The Wilms tumor suppressor WT1 directs stage-specific quiescence and differentiation of human hematopoietic progenitor cells

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WT1, a transcription factor implicated in both normal kidney differentiation and tumorigenesis, is also expressed in differentiating hematopoietic progenitors. Most human acute leukemias contain high levels of the wild-type transcript, while a minority have point mutations, raising the possibility that this tumor suppressor might have a paradoxical oncogenic effect in some hematopoietic cells. Using high titer retroviral infection, we demonstrate that *WT1* triggers rapid growth arrest and lineage-specific differentiation in primary hematopoietic progenitors and differentiation-competent leukemia cell lines, while it induces cellular quiescence in a primitive subset of primary precursors. Growth arrest by *WT1* is associated with induction of p21^{CIP1}, but expression of this cyclin-dependent kinase inhibitor alone is insufficient for either cellular differentiation or primitive cell preservation. The effects of *WT1* are enhanced by co-expression of its naturally occurring isoforms, and are correlated with the physiological expression pattern of *WT1 in vivo*. Our observations suggest a role for *WT1* in the differentiation of human hematopoietic cells, and provide a functional model that supports its capacity as a tumor suppressor in human acute leukemia.
Keywords: hematopoiesis/leukemia/tumor suppressor/ WT1

Introduction

The *WT1* tumor suppressor gene encodes a Cys–His zinc finger transcription factor that exemplifies the link between normal organ development and cancer (reviewed in Hastie, 1994). Loss of one germ-line allele is associated with genitourinary malformations, attributed to reduced gene dosage during critical stages in development, and it confers a high risk for Wilms tumor, resulting from somatic loss of the second *WT1* allele. Approximately 10% of sporadic Wilms tumors also have inactivating mutations in *WT1*, although the majority express high levels of the wild-type transcript (Little *et al*., 1992). Inactivation of WT1 in the mouse leads to an embryonic lethal phenotype, characterized by renal agenesis and aberrant development of mesothelial structures (Kreidberg *et al*., 1993). The failure of renal differentiation in WT1-null mice is attributable to widespread apoptosis of the blastemal cells that comprise the renal stem cell population, an observation that suggests a role for *WT1* in the survival of these undifferentiated cells. At the later stages of renal differentiation, which are not achieved in *wt1*-null mice, *WT1* expression peaks in specific precursors of the glomerulus, suggesting a distinct role in the differentiation of these highly specialized structures. Thus, the physiological function of *WT1* at different stages of renal differentiation, and the consequences of its inactivation in the mouse, appear complex, and have been difficult to reconcile with its presumed function as a tumor suppressor.

A contribution by *WT1* to blood cell development has been suggested by its expression in early hematopoietic precursors and its rapid downregulation following differentiation in primary blood cells and leukemia-derived cell lines (Sekiya *et al*., 1994; Baird and Simmons, 1997; Maurer *et al*., 1997; Menssen *et al*., 1997). However, the role of *WT1* in this process also appears paradoxical. Most human acute myeloid and lymphoid leukemias express high levels of wild-type *WT1* (Miwa *et al*., 1992; Menssen *et al*., 1995), which have been correlated in some studies with the development of more primitive and refractory forms of disease (Inoue *et al*., 1994; Bergmann *et al*., 1997). Yet, mutations have been reported in ~10% of cases, suggesting a role for disruption of *WT1* function in leukemogenesis that is comparable to its role in Wilms tumor (King-Underwood *et al*., 1996; Miyagawa *et al*., 1999). Attempts at defining the consequences of *WT1* expression in leukemia-derived cell lines have also produced conflicting results, reporting enhanced cellular proliferation in some systems but induction of growth arrest in others, and proposing both mediation as well as inhibition of cellular differentiation (Algar *et al*., 1996; Inoue *et al*., 1998; Smith *et al*., 1998, 2000; Svedberg *et al*., 1998; Deuel *et al*., 1999). These studies, some of which have raised the possibility of an oncogenic role for *WT1* in leukemogenesis, have relied on antisense strategies or on the selection of stably transfected clones, which may fail to detect immediate effects on cellular proliferation associated with the expression of a tumor suppressor gene. No prior studies have examined the consequences of *WT1* expression in normal hematopoietic cells.

WT1 is encoded by distinct isoforms resulting from alternative pre-mRNA splicing, of which the most functionally significant is the insertion of three amino acids, leucine–threonine–serine (or KTS), that disrupt the critical spacing between zinc fingers 3 and 4 in the DNA binding domain (Haber *et al*., 1991). WT1 proteins lacking this insertion, WT1(–KTS), bind to a 5′-GCGTGGGAGT-3′ sequence and demonstrate potent transcriptional activation of target genes including the cyclin-dependent kinase

inhibitor $p21^{CIP1}$ and the epidermal growth factor family member *amphiregulin* (Nakagama *et al.*, 1995; Englert *et al.*, 1997; English and Licht, 1999; Lee *et al.*, 1999). In contrast, the more abundant WT1(+KTS) isoform does not bind to a known DNA sequence, and while it may physically associate with WT1(–KTS), it does not appear to mediate transcriptional regulation directly (Englert *et al.*, 1995b; Moffett *et al.*, 1995; Reddy *et al.*, 1995). The localization of WT1(+KTS) within subnuclear speckles, in association with factors involved in pre-RNA splicing, has also suggested a role in RNA processing (Larsson *et al.*, 1995; Davies *et al.*, 1998). Expression of both (–KTS) and (+KTS) isoforms appears to be critical for normal *WT1* function. Frasier syndrome, characterized by glomerulo-pathy and gonadal dysgenesis (Barbaux *et al.*, 1997), results from an inherited mutation within the KTS splice donor site that disrupts the normally constant ratio between these splicing variants. Although these observations point to important roles for both (–KTS) and (+KTS) isoforms of WT1, it is unclear whether their functions are complementary or antagonistic.

To determine the relevance of *WT1* expression in normal and transformed hematopoietic cells, we used pseudotyped retroviruses to introduce the different alternative splicing variants of *WT1* at high efficiency into human primary hematopoietic progenitors and leukemia-derived cell lines. In both primary progenitors and differentiation-competent leukemic cells, *WT1* expression was sufficient to induce G_1 arrest and to trigger differentiation along the myelo-monocytic lineage. In a subpopulation of early, stem cell-enriched progenitors, *WT1* induced cellular quiescence, leading to the preservation of phenotypically primitive cells. Co-expression of the naturally occurring isoforms of *WT1* enhanced its transcriptional and physiological effects. The expression pattern of *WT1* during blood cell differentiation *in vivo* correlated with these effects: present first in the quiescent stem cell compartment, and subsequently during lineage-specific differentiation. Taken together, these observations imply a role for *WT1* in the differentiation of normal human hematopoietic cells; they argue against an oncogenic role for *WT1* in leukemogenesis and support the functional relevance of *WT1* mutations in human acute leukemias.

Results

WT1 induces myelo-monocytic differentiation in human leukemia-derived cell lines

To examine the functional properties of *WT1* in a well established model of cellular differentiation, we first expressed *WT1* in two human myeloid leukemia-derived cell lines: HL60 and U937. These lines retain the capacity for differentiation following induction with all-*trans* retinoic acid (ATRA) and tetrahydro-phorbol-ester acid (TPA) (Breitman *et al.*, 1980; Bjare *et al.*, 1988), and they differ in their expression of *WT1*: wild-type *WT1* is expressed in HL60 cells, but absent in U937 cells (Sekiya *et al.*, 1994; Svedberg *et al.*, 1998). To avoid the possibility of selection bias associated with the isolation of rare clones stably expressing *WT1*, we transduced cells with the retroviral vector MSCV-GFP (where MSCV is murine stem cell virus), which drives expression of both

WT1 and green fluorescent protein (GFP) from a single bicistronic message (Hawley *et al.*, 1992), and analyzed unselected pools of GFP-expressing cells (Figure 1A). For each experiment we conducted multiple independent transductions and analyzed results within 3–5 days following retroviral infection. Approximately 20–40% of HL60 cells and 40–80% of U937 cells expressed GFP following infection with retroviruses encoding either the (–KTS) or (+KTS) isoforms of WT1 (Figure 2A). Levels of ectopic protein expression were comparable to those of the endogenous WT1 in HL60 and other leukemic cells (Figure 1B).

Despite the difference in expression of endogenous *WT1*, the consequences of ectopic *WT1* expression in HL60 and U937 cells were virtually identical, and hence are shown for U937 cells (Figure 1). Within 48 h following retroviral infection, cells expressing *WT1(–KTS)* demonstrated a dramatic increase in the G_1-phase fraction and a corresponding reduction in S phase compared with the control vector (68% versus 46%, $P = 0.006$). This was demonstrated by both analysis of propidium iodide (PI)-stained cells (Figure 1C) and bromodeoxyuridine (BrdU) labeling (data not shown). Little or no increase in the G_1-phase fraction was observed in parallel cultures infected with *WT1(+KTS)*. The G_1 arrest mediated by *WT1(–KTS)* was accompanied by marked induction of the cyclin-dependent kinase inhibitor $p21^{CIP1}$, a known *WT1* transcriptional target gene (Figure 1B) (Englert *et al.*, 1997; English and Licht, 1999). In HL60 cells, these effects were observed despite the high levels of endogenous WT1 expression and the relatively modest levels of ectopic WT1(–KTS) expression. These cells may be sensitive to subtle alterations in the relative ratio of WT1 isoforms. Alternatively, endogenous WT1 may reside in non-functional pools in HL60 cells.

