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Transcriptional activation of c-*myc* proto-oncogene by WT1 protein

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The Wilms' tumor 1 gene (WT1) plays an essential role in urogenital development and malignancy. Through DNA binding, WT1 can either enhance or repress transcription depending on the context of the DNA-binding sites or the cell type in which it is expressed. WT1 is overexpressed in a variety of human cancers, including leukemia and breast cancer; in these diseases, the expression of WT1 is associated with a poor prognosis. To determine how WT1 affects c-*myc* expression in the context of breast cancer cells, we have examined the ability of both endogenous and exogenous WT1 proteins in breast cancer cells to bind to the c-*myc* promoter *in vivo*. Using c-*myc*-promoter-driven luciferase constructs, we found that different forms of WT1 could enhance the expression of the reporter. Unlike other studies where WT1 is reported to be a negative regulator of c-*myc*, we found that both the − and + KTS forms of WT1 could act to enhance c-*myc* expression, depending on the cell type. The WT1-binding site near the second major transcription start site of the c-*myc* promoter was confirmed to be involved in upregulation of human c-*myc* by WT1. Finally, we demonstrated that overexpression of WT1 induced a significant increase in the abundance of endogenous c-*myc* protein in breast cancer cells, consistent with the upregulation of c-*myc* transcription following WT1 induction. These observations strongly argue that in the case of breast cancer WT1 is functioning as an oncogene in part by stimulating the expression of c-*myc*.
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Introduction

The human Wilms' tumor 1 (WT1) gene resides at chromosome locus 11p13, a region frequently deleted in Wilms' tumor, a pediatric embryonic tumor of the kidney (Haber *et al*., 1990; Haber and Buckler, 1992). During embryogenesis, WT1 is expressed at high levels in the developing kidney, gonads, testis, ovary and mesothelial lining of the abdominal and thoracic cavity. Constitutional hemizygosity of WT1 is associated with malformations of the genitourinary tract (Pelletier *et al*., 1991a, b; Park *et al*., 1993a). Animals lacking WT1 have a variety of congenital abnormalities, including malformation of the kidney and absence of the spleen (Herzer *et al*., 1999). WT1 therefore appears to play an important role in the developmental program (Pritchard-Jones and Fleming, 1991; Rackley *et al*., 1993; Kreidberg, 1996).

WT1 is a modular transcription factor with the amino-terminal glutamine and proline-rich domain being involved in self-association, transcriptional repression and transcriptional activation (Scharnhorst *et al*., 2001). The four Cys–Cys–His–His-type zinc fingers in the carboxyl terminus are involved in DNA and RNA binding, nuclear localization and protein–protein interactions (Madden *et al*., 1993; Reddy and Licht, 1996; Lee and Haber, 2001). There are at least 24 different forms of WT1 that arise through alternative splicing, RNA editing and three translation-initiation starts sites, resulting in four predominant protein isoforms that differ by the presence of 17 amino acids between the activation/repression and zinc finger domains, and a three-amino-acid insert (KTS) that exists between the third and fourth zinc fingers (Haber *et al*., 1991; Telerman *et al*., 1992). The different isoforms are referred to as A, B, C and D, where A lacks both the 17 aa and KTS inserts, B contains the 17 aa insert but lacks KTS, C lacks the 17 aa but contains KTS, and D contains both the 17 aa and KTS inserts. In fetal kidney, the ratio of the isoforms is 1 : 2.5 : 3.8 : 8.3. This ratio does not appear to be regulated during development (Haber *et al*., 1991). The significance of the different quantitities of each isoform is not known.

Through its zinc fingers, WT1 can bind to GC-rich Egr1 sites (5′-GNGNGGGNG-3′) (Rauscher III, 1993), WTE sites (5′-GCGTGGGAGT-3′) (Nakagama *et al*., 1995) or (TCC)n motif (Wang *et al*., 1993). Generally, WT1 A and B isoforms are thought to possess a broader target site specificity than C and D isoforms due to the tripeptide insertion between zinc fingers 3 and 4. To date, a variety of genes involved in growth and differentiation have been identified as potential targets of WT1. These genes include (1) growth factor genes: IGF-II, PDGF-A, CSF-1, TGF-β1, inhibin-α, midkine

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and CTGF; (2) growth factor receptor genes: insulin receptor, IGF-1R and EGFR; (3) transcription factor and other genes: Egr1, PAX2, N-*myc*, c-*myc*, hTERT, Bcl-2, cyclin E, ornithine decarboxylase, multidrug resistance-1 and WT1 itself (Li *et al*., 1999; Scharnhorst *et al*., 2001; Loeb *et al*., 2002). Using reporter assays, the above genes have all been shown to be repressed by WT1. In addition, WT1 has been found to be a transcriptional activator of expression for CSF-1, amphiregulin, syndecan-1, E-cadherin, human vitamin D receptor and Bcl-2 (Mayo *et al*., 1999; Lee and Pelletier, 2001; Scharnhorst *et al*., 2001), suggesting a potential involvement of WT1 in the regulation of cell proliferation and differentiation.

c-*myc* is an immediate early growth response gene and is thought to play a pivotal role in cell proliferation and oncogenesis (Adams *et al*., 1985; Evan *et al*., 1992). Induction of c-*myc* expression stimulates quiescent cells to enter the cell cycle and is sufficient to promote G1 to S phase progression (Daksis *et al*., 1994). The enhanced expression of N-*myc* in Wilms' tumor cells (Nisen *et al*., 1986) and the presence of WT1 binding sites (5′-GNGNGGGNG-3′) in the c-*myc* promoter led us to investigate in detail whether there is a linkage between WT1 regulation and c-*myc* gene expression. Here, we observed the upregulation of c-*myc* promoter-luciferase reporter constructs in response to the four isoforms of WT1 protein in human breast cancer and leukemia cells.

