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PO Box 117
221 00 Lund
+46 46-222 00 00

Leukemia associated mutant Wilms' tumour gene 1 protein promotes expansion of human hematopoietic progenitor cells

Karina Vidovic, PhD ¹, Tove Ullmark, MD ¹, Bodil Rosberg, BSc ¹, Andreas Lennartsson, PhD ², Tor Olofsson, MD, PhD ¹, Björn Nilsson, MD, PhD ¹ and Urban Gullberg, MD, PhD¹

¹Division of Hematology and Transfusion Medicine, Lund University, Lund, Sweden;

²Department of Biosciences and Medical Nutrition, Karolinska Institute, Huddinge, Sweden.

Corresponding author:

Karina Vidovic, PhD

Division of Hematology and Transfusion Medicine

BMC, B13

S-221 84 Lund

SWEDEN

Telephone: +46-46-2220730

e-mail: karina.vidovic@med.lu.se

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Abstract

The transcription factor Wilms' tumour gene 1 (WT1) is highly expressed in the majority of leukemias, suggesting a role in leukemogenesis. Acquired WT1 mutations are reported as an independent predictor of poor clinical outcome, and mutations resulting in deletion of the entire DNA-binding zinc-finger domain (WT1delZ), is the most common type. The aim of this study was to study cellular effects of WT1(delZ) that may contribute to an oncogenic phenotype. We found that expression of WT1(delZ) supported proliferation of human hematopoietic CD34⁺ progenitor cells. Moreover, WT1(delZ) transduced cells expressed erythroid markers, including raised levels of STAT5, independently of addition of erythropoietin. At the global gene expression level, WT1(delZ) caused upregulation of genes related to cell division and genes associated with erythroid maturation, in the absence of added erythropoietin. Our results indicate that WT1(delZ) promotes cell proliferation and expansion of progenitor cells, consistent with a possible role in leukemogenesis.

Keywords: leukemia, proliferation WT1-mutant, progenitor cells, STAT5

Introduction

The WT1 gene encodes a transcription factor with four DNA-binding carboxyl terminal zinc-fingers, whereas the amino terminal part contains domains that mediate self-association, RNA recognition, and transcriptional regulation [1]. In adult human hematopoiesis, WT1 is expressed in a small subset of progenitor cells, but is undetectable in mature blood cells, consistent with a role for WT1 in hematopoiesis [2-4]. In mice, WT1 was reported as not absolutely required for murine hematopoiesis [5], but in competitive reconstitution assays, embryonic stem cells without WT1 failed to contribute to the hematopoietic system, indicating an advantage for WT1-expressing cells, as compared to WT1-null cells [6]. Recently, the role for WT1 in adult murine hematopoiesis was investigated by inducible deletion of the WT1 gene *in vivo*, leading to rapid development of aberrant hematopoiesis with complete failure of erythrocyte formation, due to intrinsic defects of erythroid progenitors [7].

Although initially characterised as a tumour suppressor in Wilms' tumour, aberrantly high expression of WT1 is detected in several forms of cancer, including breast cancer and some cases of Wilms' tumour itself [1,8], paradoxically suggesting an oncogenic role for WT1. WT1 is also highly expressed in the majority of lymphoid and myeloid leukemias [9], although the mechanism underlying this effect remains unexplained.

Acquired somatic WT1 mutations in leukemia were first reported more than 15 years ago [10-12]. In recent years, several larger studies have confirmed these early data. Thus, WT1 mutations are present in approximately 10-15% of cytogenetically normal (CN) adult AML cases at diagnosis, and in most studies the presence of WT1 mutations was reported as an independent predictor for poor clinical outcome [13-18], and reviewed in [19,20]. The overrepresentation of WT1 mutations in the subgroup of CN AMLs may suggest a driver role for the mutations in leukemogenesis. Similar types of WT1 mutations have also been found in pediatric AML [21-23].

The WT1 mutations are heterozygous frameshift mutations, or substitutions, predominantly in exon 7 and 9, respectively, predicted to encode WT1 proteins with impaired DNA-binding ability. Frameshift mutations in exon 7 are the most common class, encoding truncated

proteins lacking the DNA-binding zinc-finger domain. We and others have previously shown that WT1 with deleted zinc-fingers, WT1(delZ), may have effects on the cellular phenotype in spite of no direct regulation of target genes, due to loss of DNA-binding capacity [24,25].