Differentiation of U937 and HL60 cells induced by either retinoids or by the phorbol ester TPA is first evident by expression of characteristic cell surface markers, such as CD11b and CD14, followed by gross morphological changes (Hickstein *et al.*, 1989; Prieto *et al.*, 1994). Within 48 h following retroviral infection, 30–50% of HL60 and U937 cells expressing *WT1(–KTS)* showed spontaneous expression of CD11b in the absence of differentiation-inducing agents (Figure 2A and B). In contrast, <2% of cells infected with *WT1(+KTS)* or control vector expressed this marker. Spontaneous morphological changes accompanied CD11b expression in U937 cells transduced by *WT1(–KTS)*, which adhered to the tissue culture dish and acquired the characteristic appearance of differentiated U937 cells (Figure 2C). To confirm the functional properties of these differentiated cells, we tested their ability to reduce the substrate dye nitroblue tetrazolium (NBT), a characteristic that correlates directly with myeloid differentiation (Breitman *et al.*, 1980). In multiple experiments, 40–80% of U937 cells infected with *WT1(–KTS)* were positive for NBT, while <5% of *WT1(+KTS)*- or vector-infected populations were NBT positive (Figure 2C). Taken together, these observations indicate that expression of *WT1(–KTS)*, but not *WT1 (+KTS)*, in U937 and HL60 cells results in spontaneous differentiation within 48 h.

The mechanism by which *WT1* triggers hemato-poietic differentiation is likely to involve transcriptional
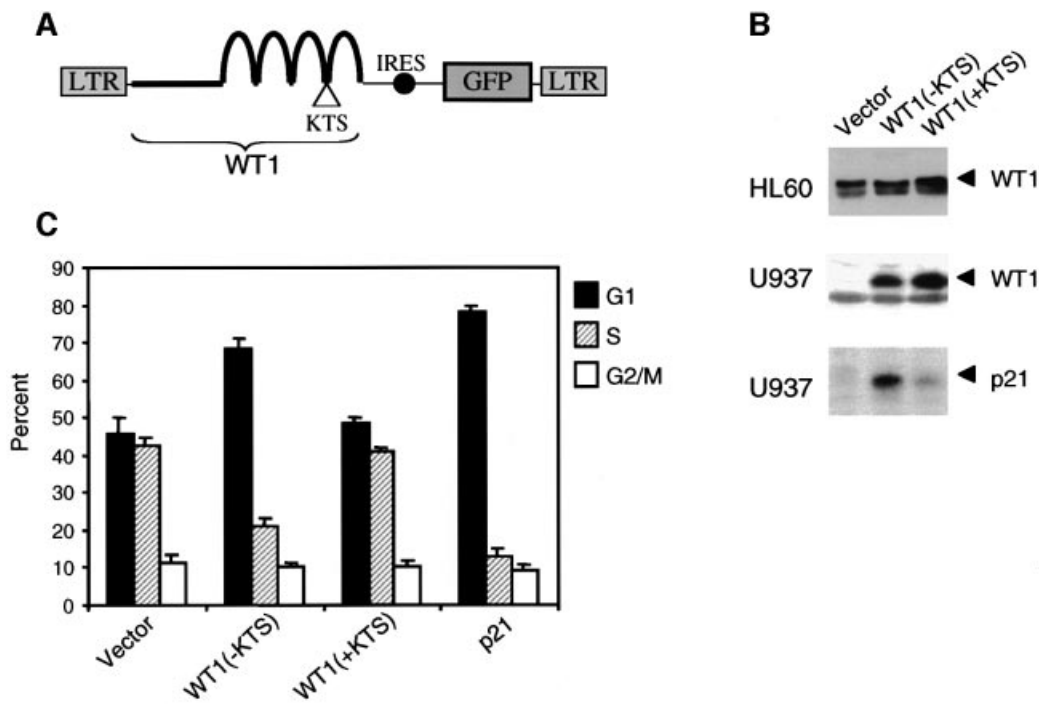
Fig. 1. G_1 arrest and induction of p21^{cip1} in U937 cells following *WT1* expression. (**A**) Schematic representation of retroviral constructs, encoding the *(–KTS)* and *(+KTS)* isoforms of *WT1*, linked by an internal ribosomal entry site (IRES) to a cDNA encoding GFP. The LTRs are derived from MSCV, which allows high levels of expression in hematopoietic precursors. (**B**) Western blots showing levels of WT1 protein in retrovirally transduced HL60 and U937 cells, and endogenous p21^{cip1} in U937 cells. Cell lysates were prepared 48 h following retroviral infection and analyzed on immunoblots probed with polyclonal anti-WT1 and anti- p21^{cip1} antibodies. Endogenous WT1 expression is not detectable in U937 cells. In HL60 cells, the lower band in the doublet is endogenous WT1 lacking alternative splice I (17 amino acids); the upper band contains both endogenous and transduced WT1 encoding this isoform. (**C**) Effect of *WT1* expression on cell cycle kinetics of transduced U937 cells. Cells were harvested 48 h after retroviral infection, stained with PI and analyzed by flow cytometry gating on the GFP-positive population. Results are derived from three independent experiments, with SE. The difference in the G_1 fraction between vector and *WT1(–KTS)*-expressing cells is significant ($P = 0.0048$), and is similar to that between cells expressing the vector or a retrovirus encoding *p21^{CIP1}*(right-most bars).

regulation of differentiation-associated target genes. One of these, *p21^{CIP1}*, is induced by *WT1(–KTS)* as well as other agents that trigger hematopoietic differentiation, and is thought to mediate a cell cycle arrest that contributes to activation of a cellular differentiation program (Zeng and el-Deiry, 1996; Steinman *et al.*, 1998). To determine whether *WT1*-mediated blood cell differentiation is simply the indirect result of a p21^{CIP1}-induced G_1 arrest, we infected U937 cells with a bicistronic MSCV-GFP retrovirus encoding p21^{CIP1}. p21^{CIP1}-expressing cells exhibited prompt G_1 arrest (Figure 1C), but little or no spontaneous expression of cell surface differentiation markers or morphological changes (Figure 2A and B). The triggering of hematopoietic differentiation by *WT1* is, therefore, likely to involve transcriptional regulation of additional target genes. Of note, induction of *p21^{CIP1}* by *WT1* may contribute to this effect, since we did observe that retrovirus-mediated expression of *p21^{CIP1}* accelerated the time course of differentiation induced by the phorbol ester TPA in U937 cells, even though it was not sufficient to cause spontaneous differentiation (data not shown).

WT1(+KTS) enhances differentiation mediated by the (–KTS) isoform

WT1(+KTS) encodes the most abundant isoform of WT1, but it has no known functional properties. While *WT1(+KTS)* was inactive in mediating cellular differentiation by itself, the observation that the WT1 isoforms are