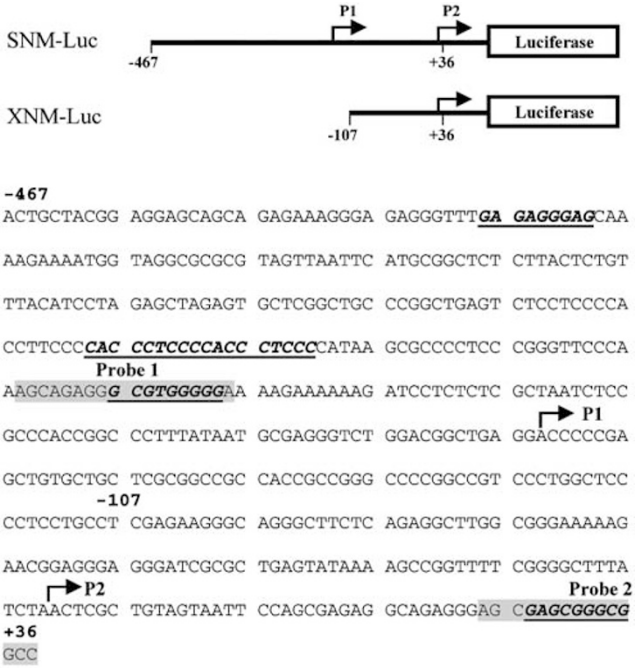

Figure 1 Schematic drawing of human c-*myc* promoter region contained in the luciferase reporter vectors. SNM-Luc, sequence −467 to +36 nt; XNM-Luc, sequence −107 to +36 nt. For the Xmut-Luc, the WT1 site is mutated to 5′-TGAGTGACC-3′. P1 and P2 are the major transcription start sites. The WT1 consensus motifs are boldfaced and underlined. The sequences of two probes used in EMSA are highlighted as indicated

Results

WT1 protein specifically activates the promoter activity of c-myc

The c-*myc* gene may be transcribed from two alternative start sites upstream of exon 1 (P1 and P2, Figure 1). Five putative WT1 binding sites with the consensus sequence 5′-GNGNGGGNG-3′ reside upstream of P2 between −467 and +36 bases. To determine the effect of these putative sites on WT1-mediated regulation of c-*myc*, we used two luciferase reporter constructs driven by the c-*myc* promoter, SNM-Luc containing bases −467 to +36 and XNM-Luc containing bases −107 to +36. The latter harbors only one WT1-binding site and the P2 transcription start site. To assay the effect of WT1 proteins on c-*myc* promoter activity in breast cancer cells, we cotransfected one of the four WT1 isoforms and luciferase constructs into MCF-7 and MDA468 cells; both cell lines express WT1 RNA and protein. Compared to control, cotransfection of WT1 isoform constructs with SNM-Luc or XNM-Luc resulted in significant enhancement of the reporter expression (Figure 2a and b). A basal level of reporter activation in control cells can be explained by the fact that those cell lines have endogenous expression of WT1. Transfection of Xmut-Luc, a mutant of XNM-Luc in which the single WT1 site was mutated, resulted in markedly reduced activity, implying that the activation of c-*myc* transcription by WT1 is dependent on that site.

To determine if the above finding was unique to these breast cancer cell lines, or could be observed in other cell lines of nonbreast origin, we cotransfected the same plasmids into K562 and Cos-7 cells that have endogenous WT1 expression and no WT1 expression, respectively. Consistent with the results from breast cancer cells, there was a significant increase in reporter activity when WT1 plasmids and wild-type reporter constructs were cotransfected. Similar to the breast cancer cell lines, no such activity was seen with the Xmut-Luc (Figure 2c and d). Of interest is the observation that XNM-Luc was consistently more active than SNM-Luc, suggesting the presence of repressor elements between bases −467 and −107 of the c-*myc* promoter domain. Moreover, compared to control, the XNM-Luc activity was increased by 7–14-fold when cotransfected with WT1 isoform vectors into Cos-7 cells. The Cos-7 cells represent an important control in that they have no endogenous WT1. This is in contrast to the studies of WT1 expressing K562 and breast cancer cell lines in which there is a significant but more modest enhancement in expression of the c-*myc* promoter-driven reporter as compared to the control reporter. This difference in the relative degree of enhancement is most likely explained by the fact that in the K562 and breast cancer cell lines the reporter construct is already being stimulated by endogenous protein, and the exogenous WT1 can only produce a fractional increase in reporter expression.