Herein, we extend our previous investigations by the use of prolonged suspension cultures and replating clonogenic assays, with and without exogenous delivery of erythropoietin. We show that expression of WT1(delZ) in primary CD34⁺ hematopoietic progenitors results in enhanced proliferation, both in the presence and absence of added erythropoietin. Moreover, we demonstrate that WT1(delZ), in the absence of added erythropoietin, entails expression of erythroid and proliferative markers, including activation of STAT5.

Materials and Methods

CD34⁺ progenitor cells and retroviral transduction

Human CD34⁺ progenitor cells were extracted from umbilical cord blood as previously described [26]

The MSCV-based retroviral vector MIG contains an internal ribosomal entry site (IRES) and enhanced green fluorescence protein (eGFP). MIG-WT1(+/-) and MIG-WT1(delZ) contain the human cDNA for WT1(+17AA/-KTS) and WT1(delZ) (+17AA isoform with deleted zinc fingers, thus encoding the N-terminal part, amino acids 1-326) as described previously [24]. Empty MIG vector was used as control. The transduction efficiencies were 40% (control), 20% (WT1) and 38% (WT1(delZ)) respectively, (mean values).

Suspension cultures

The FACS sorted cells were seeded at 400,000 cells/ml in StemSpanSFEM with added StemSpan CC100 (containing Flt3-ligand, Stem Cell Factor, IL-3, and IL-6), 100 ng/ml thrombopoietin (hTPO) and 20% fetal bovine serum, all obtained from Stemcell Technologies. Cells were split into two cultures with and without 3U/ml erythropoietin (Eprex, Janssen-Cilag, Sollentuna, Sweden) as additive. The cell cultures were carefully monitored and fresh complete medium with or without erythropoietin was added when needed to redilute the cells to a concentration of 400,000 cells/ml.

Human colony-forming cell assay

GFP⁺ sorted cells were plated to 35 mm dishes (500 cells/ml), and cultured in triplicates in methylcellulose MethoCult® H4434 Classic (containing 3U erythropoietin/ml) or MethoCult® GF+ H4535 (without erythropoietin) (Stemcell Technologies, Vancouver, Canada). Erythroid colonies, identified as hemoglobinised cells with growth characteristics of BFU-E or CFU-E, were determined as CFU-Ery. CFU-Myelo includes CFU-GM, CFU-M and CFU-G. Following scoring after 10 days, cells were resuspended in IMDM, after which total cell number and cell viability were determined by counting in Bürker chambers with trypan blue exclusion. All cells in the methylcellulose dishes were replated in new methylcellulose mixture with the same composition as previously, for determination of replating colony-forming capacity.

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) analysis was performed using a StepOnePlus™ Real-Time PCR (Applied Biosystems, Foster City, CA, USA) and standard protocols. Probes for erythropoietin receptor (EpoR) (Hs00181092_m1), Epo (Hs01071097_m1), STAT5A (Hs00559643_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) were all purchased as TaqMan® Gene Expression Assays (Applied Biosystems). Relative quantitation was calculated based on the $\Delta\Delta C_T$ method [27] using GAPDH as calibrator. Parallelism of standard curves of the test and control samples was confirmed.

Enzyme-linked immunosorbent assay (ELISA)

Cells grown in suspension cultures for 14 days were lysed after which the levels of adult hemoglobin (HbA) and fetal hemoglobin (HbF) in cell samples were quantified by ELISA as described in [28]. Statistical analysis of the results for HbA was performed with unpaired t-test.

Western Blot

A polyclonal rabbit anti-WT1 antibody, WT1(180)(sc-846), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (6C5) (sc-32233), and anti-actin (C-2) (sc-8432) were purchased from Santa Cruz Biotechnology, CA, USA. Anti-STAT5A (#06-553), anti-STAT5B (#06-

554) and anti-phospho-STAT5A/B (Tyr694/699) (#05-495) were purchased from Upstate Biotechnology, Lake Placid, NY, USA. HRP-conjugated secondary antibodies, goat-anti-rabbit IgG (H+L) and goat-anti-mouse IgG (H+L), were purchased from BioRad, Hercules, CA, USA. For quantification of band intensities, a Molecular Imager ChemiDoc XRS+ with Image Lab Software (BioRad, Hercules, CA, USA) was used.

