). We also detected the mRNA level expression in six primary Wilms' tumor samples. All of the six tumors showed higher endogenous WT1 mRNA than the normal human embryonic kidney cell line 293T did (data not shown).

Next we examined possible interactions of STAT3 and WT1 in a variety of primary Wilms' tumor cells. We found that abnormal tyrosine phosphorylation of STAT3 was clearly detected in the primary cells from the same six Wilms' tumors by Western blot (Fig. 3*D, middle*). More strikingly, tyrosine-phosphorylated STAT3 formed a complex with WT1 as indicated in the tumor cells (Fig. 3*D, top*): the tyrosine-phosphorylated STAT3 protein was coimmunoprecipitated by an anti-WT1 antibody from all six Wilms' tumor tissue samples. In normal 293T cells, which were used as a control, we did not find any association of this tyrosine-phosphorylated STAT3 and WT1 (Fig. 3*D, Con*). These findings strongly indicate that STAT3 is activated and interacts with WT1 in these primary Wilms' tumor cells, raising a possibility that activated STAT3 might be an oncogenic factor in Wilms' tumors.

WT1 promotes cell proliferation in NIH 3T3 cells expressing STAT3CA. We suspected that the presence of activated STAT3, an oncoprotein, should affect the tumor suppressor function of WT1. To determine the possible functional effect of WT1 on STAT3, we introduced STAT3CA and/or WT1 into untransformed murine fibroblast NIH 3T3 cells. To facilitate the study, stable NIH 3T3 cell lines expressing WT1A alone, STAT3CA alone, and both WT1A and STAT3CA were obtained after G418 and/or hygromycin selection (Fig. 4*A*). The proliferation potentials of these stable cells were evaluated by [^{3}H]thymidine incorporation assay. We observed that stable expression of WT1A(−17aa-KTS isoform) inhibited cell proliferation, showing a tumor suppressor function of WT1.