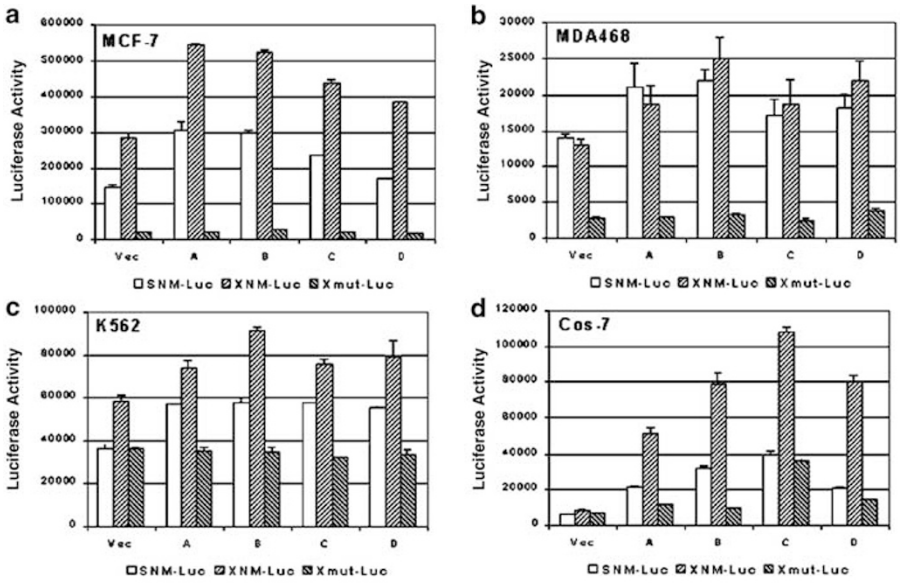

Figure 2 WT1 protein upregulated the transcriptional activity of c-*myc* promoter. (**a**) MCF-7, (**b**) MDA468, (**c**) K562 and (**d**) Cos-7 cells were cotransfected with luciferase reporter constructs (either SNM-Luc, XNM-Luc or Xmut-Luc) and expression plasmids of WT1 isoforms (either pcDNA$_3$ vector, A, B, C or D) as well as β-galactosidase expression vector, respectively, as indicated. Cells were harvested for the measurement of luciferase and β-Gal activities 2 days later. Luciferase activity was indicated at the left. The results shown are the average of three independent experiments

Overexpression of WT1 protein enhances the promoter activity of c-myc in human breast cancer cells

To determine whether a constitutively high expression of WT1 would affect the endogenous c-*myc* promoter activity, we created a series of MCF-7 and MDA468 cell lines with stable overexpression of WT1. The enhanced abundance of the A, B, C or D isoform of WT1 was verified with Western blot in the G418 selected cells (Figure 5, upper panel). These cells were then transfected with SNM-Luc, XNM-Luc or Xmut-Luc. Control cells were G418 resistant cells without exogenous WT1. As can be seen in Figure 3, enhanced reporter activity was observed in the cells overexpressing WT1. Variability in the degree of enhancement was seen among the different overexpressing isoforms; this was independent of the expression levels of WT1 proteins. For example, for the MCF-7 series greater enhancement was seen for the A, B and C isoforms, but not for the D isoform. For MDA468 cells, all isoforms demonstrated an approximate two-fold enhancement of reporter expression. The difference between SNM-Luc and XNM-Luc observed in the transient transfection assays was preserved in the stable transfectants of MCF-7 cells. In keeping with the transient transfection experiments, no enhancement of Xmut-Luc was seen.

WT1 proteins specifically bind to WT1 site(s) in the c-myc promoter domain

The above studies indicate that WT1 can modulate the promoter activity of c-*myc*. To determine whether this is through direct binding of WT1 to the c-*myc* promoter, we next examined the DNA-binding activity of potential WT1/EGR1 consensus DNA-binding sites in the promoter. Both probe 1 containing the WT1 site sequence (bases -258 to -250) in SNM-Luc and probe 2 corresponding to the WT1 site (bases $+25$ to $+33$) in XNM-Luc (Figure 1) were used to perform EMSA with *in vitro* translated WT1 proteins. The four isoforms of *in vitro* translated full-length WT1 proteins (52–54 kDa) were confirmed by Western blot (Figure 4a). In EMSA carried out under conditions that resolve WT1 protein binding to the radiolabeled probe 1, two DNA-binding complexes, P1 and P2, could be resolved in all binding reactions (Figure 4b); only the P1 complex was competed with 50-fold molar excess of cold WT oligonucleotide, but not mutated oligonucleotide, indicating that P1 is a specific DNA-binding complex. No obvious difference in DNA-binding was seen among *in vitro* translated WT1 isoforms. In EMSA carried out under conditions that resolve WT1 protein binding to radiolabeled probe 2, a single DNA-binding complex was seen using *in vitro* translated WT1 proteins (Figure 4c). The binding specificity of the complex was demonstrated with site-specific competitors of the WT1-binding site, suggesting DNA–protein complexes were dependent on this WT1-binding site.

The above experiments support the hypothesis that WT1 is able to bind to the c-*myc* promoter and mediate transactivation. To demonstrate that WT1 proteins bind to the endogenous c-*myc* promoter, we performed chromatin immunoprecipitation (ChIP) experiments. To determine if the c-*myc* promoter was brought down, we used PCR amplification with two primer pairs, MP1 and MP2, that amplify bases -443 to -217 (227 bp) and -28 to $+200$ (228 bp) of the c-*myc* promoter, respectively. As shown in Figure 4d, a single amplification band was observed when chromatins from MCF-7 and MDA468 cells were immunoprecipitated with WT1