normally co-expressed in a fixed ratio (Haber *et al.*, 1991) prompted us to examine whether simultaneous ectopic expression of these two isoforms might have functional consequences. The high retroviral infection rate achieved in U937 cells made it possible to co-infect a significant subset of cells (13%) with two different bicistronic constructs, one encoding WT1(–KTS) and GFP, and the other encoding WT1(+KTS) and the cell surface marker placental alkaline phosphatase (PLAP) (Figure 3A) (Jacob and Baltimore, 1999). In multiple experiments, fluorescence-activated cell sorting (FACS) analysis of U937 cells expressing both *WT1(–KTS)* and *WT1(+KTS)* showed a 30–50% relative increase in the number of cells positive for the differentiation marker CD11b, compared with cells co-expressing *WT1(–KTS)* and the control *PLAP* vector (Figure 3B). Cells singly infected with the control *PLAP* vector or the bicistronic *WT1(+)KTS/PLAP* construct showed little or no spontaneous CD11b expression. We also examined expression of a second differentiation marker, CD14, which is not spontaneously induced by the expression of *WT1(–KTS)*, but is strongly induced during TPA-mediated differentiation in *WT1(–KTS)*-expressing populations. Co-expression of both *WT1(+KTS)* and *(–KTS)* isoforms markedly enhanced the induction of CD14 following TPA-stimulated differentiation, compared with expression of *WT1(–KTS)* plus the control vector (Figure 3C). Moreover, the subset of co-infected cells with the highest levels of PLAP, the WT1(+KTS)
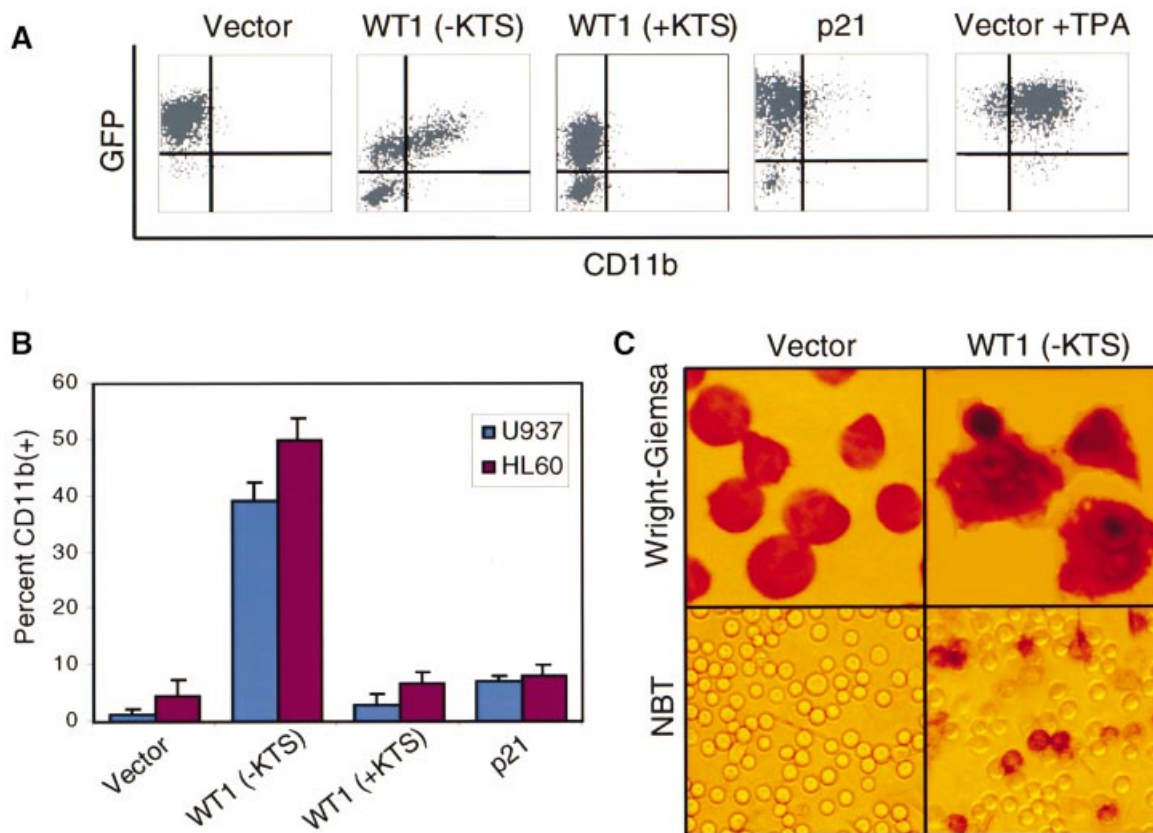
Fig. 2. Induction of spontaneous differentiation in U937 cells following *WT1* expression. (**A**) Two-color FACS analysis for GFP and CD11b on transduced U937 cells in a representative experiment. Cells were harvested 48 h after retroviral infection and stained with a PE-conjugated anti-CD11b antibody. Dot plots represent fluorescence intensity for GFP (*y*-axis) and CD11b–PE (*x*-axis), with the upper right quadrant representing infected cells expressing the differentiation marker. Differences in the percentage of GFP-positive cells are consistent, and reflect different packaging efficiencies for individual retroviral constructs as well as the growth-suppressive effect of *WT1(–KTS)*. In the right panel, vector-transduced cells cultured for 24 h in the presence of 3 nM TPA are shown as a positive control for differentiation. (**B**) Quantitation of three independent experiments showing the mean expression of CD11b among GFP-positive transduced U937 or HL60 cells. (**C**) Morphological and functional differentiation of U937 cells expressing *WT1(–KTS)*. Unsorted transduced cells were stained with Wright–Giemsa (upper panel, 250× magnification) or with 1 mg/ml NBT (lower panel, 100×), and analyzed by light microscopy. Representative fields are shown. Among *WT1(–KTS)*-transduced populations, fluorescence microscopy indicated that >90% of morphologically differentiated and NBT-positive cells were GFP positive (not shown).

surrogate marker, showed the largest increases in CD11b and CD14 staining (data not shown). Taken together, these observations indicate that WT1(+KTS) is not sufficient to trigger differentiation alone, but exhibits a potent cooperative effect in enhancing WT1(–KTS)-mediated differentiation.

WT1 enhances myelo-monocytic differentiation in primary hematopoietic progenitors

While leukemia-derived cell lines provide an important model for human hematopoietic differentiation, they are derived from cells arrested at a single stage of hemato-poiesis and capable of differentiation along a limited lineage. We therefore examined the effect of ectopic *WT1* expression on differentiation of primary CD34(+) hemato-poietic progenitor cells isolated from human umbilical cord blood. CD34(+) cells represent a heterogeneous population of undifferentiated cells, including both lineage-committed progenitors and rare uncommitted, multipotent stem cells (Civin and Small, 1995; Krause *et al.*, 1996). *WT1* expression gave rise to distinct phenotypes in these two populations: enhanced

differentiation of the more mature progenitors, and quiescence of a rare, primitive subset of CD34(+) cells.