Illumina array and gene set enrichment analysis

RNA from four independent experiments was subjected to whole-genome gene expression analysis using the Illumina platform HumanHT-12 v4 expression BeadChip Kit (Swegene Center for Integrative Biology, Lund University). Statistical analysis of differences in gene expression between WT1(delZ) expressing cells and control cells was made using the Significance Analysis of Microarrays (SAM) [29]. Illumina data are available in the GEOarchive (<http://www.ncbi.nlm.nih.gov/geo>) GEO accession number GSE47560. A heatmap showing 100 probe-sets with the highest differential gene expression (control vs WT1 (delZ) cells) was made.

To perform gene set enrichment analysis, we used the program RenderCat [30] (based on the Zhang C goodness-of-fit test). To identify marker genes for different types of blood cells, we used the d-map compendium [31], a comprehensive microarray data set containing gene expression profiles of various types of sorted blood cells from healthy individuals (Affymetrix U133A arrays). Marker genes were identified by comparing each cell type with all other cell types using Smyth's moderated t-test [32]. To match genes across the Illumina and Affymetrix microarray platforms, we used Entrez Gene IDs provided in the manufacturers' annotation files. Gene sets used in the enrichment analysis are provided as Online Supplementary Material.

Transient transfection

293T/17 cells (ATCC #CRL-11268) were transiently transfected with STAT5A plasmid (GeneCopoeia, MD, USA) and co-transfected with increasing amounts of pcDNA3/WT1 or pcDNA3/WT1(delZ). Equivalent amount of DNA was used for all transfections with input of empty pcDNA3-vector as equivalent weight, when needed. After 48 hours the cells were harvested, lysed and used for Western blot analysis. For quantification of band intensities, a Molecular Imager ChemiDoc XRS+ with Image Lab Software was used.

Statistical analysis

Analysis of the proliferation rate for the CD34⁺ cells in suspension cultures (fig 1) was performed by the use of a linear mixed model of log 10-transformed numbers with random effects for repeated measurements. This model showed a significant interaction between cell cultures (control/WT1/WT1(delZ)) and time (Fobs=2.61, ndf=16, ddf=66, p<0.0033). Post-hoc analyses using the joint normal distribution of the estimates (“single-step” in multcomp) with all three pairwise comparisons at each time point was calculated and all statistical significant differences were indicated in figure 1A and 1C. The effects on clonogenic growth after plating and replating in methylcellulose was analysed by the use of a generalized linear mixed model for the binomial response. This model showed that the log-odds differ between groups (likelihoodratio-test, p<0.001). Significant post-hoc pairwise comparisons between log-odds for control and WT1 and control and WT1(delZ) are indicated in figure 2A-D. The effects on the amount of hemoglobin A was analysed with a quasipoisson-model with log-link and log(total protein) as offset. This showed differences between groups in the amount of HbA/amount of total protein (Fobs=14.3, ndf=2, ddf=5, p=0.0085). Statistical significant differences obtained by post-hoc comparisons are indicated in figure 4A and 4B. Mixed model analyses were done using R ver 3.0.0 [33] with lme4(0.999999-0) [34]. Post-hoc analyses were done using multcomp(multcomp_1.2-17) [35].

Results

Expression of WT1(delZ) results in increased proliferation in liquid culture

Previous data indicate that WT1(delZ) has distinct effects on myeloid and erythroid growth upon expression in progenitor cells, compared to wild-type WT1 [24]. To further investigate the effects of WT1(delZ) on expansion and proliferation, transduced cells were cultured in suspension supported by early- and late-acting cytokines (Materials and Methods) to achieve a rapid and strong proliferative response of the progenitors. As a comparison to WT1(delZ) we chose full length -KTS WT1 isoform (WT1+/-) since the -KTS isoform is reported to show the most prominent DNA-binding ability. To evaluate the dependence on exogenous erythropoietin for proliferation, cultures were grown with or without added erythropoietin.