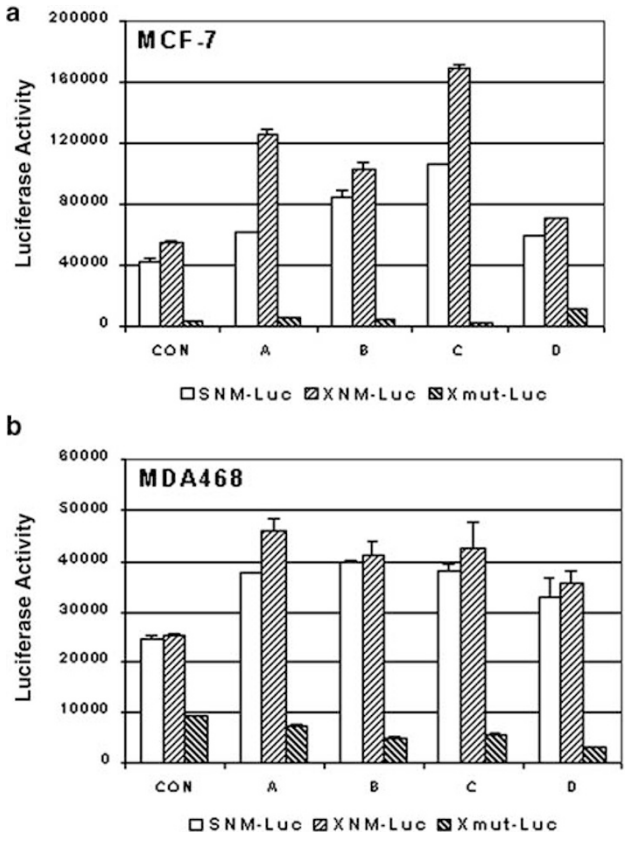

Figure 3 The promoter activity of c-*myc* is enhanced in human breast cancer cells overexpressing WT1 protein. (**a**) MCF-7 and (**b**) MDA468 cells overexpressing WT1 isoforms as well as control cells (as indicated) were cotransfected with luciferase reporter constructs (either SNM-Luc, XNM-Luc or Xmut-Luc) and β-galactosidase vector. The measurements of luciferase and β-Gal activities were performed 2 days after transfection. The results shown are the average of three experiments with the different monoclonal cell populations

antibody. This was not observed when preimmune serum (PI) or antibody against HA was used. These results indicate that in the breast cancer cells used in this study, endogenous WT1 protein is bound to the c-*myc* promoter. We also assessed the ability of overexpressed HA-tagged WT1 to bind to the c-*myc* promoter. Antibody against WT1 or the HA tag was used to precipitate crosslinked chromatins from stable transfectants of MDA468 cells in which HA/WT1 A or HA/WT1 D fusion protein is overexpressed. Antibodies against WT1 and HA-tag immunoprecipitated a single specific PCR amplification band (Figure 4e). The above results provide further support to our contention that WT1 protein can activate c-*myc* transcription by direct binding of WT1 to WT1/EGR1 sites in the c-*myc* promoter.

WT1 increases the transcription and translation level of c-myc in human breast cancer cells

To determine whether the enhanced expression of WT1 in the stable cell lines alters the amount of RNA and protein produced from the endogenous c-*myc* gene, RNA extracts and whole-cell lysates were assessed by real-time quantitative PCR and Western blot, respectively. As can be seen in Table 1, the mRNA levels of c-*myc* were enhanced 10–16-fold by overexpression of WT1 isoforms in MCF-7 cells. In keeping with the increased level of WT1 RNA, there was a 1.3–2.3-fold increase in the level of c-*myc* protein present in breast cancer cells (Figure 5). Moreover, the enhanced expression pattern of endogenous c-*myc* protein induced by different WT1 isoforms was consistent with the degree of increase in c-*myc* mRNA in the different WT1 expressing MCF-7 cells. Taken together, these results strongly argue that WT1 protein upregulates the transcription and translation level of c-*myc* in human breast cancer cell lines.

Discussion

WT1 was originally classified as a tumor-suppressor gene based on its deletion and mutation in some cases of Wilms' tumor. Transfection of WT1 into Wilms' tumor cells that lacked WT1 resulted in growth arrest, further supporting the role of WT1 as a tumor-suppressor gene. Most recently, Udtha *et al*. (2003) have confirmed that c-*myc* expression is 2.33-fold higher in Wilms' tumors carrying WT1-inactivating mutations than in WT1 wild-type tumors using cDNA microarray, elucidating the tumor repression function of WT1 in Wilms' tumors. Consistent with this observation was the finding that WT1 can repress the expression of cancer-related proteins such as c-*myc,* hTERT and bcl-2, and enhance the expression of antiproliferative proteins such as p21 in cell lines (Hewitt *et al*., 1995; Englert *et al*., 1997; Oh *et al*., 1999). Other evidence supporting the growth inhibitory effects of WT1 came from studies by Ellisen *et al*. (2001) and Svedberg *et al*. (2001), who introduced WT1 into normal hematopoietic progenitor cells. They found that forced expression of WT1 resulted in growth arrest and differentiation of the progenitor cells. It is of note that these studies were carried out by transfecting the reporter alone or along with WT1 into cells that do not normally express WT1 and are nonmalignant. Furthermore, in the studies describing WT1 as a repressor, few of the evaluations included an assessment of the endogenous protein expression.