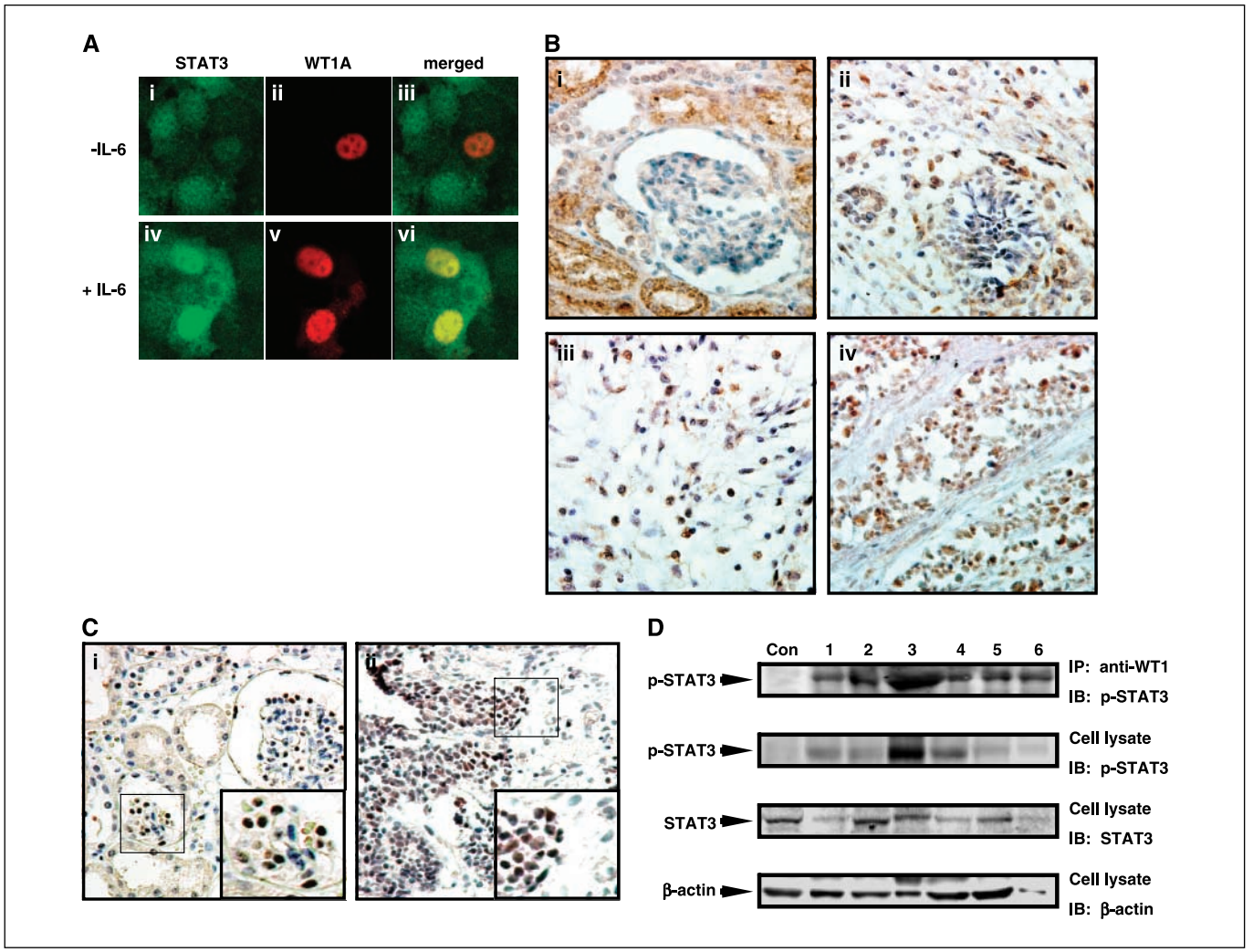

Figure 3. Interaction of activated STAT3 and WT1 in primary Wilms' tumor cells. *A,* colocalization of WT1 and STAT3 in the nucleus of mouse hepatic cells. Wild-type STAT3 mouse hepatic cells were transiently transfected with pCMV-WT1A plasmid and then the cells were starved with 0.2% serum–containing medium for another 12 hours followed by stimulation with cytokine (200 ng/mL IL-6; 30 minutes) before immunostaining and confocal microscopy. *B,* localization of STAT3 in primary Wilms' tumor cells. *i,* a demonstration of STAT3 staining in the control sample showing cytoplasmic staining of STAT3 in the normal kidney cells adjacent to tumor tissues. *ii, iii,* and *iv,* representative Wilms' tumor tissues showing the nuclear staining of STAT3. *C,* localization of WT1 in primary Wilms' tumor cells. *i,* WT1 staining in the control sample showing podocyte nuclear staining of WT1 in the normal kidney cells adjacent to tumor tissues. *ii,* representative Wilms' tumor tissues showing the nuclear staining of WT1. *D,* tyrosine-phosphorylated STAT3 was found in Wilms' tumor cells and coimmunoprecipitated with WT1. Six clinically identified Wilms' tumors were used. Whole-cell lysates from these six samples of Wilms' tumor (*lanes 1-6*) and a 293T cell control (*Con*) were analyzed for tyrosine phosphorylation of STAT3 by an anti-tyrosine-STAT3 monoclonal antibody. β-Actin was used as an internal control (*bottom*).