With no addition of erythropoietin, control cells showed a 14-fold and 900-fold expansion after 7 and 21 days, respectively (Figure 1A). The proliferation of WT1-transduced cells showed a reduced expansion rate, as compared to control cells. Cells expressing WT1(delZ), however, showed an enhanced proliferation during the latter part of the culture period: on day 21, WT1(delZ) cells showed a 4,600 fold expansion (as compared to 900-fold for control cells). The absence of early effects of WT1(delZ) (day 1-7) is consistent with previous data [24]. The viability of control cells and WT1(delZ) cells was always higher than 90% and no difference was observed in this regard, excluding cell death as the explanation for the observed differences in proliferation (Figure 1B). Cells expressing WT1, however, showed a weak but consistent decrease in viability on day 3 post-sorting (Figure 1B), possibly contributing to the observed decrease in expansion. Addition of erythropoietin to the culture medium increased proliferation of all three cell populations, but again, WT1(delZ)-transduced cells showed a higher expansion, relative to control cells: on day 21 WT1(delZ) cells showed a 33,000 fold expansion, as compared to a 7,500-fold expansion for control cells (Figure 1C). Similar to cultures without erythropoietin, expression of WT1 rather resulted in reduced expansion with initial negative effects on viability (Figure 1D). Otherwise, cultures with added erythropoietin showed equally high viability for the first 19 days of culture, after which viability of all cultures declined somewhat, probably depending on the large expansion of cells. We conclude that expression of WT1(delZ) enhances the proliferation of progenitor cells in suspension cultures both in the presence and absence of exogenous erythropoietin.

Expression of WT1(delZ) increases clonogenic growth after replating

To analyse the effect of wild-type WT1 and WT1(delZ) on extended clonogenic growth of progenitor cells and on the dependence of erythropoietin, transduced cells were grown in methylcellulose optimized for the detection and quantification of human hematopoietic progenitor cells (Materials and Methods), with or without addition of erythropoietin. Myeloid colonies (CFU-M) and erythroid colonies (CFU-E) were scored after 10 days. As shown in Figure 2A, few erythroid colonies were formed in the absence of erythropoietin. Further, the number of both myeloid and erythroid colonies were reduced as a result of expression of WT1, and to a lesser extent also of WT1(delZ). Consistently, resuspension of cells from the entire cultures showed a reduced total number of cells in the cultures with WT1-expressing cells (Supplementary data Figure S1A). Addition of erythropoietin increased, as expected, the number of erythroid colonies, and again WT1 strongly repressed clonogenic growth, in

particular formation of erythroid colonies (Figure 2B), as well as the total number of cells in the culture (Figure S1B). The inhibitory effect mediated by WT1(delZ) on colony formation was weak and formation of erythroid colonies was almost equal to that of control cells (containing empty vector), consistent with previous results, showing that inhibition of erythroid colony formation is dependent on zinc fingers [24].

To investigate long-term effects we chose to replate the cells. Following resuspension, cells were replated in methylcellulose and cultured for an additional 10 days to analyse clonogenic growth after an extended period in culture. Replated control cells without erythropoietin showed no erythroid and only few myeloid colonies, while WT1 transduced cells produced almost no clonogenic growth (Figure 2C). Interestingly, expression of WT1(delZ) resulted in a noteworthy number of erythroid colonies in spite of the lack of added erythropoietin (Figure 2C). The total number of cells was low with WT1, while cells transduced with WT1(delZ) proliferated to the same extent as the control cells (Figure S1C). These results suggest that expression of WT1(delZ) prolongs the duration of erythroid clonogenic growth, even in the absence of added erythropoietin. With erythropoietin added, replating resulted in a majority of erythroid colonies with almost the same scores generated from WT1(delZ) transduced cells, as from control cells (Figure 2D). Total proliferation was repressed by WT1, while WT1(delZ) transduced and control cells showed comparable proliferation, determined as counted total cell number (Figure S1D).

To gain further support for the notion of a prolonged clonogenic growth induced by WT1(delZ), cells were once again resuspended and replated into methylcellulose. From control cells, few colonies were formed after this second replating, but interestingly the WT1(delZ) transduced cells once again produced more erythroid colonies, in the absence (Figure 2E), as well as in the presence (Figure 2F), of exogenous erythropoietin. Total cell proliferation was repressed by WT1, but was comparable between control cells and WT1(delZ) cells, both with and without erythropoietin (Figure S1E, S1F).

Taken together, these results indicate that expression of WT1(delZ) confers an extended period of erythroid clonogenic growth, by a mechanism that is not absolutely dependent on the addition of erythropoietin.