In contrast to the notion that WT1 is a tumor-suppressor gene is the growing literature that identifies wild-type WT1 as an oncogene. High levels of wild-type WT1 are found in a variety of tumors including Wilms' tumor, renal cell carcinoma, lung cancer, melanoma, ovarian cancer, mesothelioma, breast cancer and leukemia (Bruening *et al*., 1993; Park *et al*., 1993b; Silberstein *et al*., 1997; Menssen *et al*., 2000; Loeb *et al*., 2001). High levels of WT1 expression in both breast cancer and leukemia have been associated with a poor prognosis (Miyoshi *et al*., 2002). Inhibition of WT1 RNA by antisense methodologies in lung cancer and leukemia cells leads to loss of proliferation, while overexpression of WT1 in leukemia cells blocks differentiation (Algar

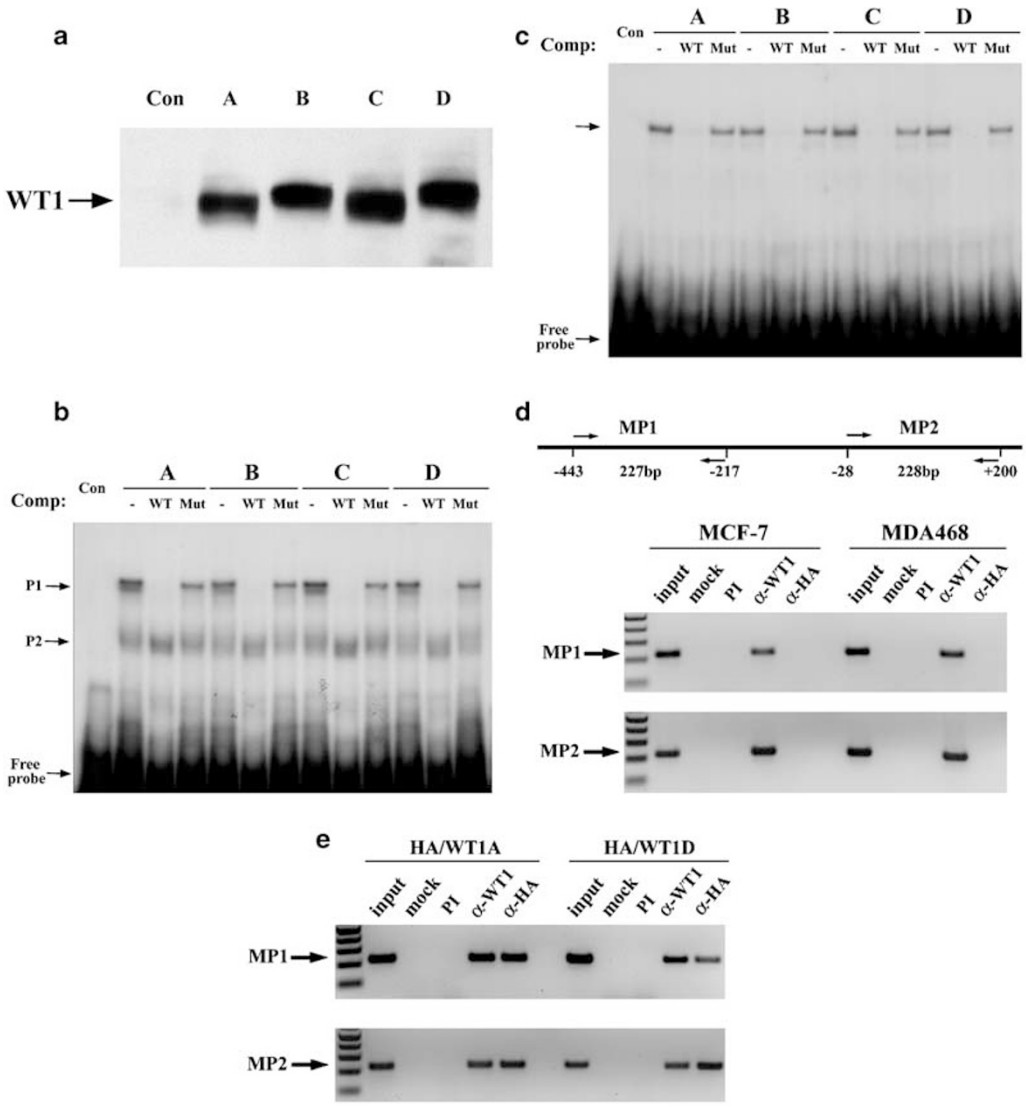

Figure 4 DNA binding of WT1 protein on c-*myc* promoter is WT1 site dependent. (**a**) Western blot with WT1 c-19 antibody shows the four *in vitro* translated WT1 isoforms (A–D). The molecular size of these WT1 proteins is 52–54 kDa. (**b**) EMSA were performed with *in vitro* translated WT1 proteins (A–D) using radiolabeled probe 1 in the absence (−) or presence of a 50-fold molar excess of cold WT or Mut oligonucleotides as indicated on the top. Migration of DNA-binding complexes (P1 and P2) is shown at the left. The left lane (Con) shows no DNA binding with unprogrammed reticulocyte lysate. (**c**) Autoradiogram of EMSA in which the bindings of *in vitro* translated WT1 proteins (A–D) to radiolabeled probe 2 were observed in the absence (−) or presence of a 50-fold molar excess of WT1-binding site wild type or site mutant cold oligonucleotides (WT, Mut) as indicated on the top. Migration of the DNA-binding complex is shown as an arrow at the left. (**d**) ChIP/PCR for the crosslinked sheared chromatins from MCF-7 and MDA468 cells. The upper panel showed the schematic representation of PCR amplification fragments MP1 and MP2 in human c-*myc* promoter. The lanes showing chromatin input, mock control and immunoprecipitation with preimmune serum, anti WT1 or anti HA antibody are indicated on the top, respectively. The 1 kb DNA ladder is shown on the left lane. Amplification bands for the endogenous human c-*myc* promoter MP1 (227 bp) and MP2 (228 bp) domains are indicated at the left. (**e**) ChIP/PCR for chromatins from stable transfectants of MDA468 cells in which HA/WT1 A or HA/WT1 D fusion protein was overexpressed is indicated on the top

et al., 1996; Svedberg *et al*., 1998). These findings suggest that in some tumor types WT1 is functioning as an oncogene.