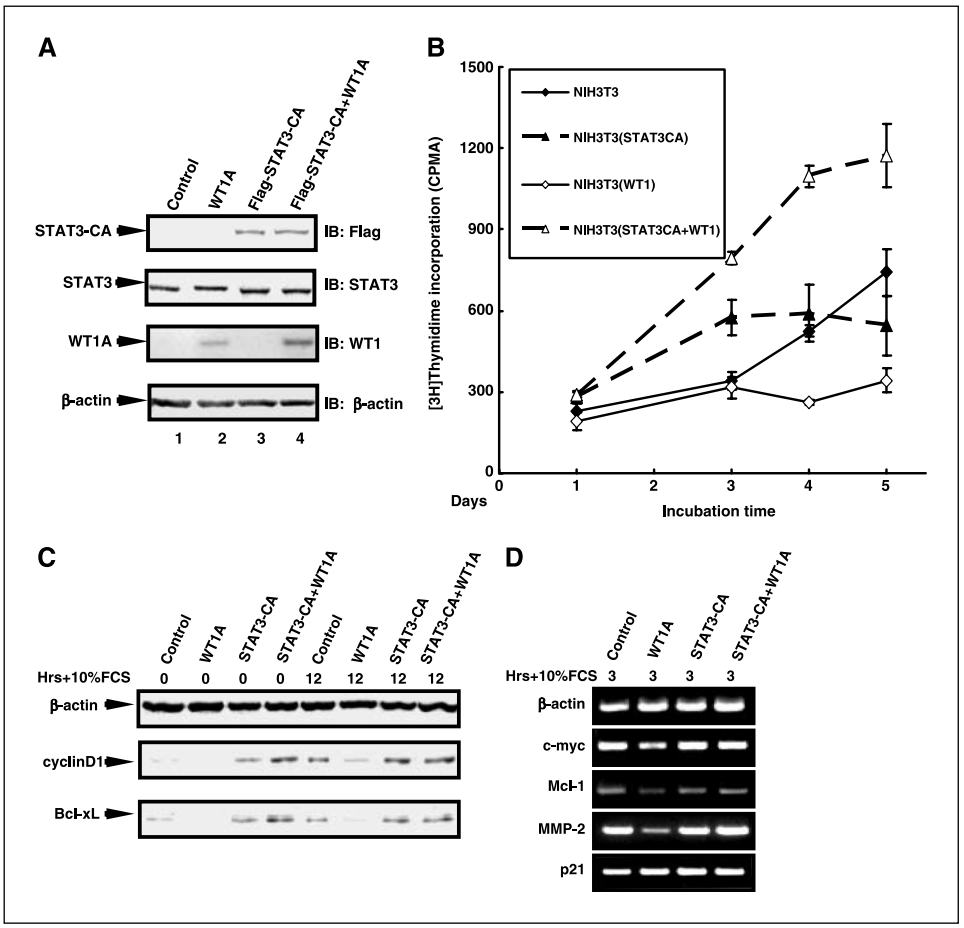

Figure 4. STAT3 modulates the function of WT1 in NIH 3T3 cells. *A,* Western blot analysis on NIH 3T3 stable cells. The four cell lines were generated by stable transfecting with pRC/CMV vector and pRC/CMV-STAT3CA, which were tagged with Flag epitope under G418 selection. The positive cell clones were further stably cotransfected with pCMV-WT1A tagged with Myc epitope and pHygro under hygromycin selection. Cell lysates from four stable cell lines were separated by SDS-PAGE and probed with indicated antibodies. *B,* [^{3}H]thymidine incorporation assay. Equal amounts (4×10^3) of NIH 3T3 stable cells [NIH 3T3, NIH 3T3(WT1A), NIH 3T3(STAT3CA), and NIH 3T3(STAT3-CA+WT1A)] were plated onto 24-well plate and cultured for 5 days in triplicate. After 24-hour serum starvation, 10% FCS plus 1 μCi [^{3}H]thymidine were added and the cells were incubated for additional 8 hours before harvesting. *C,* time-course analysis on cyclin D1 and Bcl-xL expression. NIH 3T3, NIH 3T3(WT1A), NIH 3T3(STAT3CA), and NIH 3T3(STAT3CA+WT1A) cells were grown at starvation condition for 24 hours and resupplemented with 10% FCS for additional 12 hours. Cell lysates were harvested, resolved onto 12% SDS-PAGE, and analyzed using antibodies to cyclin D1 and Bcl-xL. *D,* mRNA expression level analysis of STAT3 target genes by semiquantitative RT-PCR. NIH 3T3, NIH 3T3(WT1A), NIH 3T3(STAT3CA), and NIH 3T3(STAT3CA+WT1A) cells were grown at starvation condition for 24 hours and resupplemented with 10% FCS for additional 3 hours. Total RNA was collected and equal amounts of total RNA (0.5 μg) were added to each reaction mixture.