Retroviral titers and infection conditions were optim-ized, allowing routine infection of 20–50% of CD34(+) cells with bicistronic constructs encoding WT1 and GFP (Figure 4A, upper panel). Infected cells were cultured in the presence of the cytokines c-kit ligand (KL), FLT-3 ligand (FLT-3 L), thrombopoietin (TPO) and interleukin-3 (IL-3), and were harvested at different time points for immunophenotypic and functional analysis. Primary CD34(+) cells normally proliferate and differentiate rapidly once placed in culture, giving rise to cells that lose expression of this marker, gain lineage-specific markers and eventually cease to proliferate (Civin and Small, 1995; Traycoff *et al.*, 1996). These effects were significantly enhanced among cells expressing *WT1 (–KTS)*, and were reproduced in multiple independent experiments. Cells expressing *WT1(–KTS)* consistently showed more frequent expression of the differentiation marker CD11b compared with vector or *WT1(+KTS)*-infected cells (Figure 4). Figure 4A (lower panel) shows expression of CD11b and CD34 on transduced
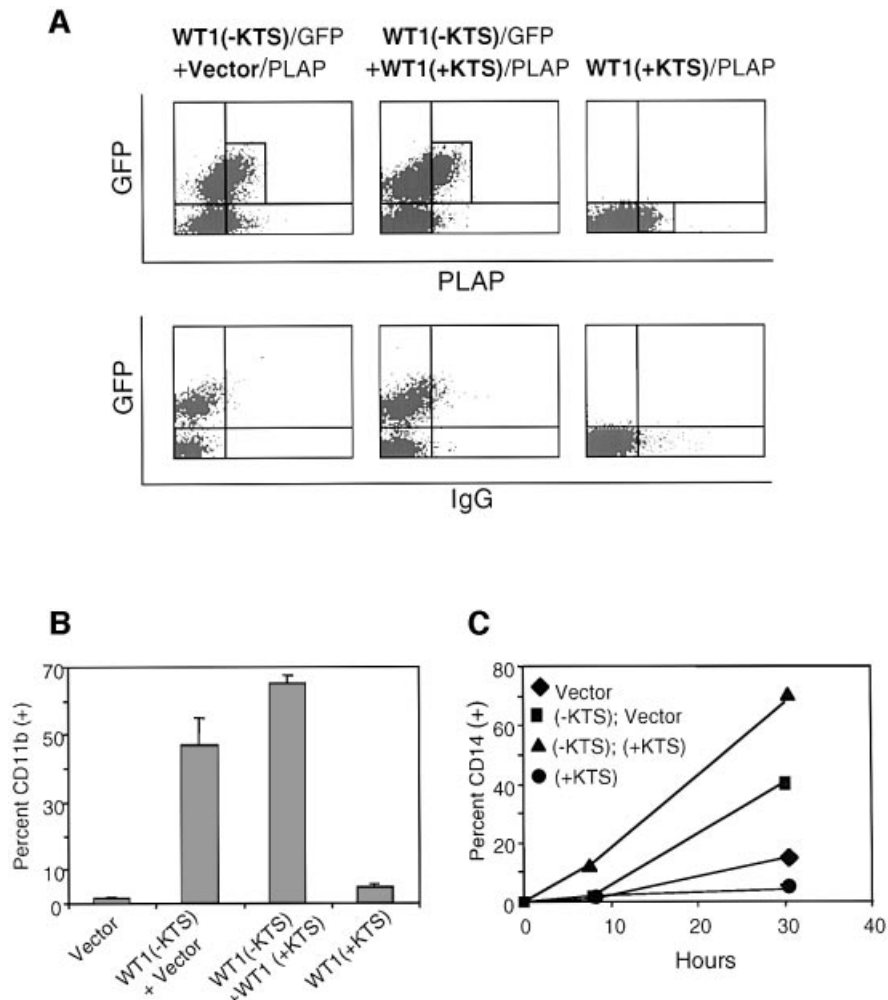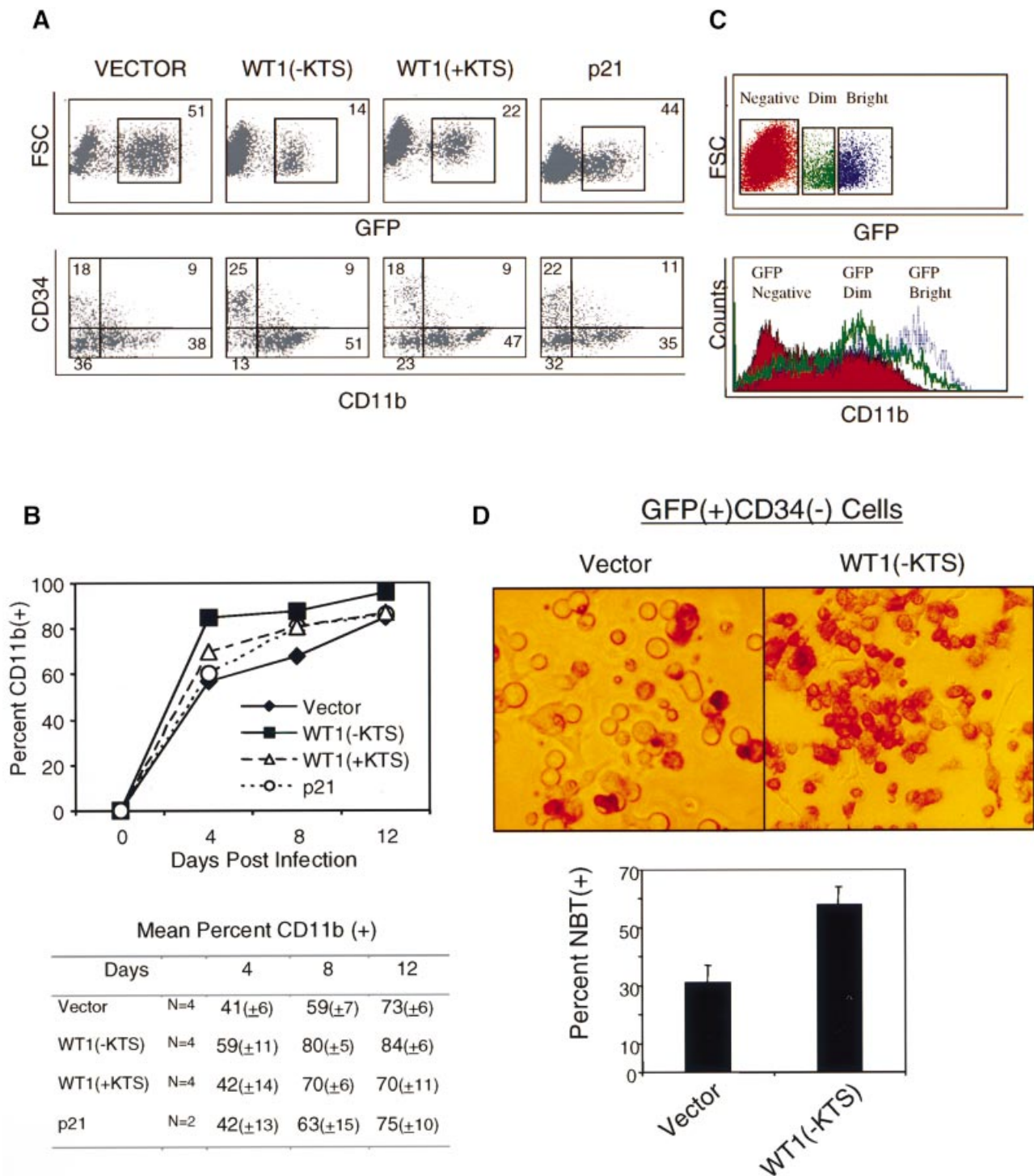
Fig. 3. Enhancement of *WT1(–KTS)*-mediated differentiation by *WT1(+KTS)*. (**A**) Two-color FACS analysis for the surrogate markers GFP [linked to *WT1(–KTS)*] and PLAP [vector alone or linked to *WT1(+KTS)*] on transduced U937 cells (upper panel). Cells were harvested 48 h after retroviral infection and stained for PLAP and CD11b or CD14. Boxes denote cell populations with comparable expression of GFP and PLAP that were selected for CD11b or CD14 analysis (see below). FACS analysis using control IgG was used to define the appropriate threshold for PLAP expression (lower panel). (**B**) Quantitative analysis of two independent experiments showing spontaneous expression of CD11b among U937 cells transduced with vector, *WT1(–KTS)*, *WT1(+KTS)* or both isoforms [see cell populations selected in (A)]. CD11b expression was determined by two- and three-color FACS analysis. (**C**) Time course of CD14 expression in U937 cells transduced with either or both *WT1* isoforms [see (A)], following TPA-induced differentiation. Cells were treated with 3 nM TPA 24 h after retroviral infection (time 0), harvested and stained at the indicated time points, then analyzed by two- and three-color FACS analysis. A representative experiment is shown.

GFP(+) cells at day 7 following retroviral infection in a representative experiment. The increased fraction of *WT1(–KTS)*-transduced cells expressing CD11b was most prominent in the population that had lost the CD34 surface marker: 79% [51/(51 + 13)] of CD34(–) cells were CD11b positive among the *WT1(–KTS)*-expressing population versus 51% [38/(38 + 36)] for the vector control population. On average, we observed a 40–50% relative increase in the proportion of CD34(–) cells expressing CD11b in *WT1(–KTS)*-infected versus vector-infected populations (Figure 4B; *P* = 0.001). This effect persisted for ~12 days of *in vitro* culture, after which time most cells in all infected populations expressed differentiation markers. Of note, the intensity of CD11b staining correlated with the level of WT1(–KTS) expression, as assessed by the surrogate marker GFP (Figure 4C). Increased functional differentiation of primary *WT1(–KTS)*-expressing cells was confirmed by NBT staining (Figure 4D).

Taken together, these data indicate that WT1(–KTS) enhances the differentiation of primary blood cell progenitors, in particular the more mature subset identified by the rapid loss of CD34. WT1(+KTS) had a less pronounced but consistent effect on *in vitro* differentiation (Figure 4).

As in leukemia-derived cell lines, *WT1*-mediated differentiation in primary hematopoietic precursors was associated with a cell cycle arrest. Within 48–72 h following retroviral infection, PI staining showed a 35% relative increase in G_o–G_1-phase cells compared with vector control or *WT1(+KTS)* (data not shown). Infection of primary hematopoietic precursors with a retrovirus encoding $p21^{CIP1}$ also led to growth arrest but did not reproduce the effect of *WT1(–KTS)* on cellular differentiation. Only a small increase in the number of CD11b(+) cells was observed after 7–12 days of ectopic $p21^{CIP1}$ expression (Figure 4B). *WT1* therefore induces a distinct

A

VECTOR   WT1(-KTS)   WT1(+KTS)   p21

C

B

Mean Percent CD11b (+)

Days		4	8	12
Vector	N=4	41(±6)	59(±7)	73(±6)
WT1(-KTS)	N=4	59(±11)	80(±5)	84(±6)
WT1(+KTS)	N=4	42(±14)	70(±6)	70(±11)
p21	N=2	42(±13)	63(±15)	75(±10)