The conflicting results and concepts presented above prompted us to re-evaluate the regulation of c-*myc* by WT1. For these experiments, we chose two breast cancer cell lines that express different levels of endogenous WT1, a human leukemia cell line K562 that expresses high levels of WT1 and has previously been shown to undergo apoptosis when WT1 expression is blocked (Algar *et al*., 1996), and Cos-7 cells that have no WT1 expression. In our studies, we found that the c-*myc* promoter, especially a region between −107 and +36, is capable of responding to WT1 with increased expression in these cells. This was found regardless of whether WT1 was produced in nontransfected cells, cells transiently transfected with WT1 isoforms or cells stably expressing one of the four isoforms. In addition, we found that cells

Table 1 Quantitative gene expression of WT1 and c-*myc* in stably transfected MCF-7 cells using RTQ–PCR

	CON	*A*	*B*	*C*	*D*
WT1: $2^{-\Delta\Delta Ct}$ ±s.d.	1±0.1	105±11	104±7	150±22	398±75
c-*myc*: $2^{-\Delta\Delta Ct}$ ±s.d.	1±0.15	16.6±2.1	15.0±2.5	14.3±2.1	10.2±2.4

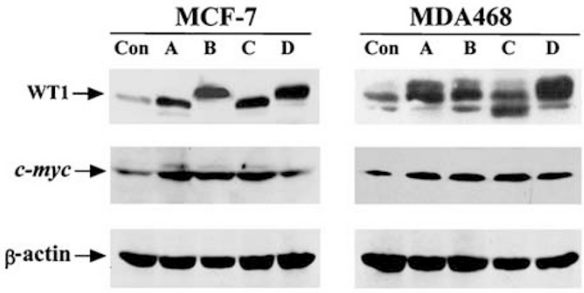

Figure 5 The representative Western blot shows that overexpression of WT1 isoforms increases abundance of c-*myc* in human breast cancer cells. Whole-cell extracts were prepared from MCF-7 or MDA468 cells stably transfected with either pcDNA$_3$ control or one of the WT1 isoforms (as indicated on the top) and subjected to Western blot analysis using either anti-WT1, c-*myc* or *β*-actin antibodies as indicated at the left. Following the normalization to *β*-actin signal, c-*myc* abundances were increased by 2.0- (WT1 A), 2.3- (WT1 B), 2.0- (WT1 C) and 1.3- (WT1 D) fold, respectively, in MCF-7 cells compared to control. For MDA468 cells, c-*myc* proteins were increased by 1.9- (WT1 A), 1.6- (WT1 B), 2.1- (WT1 C) and 1.9- (WT1 D) fold, respectively, after being normalized to *β*-actin signal

stably expressing WT1 have increased mRNA and protein levels of c-*myc*.

Disparate results for transactivation of bcl-2 by WT1 have been reported. Two groups reported that WT1 inhibited the expression of bcl-2 in transient transfection assays (Hewitt *et al*., 1995; Heckman *et al*., 1997). Those experiments were carried out in WT1-negative cells. Mayo *et al*. performed similar experiments with a somewhat longer construct in WT1 expressing Wilms' tumor cells or WT1-negative HeLa cells. They found that the −KTS but not the +KTS forms of WT1 enhanced the expression of bcl-2. A possible explanation for this is that the −KTS forms can bind to the WT1-binding site in the bcl-2 promoter, while the +KTS form of the protein is unable to bind to that site (Mayo *et al*., 1999).

Similar to bcl-2, it would also appear that whether c-*myc* is positively or negatively regulated by WT1 is dependent on the cellular context in which the experiments are performed. Hewitt *et al*. (1995) reported that WT1 represses mouse c-*myc* promoter activity in human HeLa cells using reporter transfection assays; this is contrary to our observations. In our studies, we found that the −KTS and +KTS isoforms of WT1 were able to bind to the WT1 consensus sites (5′-GAGCGGGCG-3′ and 5′-GCGTGGGGG-3′) in the human c-*myc* promoter and enhance the expression of the c-*myc* reporter construct. The difference in the effect of WT1 on c-*myc* transactivation in these two studies may be due to differences between the human and mouse genes. The WT1 site immediately upstream of P2 in the human c-*myc* gene is not present in the mouse gene. Conversely, the WT1-binding site reported to be important in the studies of Hewitt *et al*. is absent in the human gene. A second possible explanation could be that different cell lines were used.

The region upstream of human *c-myc* P1 contains four potential WT1-binding sites, and upstream of P2 there is one potential WT1-binding site. In our studies, we found that the WT1-enhancing activity can be attributed solely to the WT1 site upstream of P2, between nucleotides −107 and +36. This is reminiscent of the findings with bcl-2, where there are several potential WT1 sites but only one site actually binds and is involved in mediating the transcriptional activation by WT1 (Mayo *et al*., 1999). In the case of bcl-2, *in vitro* transcribed and translated WT1 protein was only able to bind to one of the putative WT1 sites. We do not know if WT1 is capable of binding to all or just one of the WT1 sites in the c-*myc* promoter; however, it is clear that the WT1-binding site just upstream of P2 is involved in the regulation of c-*myc* by WT1, this is evident in two ways. First, high-level reporter expression was observed with the −107 to +36 fragment. Second, mutation of the WT1 site in this fragment significantly decreased the expression of the reporter. In the two breast cancer cell lines, and Cos-7 cells the decrease was to extremely low levels, while the decrease in K562 cells was less. The reason for this difference is not known, but may indicate the role of other unknown c-*myc* regulatory factors present in K562 leukemia cells that are absent or less important in the other cell lines. We do not know the contribution of P2 to c-*myc* expression in these cell lines; however, other studies have shown that P2 contributes 80% or more of total RNA initiation in normal cells among four possible transcriptional promoters identified for c-*myc* (Taub *et al*., 1984).