However, in STAT3CA stable expression cells, exogenously expressed WT1 accelerated the rate of cell proliferation (Fig. 4*B*), suggesting that WT1A could enhance STAT3CA-induced mitogenesis. These data revealed a reverse function of WT1: in the absence of STAT3CA, WT1 repressed cell proliferation, but in the presence of STAT3CA, WT1 synergistically enhanced cell proliferation.

To further elucidate changes of the genes involved in controlling cell growth, we determined the total cyclin D1 (for cell proliferation) and Bcl-xL (for anti-apoptosis) protein levels. The data showed that exogenous WT1 inhibited both cyclin D1 and Bcl-xL expression without STAT3CA (Fig. 4*C, first and second lanes*) but enhanced these two gene expression in the presence of STAT3CA (Fig. 4*C*, comparing the third and fourth lanes). This observation was done in the absence of serum. Furthermore, when we fed the serum back to the cells for 12 hours, exogenous expression of WT1A significantly decreased expression levels of cyclin D1 and Bcl-xL in the absence of STAT3CA but it still increased the expression of these two genes in the presence of STAT3CA slightly (Fig. 4*C, last two lanes*).

Other genes related to tumorigenesis targeted by STAT3, such as Mcl-1 (apoptosis inhibitors), c-myc (positive cell cycle regulator), p21 (cell cycle inhibitor), and MMP-2 (a marker of metastasis), were also observed. The data showed that MMP-2 was inhibited by WT1 in the absence of STAT3CA but increased by WT1 in the presence of STAT3CA (Fig. 4*D*), whereas expression of p21 was not affected. Mcl-1 and c-myc had a similar expression pattern to that of MMP-2. The data consistently suggested that WT1 inhibited the cell growth–related gene expression without constitutively active STAT3 but stimulated the gene expression in the presence of constitutively active STAT3 (STAT3CA).

WT1 promotes cell proliferation in B16 cell line. We further introduced WT1A into murine melanoma B16 cells, which have previously been reported to contain endogenously activated STAT3 and used as a model for determining the effects of STAT3 on tumorigenesis (31). As expected, exogenously expressed WT1 accelerated cell proliferation significantly (Fig. 5*A*), which correlated with obviously enhanced expression of cyclin D1 and Bcl-xL (Fig. 5*B*). To address whether the WT1-stimulated proliferation was through STAT3 driving expression of target genes, we reduced STAT3 activity by introducing STAT3 siRNA to B16 cells. When endogenous STAT3 was knocked down, the expression level of cyclin D1 was reduced slightly without WT1 (Fig. 5*C*). However, at the reduction of endogenous STAT3 and in the presence of exogenous WT1, *cyclin D1* gene expression was decreased dramatically (Fig. 5*C, last lane*). Consistently, whereas the overexpression of WT1 enhanced B16 cell growth in the wild-type B16 cells and reduction of STAT3 slowed down the cell growth, overexpression of WT1 and the reduction of STAT3 inhibited cell proliferation more strongly (Fig. 5*D*). These data suggest that WT1 enhances STAT3 activity in stimulating cell proliferation.

WT1 inhibits cell proliferation without STAT3. Up-regulation of genes such as *cyclin D1* and *Bcl-xL* are believed to be a result of