D   GFP(+)CD34(-) Cells

Fig. 4. Enhanced differentiation of primary hematopoietic progenitors by *WT1*. Purified human cord blood CD34(+) cells were transduced with *GFP*-linked vector, *WT1(–KTS), WT1(+KTS)* or *p21^Cip1*, cultured in cytokine-supplemented medium, and harvested at days 4, 8 and 12 following retroviral transduction, for immunophenotypic and functional analysis. (**A**) FACS analysis of transduced cells, showing GFP staining as a marker for retroviral infection, and forward scatter (FSC), an indicator of cell size (upper panel). The percentage of infected cells (boxed) is quantitated. Analysis using anti-CD34–PE and anti-CD11b–APC on the gated GFP-positive population is shown in the lower panel. The percentage of cells in each quadrant is indicated. A representative experiment is shown for one time point (day 8). (**B**) Time course of CD11b expression during *in vitro* culture of transduced primary cell populations. A representative experiment is plotted, showing the percentage of CD11b-expressing cells within the GFP(+)CD34(–) population. Results from six independent experiments are shown in the table, indicating the mean percentage of CD11b-expressing cells (± SE). Differences in marker expression between vector and *WT1(–KTS)*-expressing cells at days 4, 8 and 12 were analyzed using the Wilcoxon matched-pairs signed ranks test ($P < 0.001$). (**C**) Correlation between the intensity of the *WT1(–KTS)*-linked marker GFP and that of CD11b in transduced cord blood cells. GFP expression was quantitated by FACS analysis (day 8) for cells stained as in (A), and subpopulations were selected as GFP-negative, dim and bright (boxed in upper panel). Expression of CD11b was measured in these subpopulations (shown as a histogram in the lower panel). (**D**) Functional differentiation of primary hematopoietic progenitors. NBT staining of FACS-sorted GFP(+)CD34(–) cells, following infection with vector or *WT1(–KTS)*-encoding retroviral constructs (day 7). Representative fields are shown by light microscopy (100×) (upper panel). Quantitation of the mean percent NBT-positive cells with SE is shown in the lower panel. The differences are statistically significant ($P < 0.001$).
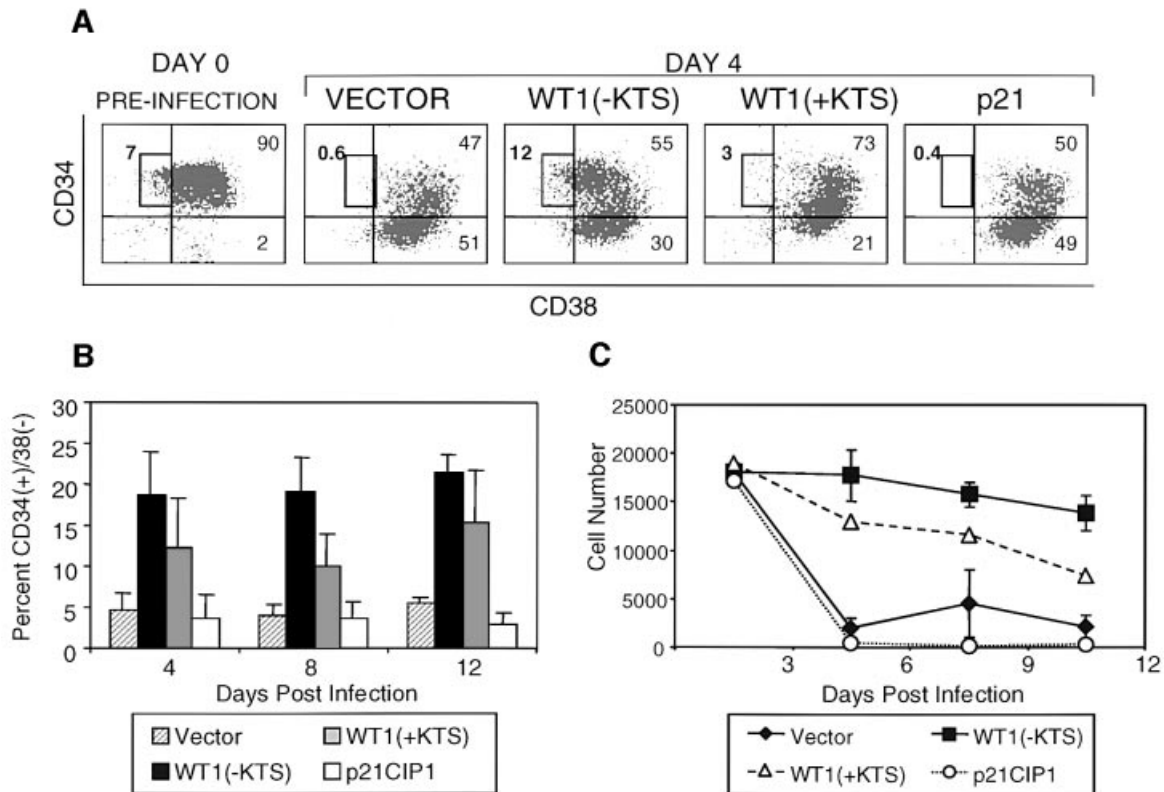
Fig. 5. Preservation of the CD34(+)CD38(–) stem cell population by *WT1*. (**A**) Expression of CD34 and CD38 markers on GFP-positive cord blood cells, on day 4 following transduction with GFP-linked constructs encoding vector, WT1(–KTS), WT1(+KTS) or p21^{CIP1}. Cells were stained with anti-CD34–PE and anti-CD38–APC and analyzed by three-color FACS analysis. Boxes denote the CD34(+)CD38(–) cell subset within the total CD34(+) population at day 0, and within gated GFP-positive transduced cells at day 4. Numbers indicate the percentage of cells in each subset. A representative experiment is shown. (**B**) Quantitation of percent CD34(+)CD38(–) cells in the GFP-positive population as a function of days in culture, shown as the mean of six independent experiments with SE. Differences between vector- and *WT1(–KTS)*-transduced cells were calculated to include observations at days 4, 8 and 12 by the Wilcoxon matched-pairs signed ranks test ($P = 0.002$). (**C**) Absolute number of CD34(+)CD38(–)CD11b(–) cells following transduction of primary progenitors with vector, *WT1(–KTS)*, *WT1(+KTS)* or p21^{CIP1}. For each time point, cells were counted and stained with anti-CD34–PerCP, anti-CD38–APC and anti-CD11b–PE, and the CD34(+)CD38(–)CD11b(–) fraction of GFP-positive cells was determined by four-color FACS analysis. Values indicate cells per milliliter of culture and represent the mean of two experiments for *WT1(–KTS)* and vector, and one experiment for *WT1(+KTS)* and *p21^{CIP1}*.

program of differentiation in hematopoietic precursors in addition to its effect on cell cycle progression.

WT1 expression promotes quiescence of the CD34(+)CD38(–) stem cell population

Coincident with the enhanced differentiation of CD34(–) cells in *WT(–KTS)*-transduced populations, we also observed a dramatic and consistent increase in the proportion of very early progenitors expressing high levels of CD34 ('CD34 bright') and lacking differentiation markers such as CD11b (Figure 4A and data not shown) (DiGiusto *et al.*, 1994). Further analysis showed that these cells also lacked surface expression of CD38, a marker of lineage-committed progenitors. CD38(–) cells normally comprise only 1–5% of CD34(+) cells in cord blood, and represent a subset that is highly enriched for hematopoietic stem cells (Terstappen *et al.*, 1991; Conneally *et al.*, 1997). Remarkably, within 4 days following infection with *WT1(–KTS)*, CD34(+)CD38(–) cells accounted for ~20% of all GFP-positive cells (Figure 5A and B). In contrast, vector- and *p21^{CIP1}*-infected populations showed only the expected small fraction (mean 3–5%) of cells with this phenotype ($P = 0.002$). The consistent increase in the fraction of cells within this subset persisted throughout the

12 day time course of *in vitro* culture. Quantitative analysis showed no expansion in the absolute number of CD34(+)CD38(–) cells, but rather a relative increase due to their slower decline and the rapid differentiation of committed progenitors in the *WT1(–KTS)*-transduced population (Figure 5C). Detailed phenotypic analysis of these *WT1*-expressing CD34(+)CD38(–) cells was consistent with uncommitted progenitor cells (Dorrell *et al.*, 2000), including absent staining for the cell surface markers CD11b, CD13, CD15 and CD33 (data not shown). While *WT1(–KTS)* had the strongest effect, expression of the *WT1(+KTS)* isoform also led to an increase in the fraction of cells within this hematopoietic progenitor compartment (Figure 5B and C).

The persistence of CD34(+)CD38(–) cells in *WT1 (–KTS)*-infected cultures could represent either quiescence associated with reduced differentiation, or alternatively, enhanced proliferation coupled with self-renewal or apoptosis. To examine the proliferative activity of *WT1*-infected CD34(+)CD38(–) cells, we stained cells using the vital fluorescent membrane dye PKH26, whose progressive dilution serves to monitor cell division (Lansdorp, 1996; Ladd *et al.*, 1997). Using three- and four-color cytometric analysis, we determined the change in PKH26
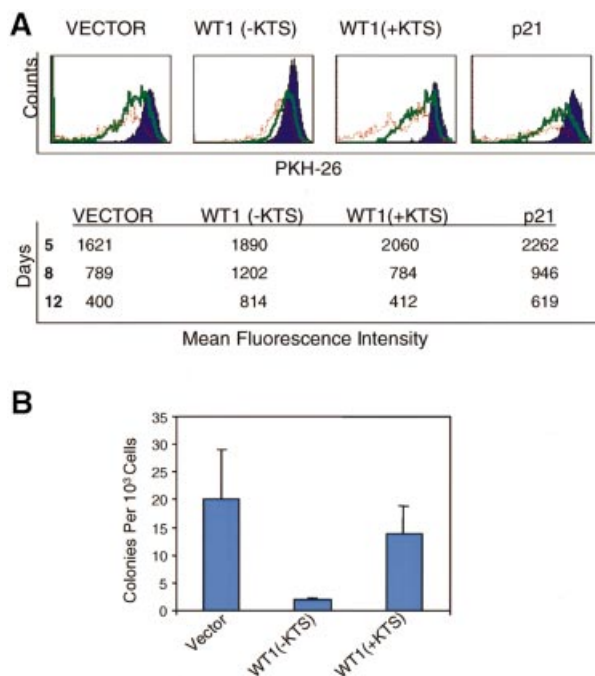
Fig. 6. Quiescence of uncommitted progenitors induced by *WT1(–KTS)*.
(**A**) Reduced proliferation of *WT1(–KTS)*-expressing primary
hematopoietic progenitors, demonstrated by retention of the dye
PKH26. GFP-positive transduced cells were purified by FACS on day 4
following retroviral infection, stained with PKH26, then cultured and
harvested on days 5, 8 and 12. PKH26 expression was quantitated in
CD34(+) cells and is shown as a histogram [upper panel; day 5 (blue),
day 8 (green) and day 12 (red)]. For each transduced construct, the
table shows the absolute mean fluorescence intensity at each time point
in a representative experiment. Immediately after labeling at day 4, the
mean fluorescence intensity of PKH26 in all four samples was
comparable (~2000). (**B**) Effects of *WT1* on colony-forming ability of
primary hematopoietic progenitors. CD34(+) cells expressing GFP-
linked vector, *WT1(–KTS)* or *WT1(+KTS)* were purified by FACS after
8–10 days of liquid culture. Sorted cells were plated in triplicate, at a
density of 1000 cells/ml in methylcellulose supplemented with IL-3,
KL and EPO. Colony counts at day 14 are shown as the average of
three independent experiments with SE. The difference between
WT1(–KTS)- and vector-transduced populations is statistically
significant (P <0.003).

fluorescence intensity over time in each phenotypic subset
of infected cells, including total CD34(+) and CD34(+)
CD38(–) populations. *WT1(–KTS)*-expressing CD34(+)
CD38(–) cells demonstrated a high level of dye retention,
indicating profound growth arrest compared with vector-
infected cells (Figure 6A). The inhibition of proliferation
in this stem cell-enriched population by *WT1(–KTS)* was
considerably more pronounced than that mediated by
$p21^{CIP1}$, whose ectopic expression only modestly and
transiently decreased proliferation of CD34(+) cells.
Despite their profound unresponsiveness to growth and
differentiation signals, the viability of this cell population
was confirmed, and no differences in apoptosis were
observed among *WT1(–KTS)*, *WT1(+KTS)*, $p21^{CIP1}$ or
control vector-transduced cells (data not shown).