In summary, we show that WT1 can affect levels of c-*myc* RNA and protein in cancer cell lines in a direct manner; this is supported by the following observations. First, the four major WT1 isoforms translated *in vitro* are specifically bound to WT1 sites, that is, the EGR1 consensus sequence, in the promoter domain of the human c-*myc* gene. Second, both endogenous and overexpressed WT1 proteins are capable of binding to the c-*myc* promoter in breast cancer cell lines. Third, transcription mediated by the c-*myc* promoter region between −107 and +36 was strongly activated by WT1 in a WT1-binding site-dependent manner. Finally, c-*myc* expression was significantly upregulated in breast cancer cells overexpressing WT1 isoforms.

Materials and methods

Cell culture

The human breast cancer lines MCF-7 (ATCC HTB22) and MDA 468 (ATCC HTB132), human erythroleukemia K562,

and Cos-7 cells were cultured in minimal Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (penicillin/streptomycin/fungizone) in a humidified atmosphere of 5% CO_2 at 37°C. The breast cancer cells overexpressing WT1 isoforms were cultured in G418-containing selective medium.

Plasmids

The expression plasmids for the four mouse WT1 isoforms with and without HA-tag were constructed by cloning the coding region of full-length WT1 into pcDNA$_3$ vector under a CMV promoter. The human c-*myc* promoter-luciferase reporter construct SNM-Luc was generated by subcloning the 504-bp *Sca*I-*Bam*HI fragment from the human c-*myc* promoter into the *Sma*I-*Bgl*II sites of pGL2 basic vector (Promega). The shorter XNM-Luc was constructed by subcloning the 144-bp *Xho*I-*Bam*HI fragment from the c-*myc* promoter into the *Xho*I-*Bgl*II sites of pGL2 vector. Mutagenesis *in situ* of the XNM-Luc plasmid (Xmut-Luc) was performed using a GeneEditor *in vitro* site-directed mutagenesis system (Promega) in accordance with the manufacturer's instructions. The mutagenic oligonucleotide used to produce nucleotide substitution within the c-*myc* promoter was 5′-pAGCGAGAGGCAGAGGGAGC*TGAGTGACC*GCCCC GGATCTAAGTAG-3′. The mutagenesis of the WT1/EGR1-binding site is italicized and underlined. The selection oligonucleotide was 5′-pGATAAATCTGGAGCCTCCAA GGGTGGGTCTCGCGG-3′. Mutation was confirmed with the direct nucleotide sequencing.

Transfection and luciferase assays

The cells were transiently cotransfected using Lipofectin (Life Technologies) into triplicate 60-mm plates with 1 μg of luciferase reporter (pGL2 basic vector as control), 0.1 μg of β-galactosidase internal control vector and 3 μg of expression plasmid of either WT1 isoform (pcDNA$_3$ as control) based on the manufacturer's recommended protocol. After 48 h, cells were washed twice with PBS and harvested for the assay of luciferase and β-Gal activities according to standard methods (Promega). Luciferase activity was determined by subtracting machine background and normalizing each plate to β-galactosidase activity. The results of all assays are the average of at least three independent experiments.

For stable transfection, the breast cancer cells were cultured in G418-containing selective medium for at least 2 weeks after transfection with one of the WT1 isoforms or the pcDNA$_3$ blank vector (control). Single colonies from the transfected cell pool were picked and expanded. The overexpressions of either WT1 isoform were confirmed with Western blot.

Preparation of cellular extracts and in vitro translated WT1 proteins

The cells were washed twice with PBS prior to harvesting. The cell pellets were lysed in RIPA buffer (50 mM Tris-Cl, pH7.5, 150 mM NaCl, 0.5% sodium deoxycholicate, 0.1% SDS, 1% NP-40 and proteinase inhibitor cocktails) on ice. The supernatants were saved as cellular extracts after cell lysates were centrifuged at 12 000 g for 10 min at 4°C. The cellular extracts were normalized for protein amounts determined by the Bradford assay using BSA as a standard (Bio-Rad). *In vitro* translated WT1 proteins were prepared using TNT Quick Coupled Transcription/Translation Systems (Promega). Briefly, 1 μg of expression plasmid of either full-length WT1 isoform (pcDNA$_3$ as control) was incubated with 40 μl of TNT Quick Master Mix and 20 μM methionine in a 50-μl final volume for 90 min at 30°C. The translated full-length WT1 isoforms were confirmed with Western blot.

Electrophoretic mobility shift assays (EMSA)

In vitro translated WT1 proteins (5 μl of reaction mixture) were incubated with 40 000 cpm of ^{32}P-labeled WT1-binding site wild type (WT) duplex oligonucleotide probe and 1 μg of poly(dA-dT) in a buffer containing 8% glycerol, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT and 0.1 mg/ml PMSF in a final volume of 20 μl for 15 min at room temperature. The complexes were fractionated on 6% native polyacrylamide gels run in 1 × TBE buffer (25 mM Tris, 25 mM boric acid and 0.5 mM EDTA), dried and exposed to Kodak X-AR film at −70°C. Competition was performed by the addition of a 50-fold molar excess of nonradioactive double-stranded oligonucleotide competitor at the time of addition of radioactive probe. The employed probe 1 sequence is: WT 5′-AGCA GAGG*GCGTGGGGG*A-3′, Mut 5′-AGCAGAGG*TGAGTGA CC*A-3′; and probe 2: WT 5′-AGC*GAGCGGGCG*GCC GGCT-3′, Mut 5′-AGC*TGAGTGACC*GCCGGCT-3′. The wild type and mutant of the WT1-binding site is italicized and underlined.