Increased levels of STAT5 in WT1(delZ) transduced cells

The above results indicate that WT1(delZ) stimulates erythroid clonogenic growth by mechanisms that are independent of erythropoietin, suggesting that signalling pathways downstream of the erythropoietin receptor (EpoR) are affected. Signal transducers and activators of transcription proteins (STATs) mediate cytokine signalling into the nucleus. STAT5 is a critical mediator downstream of EpoR-signalling in erythropoiesis [36,37]. Furthermore, STAT5 is involved in proliferative signalling in several types of cancer [38]. We therefore chose to determine the levels of STAT5A and STAT5B protein in transduced cells. As shown in Figure 3 addition of erythropoietin resulted in strong upregulation of STAT5A and STAT5B protein. Also the level of phosphorylated STAT5A/B was increased in erythropoietin treated cells, indicating high levels of transcriptionally active STAT5A/B. Most interestingly, however, in cells transduced with WT1(delZ), the levels of STAT5A, STAT5B and phosphorylated STAT5A/B were high also in the absence of erythropoietin, indeed comparable to levels in erythropoietin-treated cells (Figure 3). Thus, the presence of WT1(delZ) increases the amount of STAT5 in the cells, giving a possible mechanistic explanation for positive effects on erythroid clonogenic growth. WT1 expression did also slightly raise STAT5 protein, although to a lesser extent than WT1(delZ) did (Figure 3). When the mRNA of STAT5A were analysed in transduced cells, however, mRNA levels did not show any clear correlation to the amount of STAT5 protein (Online Supplementary Figure S2) indicating, at least partially, regulation of STAT5 to be on a posttranscriptional level. To seek further support for a WT1(delZ)-induced increase of STAT5 protein, we transiently overexpressed WT1, WT1(delZ) and STAT5 in 293T/17 cells, after which protein levels were determined by Western blotting. Increasing amounts of WT1(delZ) protein resulted in elevated STAT5 protein levels (Online Supplementary Figure S3), consistent with results seen in cultured progenitor cells (Figure 3). Although to a weaker extent, WT1 also increased STAT5 protein, again consistent with data from progenitor cells.

We conclude that in the absence of erythropoietin, the presence of WT1(delZ) confers an increase of STAT5 protein, probably involving posttranscriptional mechanisms, the identity of which remain to be identified.

WT1(delZ) enhances levels of the erythroid marker HbA via an erythropoietin-independent mechanism.

Results shown above from experiments with replating of cells in methylcellulose cultures indicate that WT1(delZ) increases erythroid colony formation, also in the absence of added

erythropoietin (Figure 2). To gain additional support for a WT1(delZ)-induced erythroid phenotype, we analysed the amount of fetal and adult hemoglobin (HbF and HbA) in cell cultures by ELISA (Material and Methods). After 14 days of culture in the absence or presence of exogenous erythropoietin, the amount of HbF was very low or below detection limit (data not shown). When cells cultured in the absence of added erythropoietin were analysed for HbA, the amount of HbA was highest in WT1(delZ)-expressing cells, giving further support for a positive effect of WT1(delZ) on erythroid maturation, independent of added erythropoietin (Figure 4A). As expected, addition of erythropoietin to cultures resulted in strongly increased HbA levels (11% of total protein in control cells, as compared to 0.4% in control cells without erythropoietin) (Figure 4B). With added erythropoietin, WT1-transduced cells showed the highest HbA levels, an effect that was not detected in WT1-transduced cells in the absence of erythropoietin. The mechanism by which WT1 stimulates HbA levels in the presence of erythropoietin could hypothetically involve the previously reported WT1-induced transcriptional upregulation of the erythropoietin receptor [39]. To investigate possible effects on expression of the EpoR, we analysed its mRNA levels after 7 days of culture. Regardless of added erythropoietin, WT1, but not WT1(delZ), slightly enhanced the expression of EpoR (Supplementary data Figure S4), consistent with the previous report [39], thus giving a possible explanation for WT1-induced HbA increase in the presence of erythropoietin. Importantly, expression of EpoR was neither in the presence, nor in the absence of erythropoietin, higher in WT1(delZ)-transduced cells, as compared to control cells (Supplementary data Figure S4), lending further support to the notion that the mechanism by which WT1(delZ) affects the cells is independent of EpoR signaling. Erythropoietin mRNA levels, as determined by RT-qPCR, was close to, or below, the detection limit in both control, WT1 and WT1(delZ) expressing cells, either in the absence or presence of added erythropoietin (data not shown). These results indicate that WT1(delZ) enhances the levels of the erythroid marker HbA via an erythropoietin-independent mechanism.