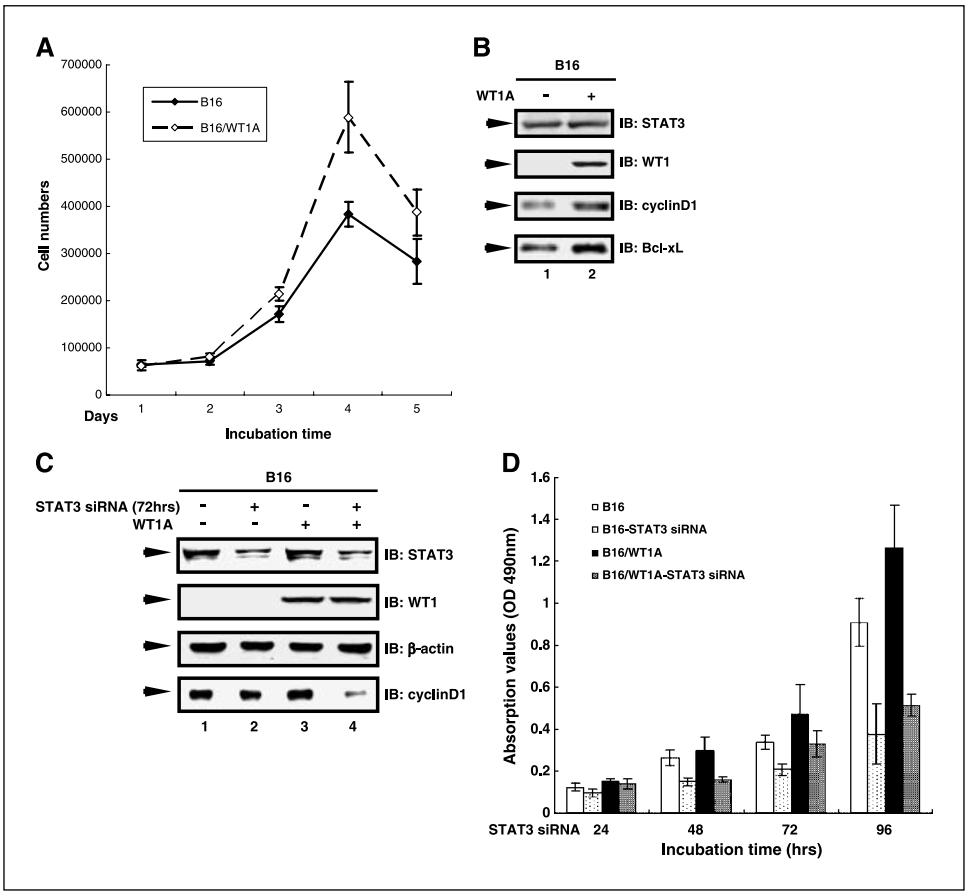

Figure 5. WT1 promotes cell proliferation in B16 cells, which correlates with enhanced expression of cyclin D1 and Bcl-xL. *A,* WT1 promotes cell proliferation in B16 cells. WT1 expression colonies were isolated and used for the experiment. Mock B16 cells were used as control. *B,* expression of WT1 in B16 cells increased expression of cyclin D1 and Bcl-xL. Cultured cells were starved for 16 hours and re-treated with serum-containing medium for 24 hours and then cell lysates were harvested and analyzed using antibodies to cyclin D1 and Bcl-xL. *C,* STAT3 siRNA reduced STAT3 expression in B16 cells (*top*), which correlated with significantly decreased expression of cyclin D1 in WT1-expressing cells (*bottom, lane 4*). Cultured cells were starved for 16 hours and re-treated with serum-containing medium for 24 hours before harvesting. *D,* reduction of cell proliferation rate by STAT3 siRNA on mock B16 and B16/WT1A cell line.

the oncogenic function of STAT3 (25, 32–34). Furthermore, the tumor suppression function of WT1 could be abrogated by action of cyclins (35). We reasoned that STAT3 may convert WT1 to become an activator of transcription when both bind to the same promoter. To examine this possibility, we first determined whether the *cyclin D1* promoter can be co-occupied by WT1 and STAT3 using a chromatin immunoprecipitation assay. The data showed that STAT3 binds the region −337 to −787 of the *cyclin D1* promoter (Fig. 6*A, lane 4*), which contains two well-conserved STAT3 binding sites and no characterized WT1 binding site. In the same assay, c-Myc-tagged WT1 could bind to the same region (Fig. 6*A, lane 5*).

We next observed the roles of WT1 in STAT3$^{-/-}$ cells, which were originally isolated from STAT3 knockout mouse hepatocytes.[5] As expected, expression of WT1 suppressed cell proliferation in these STAT3-deficient cells (−/−; Fig. 6*B*), correlated with G_1 cell cycle arrest (Fig. 6*C*), and diminished expression of cyclin D1 and Bcl-xL (Fig. 6*D, lanes 2* and *4*). Taking all the data together, we suggest that whereas WT1 alone could suppress cell proliferation, the presence of activated STAT3 may abolish the growth suppressor function of WT1. Expression of STAT3 and WT1 jointly may have a promoting effect on the induction of genes such as *cyclin D1* and *Bcl-xL*, resulting in increased cell growth and survival.