The profound cellular quiescence induced by constitu-
tive *WT1* expression in this stem cell-enriched population
was further supported by reduced activity in short-term
colony-forming (CFU) assays and in long-term culture
with primary bone marrow stroma (Ploemacher *et al.*,
1989; Sutherland *et al.*, 1990). Sorted GFP(+)CD34(+)

cells expressing *WT1(–KTS)* cells showed a 10-fold
decrease in clonogenic efficiency compared with control
vector and *WT1(+KTS)* ($P = 0.027$; Figure 6B), and very
low or undectable proliferative activity at weeks 1–5
following plating onto stroma, despite preserved viability
(data not shown).

Biphasic in vivo expression of WT1 during human hematopoiesis

The distinct effects of retrovirally mediated *WT1* expres-
sion on hematopoietic precursors suggest that it may have
different functions at early and late stages of blood cell
differentiation. To correlate these stage-specific effects
with endogenous *WT1* expression during *in vivo* hemato-
poiesis, we quantified *WT1* expression in individual cells
representing different stages of hematopoietic differenti-
ation, isolated from FACS-sorted populations of human
hematopoietic cells. Previous studies using direct RT–
PCR analysis had indicated variable expression of *WT1* in
CD34(+) populations (Menssen *et al.*, 1995; Maurer *et al.*,
1997). To determine the expression pattern of WT1 in
different subsets of CD34(+) cells, we used a quantitative
method involving representational amplification of all
polyA(+) RNA from single cells, followed by blot
hybridization using a *WT1* probe and controls. This
approach, performed in multiple replicates, has proved to
be highly accurate in measuring gene expression from rare
populations of hematopoietic precursors (Cheng *et al.*,
1996). Because this technique examines expression in
individual cells within heterogeneous populations, expres-
sion levels vary from cell to cell (each lane in Figure 7A
represents cDNA from a single cell), and these are
therefore averaged for each population studied
(Figure 7B). *WT1* expression in human bone marrow
and peripheral blood cells showed a biphasic pattern. *WT1*
was present in some quiescent primitive progenitors,
absent in lineage-committed precursors, then present again
in a subset of the more differentiated populations
(Figure 7A and B). The early progenitors expressing
WT1 comprised the rare population of non-lineage-com-
mitted precursors that are in G_0, isolated by treatment of
CD34(+) cells with the anti-metabolite 5-fluorouracil
(5-FU) to eliminate cycling cells. This cell population
represents a subset of CD34(+)CD38(–) cells that is highly
enriched for functional stem cells capable of producing
both myeloid and lymphoid progeny (Berardi *et al.*, 1995;
Gothot *et al.*, 1998). In contrast, *WT1* mRNA was below
detection using this assay in the bulk population of both
lineage-committed and uncommitted CD34(+) precursors.
WT1 expression reappeared at late stages of hematopoietic
differentiation, in a pattern consistent with the lineage-
specific differentiation induced by *WT1* in primary
hematopoietic precursors. In the more abundant differen-
tiated populations, similar results were obtained using
either the single-cell technique or direct RT–PCR ampli-
fication of the *WT1* transcript, a more commonly used but
less quantitative approach (Figure 7C). Among myeloid
cells, *WT1* expression was restricted to cells expressing the
granulocytic/monocytic marker CD11b, and was absent in
cells expressing erythroid as well as megakaryocytic
markers. *WT1* was also present in B lymphocytes express-
ing the CD19 antigen. The correlation between *WT1*
expression *in vivo* and the effects of its ectopic expression,
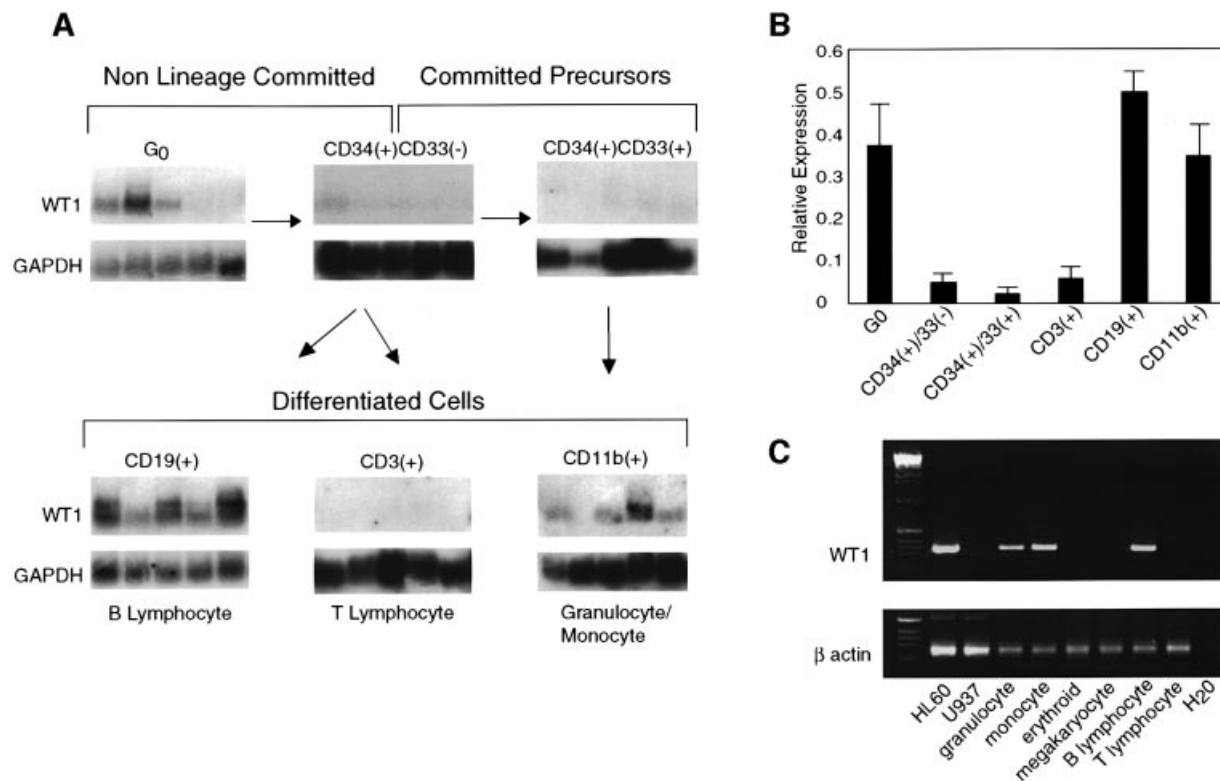
Fig. 7. Biphasic expression of *WT1* during human hematopoiesis *in vivo*. (**A**) Quantitation of *WT1* expression in individual hematopoietic precursor cells. Single-cell RT–PCR was used to amplify total cellular mRNA from individual cells isolated by FACS or micromanipulation from human bone marrow and peripheral blood as described in Cheng *et al*. (1996). G_0, stem cell-enriched quiescent cells were obtained by treatment of CD34(+) cells with the anti-metabolite 5-FU to eliminate cycling cells (Berardi *et al*., 1995). Southern blots of amplified cDNA were then hybridized with *WT1* or *GAPDH* cDNA probes and quantified by densitometry. Representative blots are shown, and the phenotype of each cell type examined and their relationship to each other are indicated. The CD34(+)CD33(–) population contains both lineage-committed and uncommitted precursors. Each lane represents cDNA from a single cell; thus, differences in expression between cells in each sorted population reflect the heterogeneity of each population. The size distribution of the bands detected is that of the total pool of amplified *WT1* or *GAPDH* cDNA, and therefore does not correspond to a precise message size. (**B**) Quantitation of *WT1* expression levels for each cell population examined in (A). Results are expressed as the mean ratio of *WT1*/*GAPDH* expression for at least 15 lanes examined from each population, with SE. (**C**) Expression of *WT1* in differentiated hematopoietic populations. Ethidium bromide-stained gel showing products of direct RT–PCR amplification of the *WT1* and *β actin* transcripts from leukemia cell lines and FACS-isolated populations of cord blood cells. Populations were isolated by two-color cell sorting using the following surface markers: CD11b(+)/CD14(–), granulocyte; CD11b(+)/CD14(+), monocyte; glycophorin A (GPA)(+)/CD41(–), erythroid; GPA(–)/CD41(+), megakaryocyte; CD19(+)/CD3(–), B lymphocyte; CD19(–)/CD3(+), T lymphocyte. The 284 bp *WT1* PCR fragment shown corresponds to a portion of exons 1–4.

both in primitive uncommitted progenitors and in lineage-committed cells, underscores the physiological relevance of these effects.