Effects on gene expression profiles, marker genes associated with proliferation and erythropoiesis upregulated

To further characterise the phenotypic effects of WT1(delZ), we performed gene expression profiling of WT1(delZ)-transduced cells and control cells after 14 days in suspension culture without erythropoietin. Comparing the expression profiles of WT1(delZ)-containing cells

with controls, we obtained a gene list with a striking over-representation of genes that encode proteins that are known to be expressed on erythrocytes, or to be associated with erythroid development, including A4GALT, HMBS, XK, CA1, CPOX, RHAG, SPTA1, GYPA, NFE2, and also of genes associated with proliferation such as MYC, CCNG1, CCNE1, CCNC, MCM4, and SKP2 (complete gene list Supplementary data). This observation confirms findings above and indicates that differentiation is skewed in favour of the erythroid direction combined with maintained proliferation. To test whether the enrichment of erythroid markers was statistically significant, and to identify additional differentiation effects, we tested for enrichments of sets of marker genes for all the major blood cell types in the WT1(delZ)-vs-control signature (as described in Material and Methods). As shown in Figure 5, this analysis revealed a strong enrichment not only for erythroid markers but also for markers of megakaryocyte-erythrocyte progenitors (MEPs), confirming the erythroid phenotype and consistent with extended erythroid clonogenic growth. The gene list (Supplementary data) also indicated down regulation of genes related to myeloid differentiation, including DEFA4, CD14, LYZ, FCGRT, CEBPD, CTSG, BPI, CEBPE, DEFA3, AZU1, and PRTN3, in WT1(delZ) transduced cells as compared to control cells. Consistent with this observation, statistically significant enrichment of granulocyte and monocyte marker genes among genes down-regulated in WT1(delZ) expressing cells were found (Figure 5). (Complete analysis of 16 marker gene sets in Supplementary Data). Together, these data on gene expression in the WT1(delZ) expressing cells further support the notion of cell proliferation and erythroid development at the expense of the formation of monocytes and granulocytes. Figure 6 shows a heatmap of the 100 probe-sets with the highest differential gene expression (control vs WT1(delZ) cells).

Discussion

Heterozygous mutations of WT1 are present at diagnosis in approximately 10-15% of cytogenetically normal AML and are reported as predictors of a poor clinical outcome [13-18]. and reviewed in [19,20]. The most common leukemia-associated mutations of WT1 encode a truncated protein devoid of DNA-binding capacity. Mutations therefore might result in loss of WT1 function, due to haplo-insufficiency or to dominant negative effects, implying that wild-type WT1 acts as a tumour suppressor in leukemia. However, the frequent overexpression of wild-type WT1 in leukemias [9] and results from animal models [6,40] demonstrate an oncogenic role for wild-type WT1. These observations contradict WT1 being a tumour

suppressor in leukemia. Therefore, current notions strongly argue against a tumour suppressor role, and therefore also against dominant negative effects or haplo-insufficiency as mechanisms by which mutant WT1 proteins exert oncogenic effects. It has previously been shown that endogenous WT1 is rapidly down modulated in hematopoietic progenitors cultured *in vitro* [2-4]. The present experimental model also shows undetectable amounts of endogenous WT1 protein (Figure 3), lending support to the conclusion that the observed effects of WT1(delZ) are independent of endogenous WT1 protein.

Previously, we have reported that expression of WT1(delZ) does not affect erythroid clonogenic growth, while inhibiting myeloid clonogenicity. Our current results from replating methylcellulose cultures indicate that forced expression of WT1(delZ) extends the replating efficiency of erythroid progenitor cells: the number of erythroid colonies were increased after the second and third plating in the absence of added erythropoietin, and after the third plating in presence of erythropoietin. This may suggest that WT1(delZ) preserves the presence of progenitors with erythroid characteristics *in vitro*. Our finding that expression of WT1(delZ) results in a strong enrichment not only of erythroid markers but also markers of megakaryocyte-erythrocyte progenitors (MEPs), are consistent with prolonged preservation of erythroid progenitors. Moreover, our finding that enhancement of clonogenicity was pronounced also in the absence of added erythropoietin, suggests that WT1(delZ) to some extent substitutes for EpoR signalling. However, since neither erythropoietin, nor erythropoietin receptor expression was affected by WT1(delZ), the effects of WT1(delZ) appear to be exerted downstream of the erythropoietin receptor, thus to some degree bypassing erythropoietin signalling.