Discussion

Studies indicated that Wilms' tumor arose from mesenchymal blastemal cells that fail to differentiate into metanephric epithelial structures but continue to proliferate (5). There are several unsolved puzzles in the pathogenesis of Wilms' tumor and functions of WT1. First, what are the mechanisms for >80% of sporadic Wilms' tumors that do not have a WT1 mutation? It is highly likely that other factors besides WT1 are involved in these tumors. Second, many of these tumors still express the WT1 wild-type gene. Therefore, it should be asked whether the wild-type gene of WT1 has any role in tumorigenesis. Third, WT1 is overexpressed in many other cancer cells besides Wilms' tumor and is believed to be an oncogene in these cells. What are the possible mechanisms by which a tumor suppressor is converted to be an oncoprotein? Previous studies in many laboratories have provided some clues to these questions by showing that other factors, such as Ras, p53, E1B, and Par-4 (36–39), are partners of WT1 and possibly involved in tumorigenesis of Wilms' tumor.

On the basis of results presented in this report, we suggest a novel hypothesis that WT1 and activated STAT3 synergistically enhance tumor cell growth and cell survival. In this way, WT1 functions as an oncoprotein rather than as a tumor suppressor. This hypothesis suggests an important molecular mechanism for the pathogenesis of Wilms' tumor and possibly also of tumors involving both constitutively activated STAT3 and overexpression of WT1.

Our studies have provided for the first time the biochemical and functional evidence of interactions between STAT3 and WT1, both

[5] X-Y. Fu et al. unpublished results.