Discussion

WT1 has a well defined role in kidney development and malignancy, but its potential function in human hemato-poiesis remains controversial. Prior studies of cloned leukemic cell lines stably transfected with *WT1* constructs have typically shown defects in the response to differentiation agents, suggesting that inhibition of differentiation by wild-type *WT1* may in fact contribute to leukemo-genesis (Inoue *et al*., 1998; Svedberg *et al*., 1998; Deuel *et al*., 1999). Other studies have argued for a pro-differentiation effect of the WT1(+KTS) isoform in hematopoietic cells (Smith *et al*., 1998). Here, introduction of *WT1* by high titer retroviral infection has allowed us to examine unselected populations of hematopoietic cells at early time points following *WT1* expression, and to examine the fate of cells belonging to different lineages and developmental stages. In primary hematopoietic

precursors and in differentiation-competent leukemic cell lines, *WT1* expression is associated with growth arrest and spontaneous differentiation. Given the rapid and potent effect of WT1 on cellular differentiation, prior conflicting results are likely to be explained by the selection of individual clones whose resistance to differentiation signals may allow for stable expression of *WT1*.

Phenotypes resulting from ectopic expression of *WT1* warrant cautious interpretation, but several points support the physiological relevance of our observations. First, the levels of retrovirally expressed WT1 protein are similar to those in leukemic cells. Secondly, the lineage-specific differentiation induced by *WT1* in primary hematopoietic precursors correlates with its lineage-specific expression among myeloid cells *in vivo*. Thirdly, although relatively few bona fide transcriptional target genes of WT1 have been identified, one of these, *p21^{CIP1}*, is induced by WT1 in our assays and appears to mediate a portion of the observed effects. Nonetheless, additional studies will be required to conclusively demonstrate a role for *WT1* in normal hematopoiesis. Inactivation of *WT1* in the mouse is not associated with gross hematological deficits, although

embryonic lethality at day 13–14 precludes evaluation of adult hematopoiesis. Species-specific differences may also need to be considered, since *WT1* inactivation in the mouse has not been linked to renal tumorigenesis, and murine leukemia cells do not commonly express endogenous *WT1*, unlike their human counterparts.

Like sporadic Wilms tumor, only 10–15% of human acute leukemias harbor mutations in *WT1*, while the majority express high levels of the wild-type transcript (Miwa *et al.*, 1992; Menssen *et al.*, 1995; King-Underwood *et al.*, 1996; Miyagawa *et al.*, 1999). Some clinical studies have suggested a correlation between *WT1* expression in acute leukemias and a more primitive histology associated with an adverse prognosis (Inoue *et al.*, 1994; Bergmann *et al.*, 1997), while others have not found such an association (Gaiger *et al.*, 1998, 1999). Our demonstration that *WT1* induces growth arrest and differentiation in hematopoietic cells argues against an oncogenic role for *WT1* in human leukemia, and instead suggests a parallel with sporadic Wilms tumor. Thus, disrupted *WT1* function may contribute to malignancy in a subset of leukemias, while the majority of cases have acquired other mutations that may render them insensitive to the effects of wild-type *WT1*. This is consistent with our finding that ectopic *WT1* expression fails to induce differentiation in common myeloid leukemia-derived cell lines, such as KG1 and SR-91, that do not respond to standard differentiation agents (data not shown).

Early studies of ectopic *WT1* expression in immortalized cell lines revealed its ability to induce a cell cycle arrest as well as trigger apoptosis (Englert *et al.*, 1995a; Kudoh *et al.*, 1995). However, the cell fates that can be studied in most cancer-derived cell lines are limited. Our analysis of primary hematopoietic cells has allowed us to demonstrate a direct effect of WT1 on cellular differentiation, consistent with its presumed role in renal organogenesis. Using this *in vitro* system we have demonstrated cooperative roles in differentiation for two WT1 isoforms. In contrast to WT1(–KTS), the more abundant WT1(+KTS) isoform does not possess sequence-specific DNA binding activity (Rauscher *et al.*, 1990). WT1(+KTS) has appeared to be inactive when expressed in most cell lines, and variable effects have been noted in other cell types that do not tolerate expression of WT1(–KTS) (Murata *et al.*, 1997; Smith *et al.*, 1998). Nonetheless, the physiological importance of the WT1(+KTS) isoform is underscored by the severe developmental abnormalities that result from its decreased expression in patients with Frasier syndrome (Barbaux *et al.*, 1997). Indeed, we did observe a subtle but reproducible effect of WT1(+KTS) alone on the differentiation of primary hematopoietic progenitors (Figure 4C), and some enhancement of induced differentiation of leukemic cells (Figure 3C). These effects are consistent with an ability of WT1(+KTS) to modulate but not initiate differentiation. The precise mechanism by which the WT1(+KTS) and (–KTS) isoforms cooperate to induce differentiation remains to be determined, and a complete understanding of WT1 function is likely to require analysis of cooperation among all four expressed isoforms.

The effect of *WT1* on hematopoietic precursor subsets appears to be stage specific: cellular differentiation is induced in lineage-committed precursors, while cellular quiescence is enhanced in more primitive cells. These effects may provide insight into the potentially analogous role of *WT1* in kidney development, a complex process that is not as readily analyzed *in vitro*. The biphasic expression of *WT1* during hematopoiesis is remarkably similar to that in the developing kidney, where low levels of the transcript are present in undifferentiated blastemal stem cells and very high levels appear in subsequent stages of differentiation, as epithelial cells give rise to the podocytes of glomeruli (Pritchard-Jones *et al.*, 1990). *WT1* has been postulated to be essential for the survival of blastemal cells, based on widespread apoptosis of these cells in *wt1*-null mice (Kreidberg *et al.*, 1993), a phenomenon that is consistent with the preservation of the hematopoietic stem cell compartment by *WT1*. We have demonstrated recently that $p21^{Cip1}$ is required for the long-term maintenance of hematopoietic stem cells in the mouse (Cheng *et al.*, 2000), raising the possibility that induction of $p21^{Cip1}$ by WT1 itself may contribute to this effect. A subsequent role for *WT1* in the differentiation of glomerular podocytes may also be analogous to its ability to induce myelo-monocytic differentiation in more mature hematopoietic precursors. Other genes are also known to have dual roles in blood and kidney development. For instance, treatment of cultured primary metanephric mesenchyme with leukemia inhibitory factor (LIF)/IL-6 triggers early stages of renal differentiation *in vitro* (Barasch *et al.*, 1999), and *GATA-2*-null mice display severe genitourinary defects following rescue of their hematopoietic deficits (Zhou *et al.*, 1998). Thus, *WT1* may demonstrate a role within convergent hematopoietic and renal differentiation pathways, consistent with its mutational inactivation in both Wilms tumor and acute leukemia.

Materials and methods

Retroviral expression constructs

cDNAs encoding *(+KTS)* and *(–KTS)* isoforms of *WT1* have been described elsewhere (Englert *et al.*, 1995a), and the human $p21^{CIP1}$ cDNA was generated by PCR amplification of cDNA from primary human fibroblasts using coding region-specific primers. cDNA fragments were subcloned into the multicloning site of the retroviral vector MSCV-GFP (Hawley *et al.*, 1992) (Figure 1A), or into MSCVpuro IRES PLAP (kindly provided by J.Jacob) (Jacob and Baltimore, 1999). Both of these vectors drive expression from the 5′ long terminal repeat (LTR) of a single biscistronic mRNA encoding the protein of interest and either an enhanced GFP (Clontech Laboratories, Palo Alto, CA), or a catalytically inactive mutant PLAP, linked by an IRES.

Cells and cell culture

The human myeloid cell lines HL-60 and U937 were obtained from the American Type Culture Collection. Parental and transduced cell lines were maintained in RPMI medium supplemented with 10% fetal calf serum (FCS; Sigma) (R10). To induce cellular differentiation, 3 nM TPA (Sigma) was added to HL-60 or U937 cells in R10. Human embryonic kidney-derived 293T cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS. All cells were cultured at 37°C under 5% CO_2. Cord blood samples were obtained from the Pediatric Research Institute, University of St Louis, MO, according to the guidelines established by the Human Investigation Committee. Mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation, and CD34(+) cells were purified by immunomagnetic bead selection (Milteny Biotec, Auburn, CA), according to the manufacturer's instructions. Transduced CD34(+) cells were grown in Iscove's modified Dulbecco medium (IMDM) (Mediatech Inc., Harndon, VT) containing 10% FCS (IMDM10) and supplemented with KL (20 ng/ml), TPO (20 ng/ml), FLT-3 L (20 ng/ml) and IL-3 (20 ng/ml)

(R & D, Minneapolis, MN). Cells were initially cultured in 1 ml final volume at a density of $0.5–1 \times 10^6$/ml in 24-well plates. Fresh media and cytokines were added every 3–4 days. Clonogenic progenitor assays were performed with cells harvested from the liquid cultures. Harvested cells were cultured in methylcellulose (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with IL-3 (50 ng/ml), KL (50 ng/ml) and erythropoietin (EPO) (Amgen, Thousand Oaks, CA) according to the manufacturer's recommendations. Colonies were evaluated by phase microscopy and scored according to standard morphological criteria at day 14.