The increased proliferation induced by WT1(delZ) was not only associated with increased replating capacity for erythroid progenitors, but also with increased levels of the erythroid phenotypic marker HbA. Similarly, our previous results show that when driven into erythroid differentiation by uni-lineage cytokines, expression of WT1(delZ) slightly enhanced expression of the erythroid marker GPA [24]. In the present work, our analysis of the transcript profile in WT1(delZ)-expressing cells demonstrates a highly significant increase in the expression of erythroid- and MEP-associated genes. These results may suggest that WT1(delZ) induce differentiation into the erythroid lineage. However, the observation of an enhanced proliferation indicates that cells were not committed into a complete terminal erythroid differentiation program. Consistently, genes involved in cell division were found to be differentially expressed in WT1(delZ) containing cells, as compared to control. Notably, c-

myc (MYC) was among the 100 most differentially expressed genes, consistent with a proliferative phenotype. These findings are consistent with the increased proliferation of WT1(delZ) expressing cells observed in suspension culture (Figure 1). Taken together, our data demonstrate that WT1(delZ) confers an enhanced proliferative phase, also in the absence of erythropoietin combined with perturbation of differentiation with skewing towards an erythroid phenotype.

How is the effect of WT1(delZ) mechanistically mediated? As mentioned, WT1(delZ) lacks DNA-binding zinc-fingers, predicting that direct transcriptional control is excluded. In accordance with this, we show that mRNA expression of erythropoietin receptor, reported as a direct target gene of WT1, was unaffected by WT(delZ) transduction. Nevertheless, enhanced expression of erythroid markers suggests involvement of mechanisms downstream of the erythropoietin receptor. Ligand-binding to the erythropoietin receptor normally leads to phosphorylation and activation of STAT5, which therefore is central in the signalling from the erythropoietin receptor, as well as from certain other cytokine receptors [41]. Consistent with a positive role in proliferative signalling downstream of cytokine receptors, STAT5 is commonly overexpressed in leukemias and also in other forms of cancer [42], indicating that the pathogenetic role of STAT5 is not limited to erythroid malignancies. Importantly, an increase of STAT5 could therefore play an oncogenic role in leukemias of different phenotypes, the nature of which is determined rather by other concurrent strong oncogenes. Here, we demonstrate that WT1(delZ) affects STAT5 protein. In the absence of erythropoietin, WT1(delZ)-transduced cells showed raised levels of STAT5 and phospho-STAT5, comparable to the levels in cells cultured with erythropoietin. The mechanism by which WT1(delZ) increases STAT5 protein is not clear, but our data suggests involvement of post-transcriptional mechanisms. Our finding of a dose-dependent increase of STAT5 by WT1(delZ) in 293T/17 cells may support the notion of WT1(delZ)-induced stabilization of STAT5 protein. Interestingly, our observation that the proproliferative effect of WT1(delZ) is clearly evident after 14 days of culture, may indicate that it is dependent on cellular context. We can only speculate on that one or several proteins expressed in more mature progenitor cells, present at later time points in the culture, are cooperating with WT1(delZ). The exact mechanism by which WT1(delZ) affects STAT5, and its precise role, remains however to be determined.

In conclusion, we demonstrate that upon expression in hematopoietic progenitor cells, mutant WT1 lacking zinc-fingers confers a proliferative phenotype including erythropoietin-

independent acquisition of erythroid phenotypic markers, possibly by mechanisms involving STAT5. Our results thus demonstrate prominent cellular effects of WT1 that are independent of direct DNA-binding, and are consistent with the notion of an oncogenic role of this form of WT1 mutation in leukemia, whose incidence seems to be linked to a worse clinical outcome.

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Conflict of interest

The authors declare that they have no competing interests.

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Authors' contributions. K.V. designed and performed experiments, analysed data and wrote the paper. T.U. performed experiments, analysed data and prepared the manuscript. B.R, carried out experiments, gave technical support and conceptual advice. A.L, made substantial contribution to concept and design of microarray analysis, and edited the manuscript. T.O, designed and carried out FACS-analysis and gave conceptual advice. B.N, conducted all bioinformatics analyses, interpretation of microarray data, and were involved in drafting the manuscript. U.G, made substantial contribution to concept and design, supervised analysis and interpretation of data, drafted and revised the manuscript.