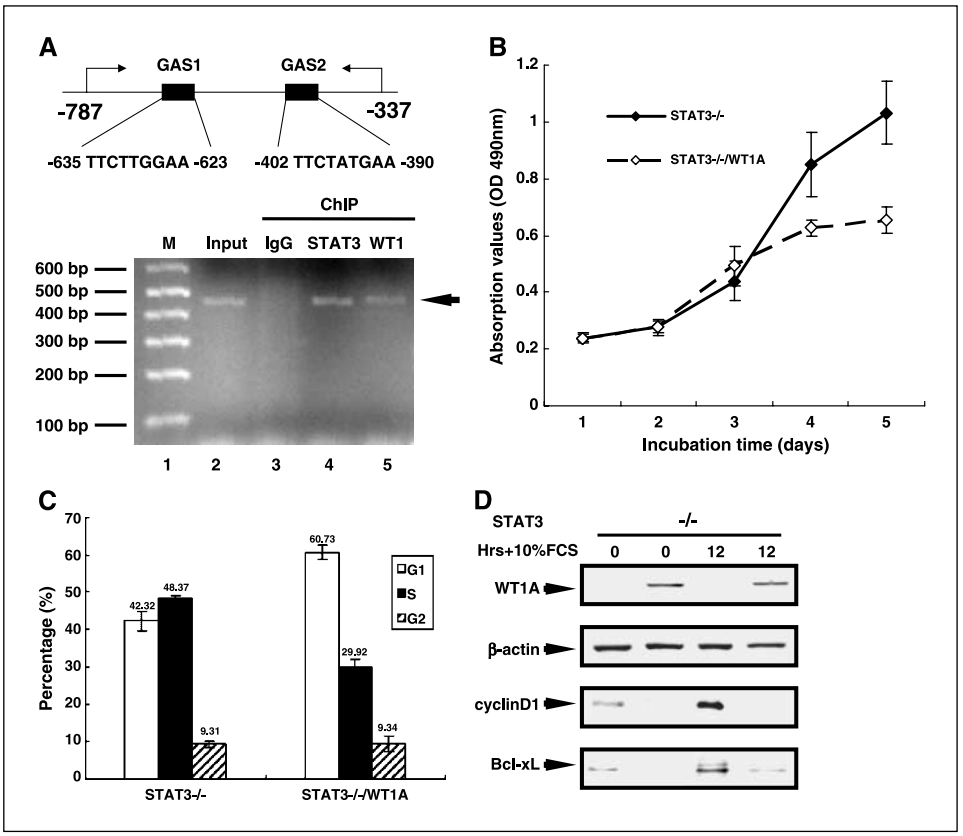

Figure 6. WT1 inhibits cell proliferation and related gene expression in STAT3-deficient cells. *A,* chromatin immunoprecipitation analysis of STAT3 and WT1 binding to the *cyclin D1* promoter. Two putative STAT binding sites (*GAS*) are located in the position −635 to −623 (TTCTTGGAA) and −402 to −390 (TTCTATGAA) in human *cyclin D1* promoter. Binding assay was done by soluble chromatin immunoprecipitation with anti-rabbit IgG (*lane 3*), anti-STAT3 antibody (*lane 4*), or anti-c-Myc antibody (*lane 5*). *B* and *C,* in STAT3-deficient cells, expression of WT1 could suppress cell proliferation (*c*), which correlated with a significant G_1 arrest. *D,* in STAT3-deficient mouse hepatic cells (KO), expression of WT1 diminished expression of cyclin D1 and Bcl-xL. The two stable cell lines were generated by stably transfecting with cotransfected pCMV empty vector or pCMV-WT1A and pHygro under hygromycin selection. The KO and KO(WT1A) cells were grown at starvation condition overnight and resupplemented with 10% FCS for additional 12 hours. Cell lysates were harvested, resolved onto 12% SDS-PAGE, and analyzed using antibodies to cyclin D1 and Bcl-xL.

in vitro and *in vivo*, including primary Wilms' tumor cells. This interaction was revealed by using yeast two-hybrid screening and then proved by coimmunoprecipitation assays using mammalian cells. Our data provide evidence that WT1 could enhance the transcriptional activity of STAT3 in the cases of cytokine-induced or c-Src-induced activation of STAT3, and WT1 also increased the activity of constitutive activated STAT3CA. Reciprocally, cytokine-induced or Src kinase–induced STAT3 activation and overexpression of constitutive activated STAT3CA (data not shown) had little effect on transcriptional activity of WT1 using a reporter with the WT1 binding site. Electrophoretic mobility shift assays showed that WT1 did not affect the DNA binding ability of STAT3. Interestingly, chromatin immunoprecipitation experiments indicated that WT1 bound to the STAT3 binding region in the *cyclin D1* promoter. All the data imply that WT1 functions as a transcriptional coactivator for activated STAT3.

Most importantly, our results support a model that WT1 activity can be modulated to be either a suppressor or a stimulator for cell growth under cell context–specific control and by activated STAT3. In NIH 3T3 cells originated from normal mouse fibroblasts, ectopic expression of WT1 caused decreased cell proliferation, consistent with reduced expression of oncoproteins such as cyclin D1 and Bcl-xL. In contrast, in STAT3CA-expressing NIH 3T3 cell line and mouse melanoma cell line B16, in which STAT3 is constitutively activated, ectopic expression of WT1 resulted in further increased expression of cyclin D1 and Bcl-xL, leading to increased cell proliferation. Importantly, we confirmed the essential function of STAT3 in modulating WT1 using a STAT3 knockdown experiment, as well as in STAT3-deficient cells. We observed that expression of cyclin D1 and Bcl-xL by WT1 was diminished with reduced or abolished STAT3. These data suggest a model that the function of WT1 as tumor suppressor or stimulator is dependent on STAT3 activities in the cell: without activation of STAT3, WT1 functions as a tumor suppressor, but when STAT3 is activated, WT1 enhances its activities of tumorigenesis, thereby functioning as an oncoprotein.

STAT3 is constitutively activated in a variety of human malignancies, including prostate, lung, brain, breast, and squamous cell carcinomas (25, 26, 40). Similarly, we showed that STAT3 is abnormally tyrosine phosphorylated and translocated to the nucleus in primary Wilms' tumor cells. Strikingly, activated STAT3 formed a complex with WT1 in these primary tumor cells. The future work is to study the mechanism on how abnormally activated STAT3 occurs in primary Wilms' tumor. We expect that our findings of the functional interaction of STAT3 with WT1 *in vitro* and *in vivo* will provide critical information for development of effective therapies against this lethal childhood tumor.

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