Retroviral generation and transduction

Retroviral generation and transduction were performed essentially as described (Carlesso et al., 1999). Vectors were co-transfected with pKat, an amphotropic packaging plasmid (kindly provided by M.Finer, Cell Genesys Inc., Foster City, CA), and pCMV-VSV-G, a plasmid encoding the vesicular stomatitis virus G-glycoprotein (kindly provided by T.Friedmann, University of San Diego, CA), into 293T cells using calcium phosphate precipitation (CalPhos reagent; Clontech). Supernatants containing pseudotyped retrovirus were collected at 36 h and used to infect primary CD34(+) cells, HL-60 and U937 cell lines. CD34(+) progenitors were cultured in IMDM10 supplemented with KL (50 ng/ml), TPO (20 ng/ml), FLT-3 L (20 ng/ml) and IL-3 (20 ng/ml) (R & D) for 24 h prior to infection, in order to induce cells to enter into cell cycle. HL-60, U937 or stimulated CD34(+) cells were washed in phosphate-buffered saline (PBS; Sigma) and resuspended at $1–2 \times 10^5$ cells/ml in 1 ml of 50% retroviral supernatant and 50% fresh complete medium plus Polybrene (final concentration 8 μg/ml; Sigma). Cells in suspension were placed in a 48-well plate (Becton Dickinson Inc., Franklin Lakes, NJ), spinoculated at 650 g for 50 min, then incubated at 37°C under 5% CO_2 for an additional 8–12 h, washed, and resuspended in fresh medium overnight. A second and a third infection were conducted on the following days using an identical procedure. Infection efficiency was evaluated by GFP expression 4 days after the first infection.

Antibodies, cell staining, FACS analysis and cell sorting

Western blots were carried out using commercial polyclonal antibodies against WT1 (sc-192) and p21^{CIP1} (sc-397) (Santa Cruz). Fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein (PerCP)-coupled control immunoglobulins or specific monoclonal antibodies directed to CD34, CD38, CD33, CD13, CD11b, CD14 and CD15 (Becton Dickinson, San Jose, CA) were used in three- or four-color labeling to determine hematopoietic cell subsets in conjunction with GFP. Cells were incubated with 0.5% human IgG (Sigma) in PBS for 20 min at 4°C to block the Fc receptor prior to staining with antigen-specific antibodies. For PLAP staining, cells were incubated with polyclonal rabbit anti-PLAP or control Ig (Zymed, San Francisco, CA), then washed and incubated with biotin-conjugated polyclonal goat anti-rabbit (PharMingen), and then washed and incubated with APC-conjugated streptavidin (PharMingen) plus anti-CD11b–PE or anti-CD14–PE. Flow cytometric analysis was performed using the FACScalibur instrument (Becton Dickinson, San Jose, CA) and Cell Quest program (Becton Dickinson). A minimum of 70 000 events were analyzed in each sample. For cell sorting, GFP(+) or GFP(+)CD34(+) fractions were purified from cord blood CD34(+) transduced cells at day 4 or 7 of culture by FACS on FACS Vantage (Becton Dickinson). For each sorting, $4 \times 10^5–2 \times 10^6$ harvested cells were used. GFP(+), GFP(+)CD34(+) and GFP(+)CD34(–) cells were isolated at 90–95% purity. Sorted populations were then used for PKH26 labeling, colony assay, long-term culture and NBT staining.

Cell cycle analysis

Cell cycle analysis was performed using two-color FACS analysis for GFP and the DNA binding dye PI (Molecular Probes, Eugene, OR). Cells were fixed in PBS/1% formaldehyde for 30 min, permeabilized in 0.1% Triton X-100 (Sigma) for 30 min at room temperature, washed and resuspended in PBS/1 mg/ml RNase A (Sigma) and 10 μg/ml PI for 30 min at 37°C prior to FACS analysis. S phase fractions were analyzed by BrdU (Sigma) incorporation in asyncronous populations. BrdU-pulsed cells were fixed in 70% ethanol at –20°C overnight and denatured by 2 N HCl, 0.5% Triton X-100 for 30 min at room temperature, followed by neutralization with borate buffer pH 8.5. Treated cells were subjected to dual color staining with a monoclonal anti-BrdU antibody (Becton Dickinson) followed by goat anti-mouse FITC and 10 μg/ml PI. Analyses were carried out using the FACScalibur flow cytometer, Cell Quest and Modefit software (Becton Dickinson).

PKH26 and NBT staining

Total transduced or sorted GFP(+) cells were stained with PKH26 (Sigma ImmunoChemicals) between days 4 and 7 of liquid culture following the manufacturer's instructions and as previously described (Ladd et al., 1997). Briefly, cells were labeled in 2 μM PKH26 in diluent for 10 min at room temperature. After FBS blocking and extensive washing, cells were resuspended and cultured in IMDM10 supplemented with cytokines at a density of 2.5×10^5/ml. Samples collected immediately after labeling and at different time points of liquid culture were then stained with anti-CD34–APC or anti-CD34–PerCP and anti-CD38–APC. Labeled cells were fixed in 1% paraformaldehyde and analyzed by three- or four-color FACS analysis of the PKH26 red fluorescence intensity on GFP(+)CD34(+) and GFP(+)CD34(+)CD38(–) subsets. For NBT staining, transduced U937 cells were aliquoted into 24-well plates at 2×10^5 cells/well, and treated with 1 mg/ml NBT and 3 nM TPA in complete medium for 60 min at 37°C. Transduced cord blood cells were stained with anti-CD34–PE after 7 days in culture, and GFP(+)CD34(–) cells were sorted and plated in growth factor-supplemented medium for an additional 2 days prior to staining. Percent NBT positivity was scored by light microscopy, and quantitated by counting at least three representative fields of 200 or more cells.

Determination of endogenous WT1 expression

For single-cell expression studies, FACS-isolated cell populations from human bone marrow [CD34(+)CD33(–), CD34(+)CD33(+)] or peripheral blood [CD11b(+), CD19(+), CD3(+)] were plated by micromanipulator selection into 96-well plates. G_0 or 'cytokine non-responsive' stem cells were obtained by culturing purified CD34(+) cells in IMDM10 supplemented with 200 mg/ml 5-FU (Pharmacia Inc., Kalamazoo, MI), KL (100 ng/ml) and IL-3 (100 ng/ml) for 7 days, then sorting by FACS based on Annexin V (Caltag Laboratories, San Francisco, CA) and PI or 7-amino actinomycin D (7-AAD) (Calbiochem, La Jolla, CA) staining. RT–PCR and Southern blotting were performed as described previously (Cheng et al., 1996). Briefly, single cells were lysed, subjected to reverse transcription with murine Moloney reverse transcriptase (Gibco-BRL) and avian reverse transcriptase (Boehringer Mannheim), and then poly(A)-tailed with terminal transferase (Boehringer Mannheim). PCR was performed using a $(dT)_{24}$ primer and AmpliTaq polymerase (Perkin-Elmer). Cell- and RT-free samples were used as negative controls. Samples were amplified for a total of 50 cycles (42°C annealing temperature). The PCR product was electrophoresed through 1.2% agarose, transferred to a nylon membrane with vacuum blotter (Pharmacia), immobilized by UV (Stratagene) and hybridized to the indicated ^{32}P-radiolabeled cDNA probes in Express Hybridization Solution (Clontech). Washed blots were analyzed by autoradiography and Molecular Dynamics Computing Densitometer and Image Quant Software v3.22. At least three separate blots were hybridized for each cell population analyzed. For direct RT–PCR analysis, total RNA was prepared and reverse transcribed from 3×10^4 FACS-isolated cord blood cells, and 10% of the cDNA was subjected to PCR analysis using a nested strategy. A portion of WT1 exons 1–4 was amplified using forward 5′-CCTACCTGCCCAGCTGCCTC-3′ and reverse 5′-CTCCTAAGT-TCATCTGATTCC-3′ primers for 20 cycles (56°C annealing temperature), followed by nested forward 5′-AGAGCCAGCCCGCTATTCG-3′ and reverse 5′-GGTCATGCATTCAAGCTGG-3′ primers for 30 cycles (58°C annealing temperature). For β-actin quantitation, identical samples were amplified for 35 cycles with actin-specific forward and reverse primers.

Statistical analysis

Equality of distributions for matched pairs of observations was tested using the Wilcoxon matched-pairs signed ranks test and the two-tailed t-test. The analyses were performed using the software STATA (r) (Stata Corporation, College Station, TX).

Acknowledgements

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