Chromatin immunoprecipitation (ChIP) and PCR amplification

ChIPs were performed using the Chromatin Immunoprecipitation Assay Kit (Upstate Biotech) with a slight modification. Approximately 1×10^7 cells were crosslinked with 1% formaldehyde in growth medium at 37°C for 15 min. After crosslinking was quenched by 0.125 M glycine at room temperature for 10 min, cells were collected by scraping and centrifuged at 4000 *g* for 4 min at 4°C. Pelleted cells were resuspended in 200 μl SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) and incubated on ice for 15 min. Chromatin was then sonicated on ice to an average length of 600 bp using a Fisher Scientific Dismembranator 100 with 7 × 15 s pulses at a 4 W power output setting. Cell debris was removed by centrifugation at 12 000 *g* for 15 min at 4°C. The sheared chromatins were precleared with salmon sperm DNA/protein A agarose for 30 min at 4°C and subjected to immunoprecipitation with 2 μg of either anti-WT1 (C-19) antibody (Santa Cruz Biotech) or anti-HA antibody (Roche) overnight at 4°C. A no chromatin mock control and a preimmune serum control were prepared in parallel. After incubation with the secondary antibody for the additional 1 h at 4°C and collected with salmon sperm DNA/protein A agarose, immunoprecipitated chromatin complexes were washed five times according to Upstate protocol and eluted with the elution buffer (50 mM $NaHCO_3$ and 1% SDS). The supernatant of preimmune serum sample was taken as input. Following the reversal of crosslinks by an incubation with 0.3 M NaCl for 4 h at 65°C and proteinase K digestion for 2 h at 45°C, DNA was extracted with phenol/chloroform, precipitated with ethanol and resuspended in 30 μl water. PCR reactions containing 3 μl of the above DNA preparation, primers and Platinum Taq in a 50-μl final volume were performed with the initial denaturation at 95°C for 4 min, followed by 30 PCR cycles (95°C for 45 s, 55°C for 30 s and 68°C for 1 min) and a final extension at 72°C for 10 min. The primer sequences for endogenous human c-*myc* promoter were as follows: MP1, FP 5′-AAGGGAGAGGGTTTGAGAGG′ -3′; RP 5′-CGGAGATTAGCGAGAGAGGATC-3′. MP2, FP 5′-TCGGGGCTTTATCTAACTCG'-3′; RP 5′-GCTGC TATGGGCAAAGTTTC-3′. DNA specificity of the primers was demonstrated by sequencing the single amplification product band that appeared on agarose gel.

Real-time quantitative PCR (RTQ-PCR)

The total RNA was extracted from either WT1 overexpressing or control MCF-7 cells using the RNeasy Mini Kit (Qiagen) and converted into cDNA with reverse transcriptase (Gibco) at 48°C for 30 min. The PCR reactions containing SYBR Green Master Mix (PE Applied Biosystems), 100 nM primer and template cDNA in a 25-μl final volume were performed in the ABI PRISM 7799 sequence detector. The SYBR Green PCR program consisted of the initial denaturation at 95°C for 10 min, followed by 40 PCR cycles: 95°C for 15 s, 60°C for 60 s with the continual measurement of fluorescence. The primer sequences for gene amplification were as follows: WT1, FP 5′-CAAGGATACAGCACGGTCAC-3′; RP 5′-GGTCCT CGTGTTTGAAGG-3′. c-*myc,* FP 5′-TTCGGGTAGTGG AAAACCAG-3′; RP 5′-CAGCAGCTCGAATTTCTTCC-3′. GAPDH, FP 5′-GAAGGTGAAGGTCGGAGTC-3′; RP 5′-GAAGATGGTGATGGGATTTC-3′. DNA specificity of the primers was demonstrated by a lack of amplification in the samples of NTC (no template control) and NAC (no amplification control), as well as by sequence of the single amplification product band on agarose gel. Quantitation of gene expression is performed using the comparative threshold cycle (Ct) method (PE Applied Biosystems) and represented as mean Ct and standard deviation (s.d.) of at least three experiments. ΔCt is the difference between the mean Ct values of WT1 samples and those of GAPDH, while $\Delta\Delta$Ct is the difference between the mean ΔCt values of samples from WT1 transfected cells and those of samples from control cells. The relative quantitation value is expressed as $2^{-\Delta\Delta Ct} \pm$s.d. The results represented the average of at least three independent experiments.

Western blot

In all, 10 μl of reaction mixtures of *in vitro* translated proteins or 200 μg of cellular extracts was boiled in Laemmli buffer, separated through a 10% SDS–PAGE, and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were then blocked in PBS containing 8% milk and blotted with either affinity-purified rabbit polyclonal anti-WT1 (C-19) antibody (reactive with aa 431–450, Santa Cruz Biotech), anti c-*myc* antibody (reactive with full-length c-*myc* protein, Upstate Biotech) or anti-β-actin antibody (Sigma). Filter bound immune complexes were detected by binding goat anti-rabbit IgG conjugated to horseradish peroxidase followed by reaction in the enhanced chemiluminescence assay (ECL, Amersham International) according to the manufacturer's recommendations. The band quantitation was performed with a densitometer (Molecular Dynamics).

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