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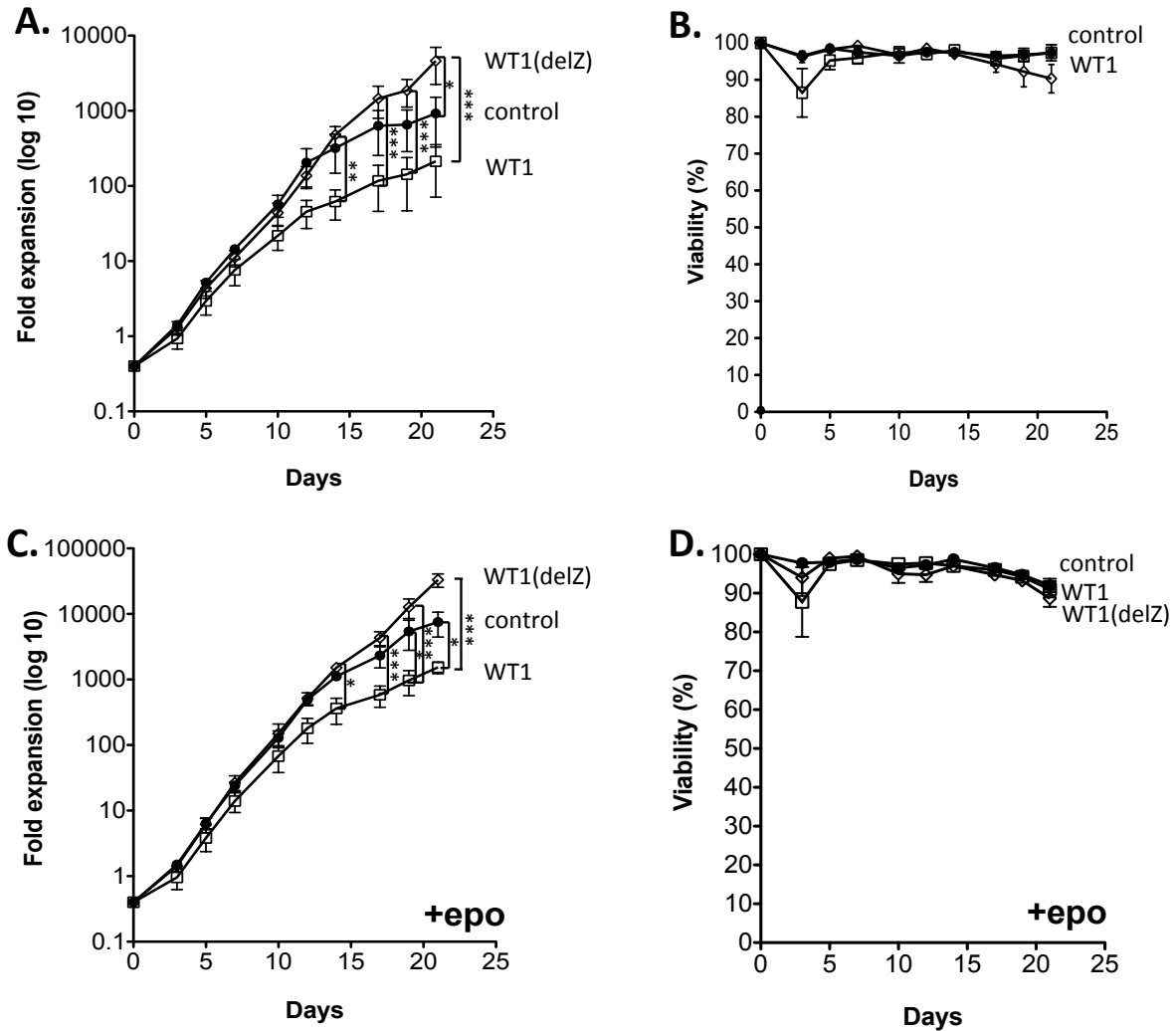


Figure 1: Effects on proliferation in suspension culture. CD34⁺ cord blood cells were transduced with control vector, with WT1 or with WT1(delZ). Sorted GFP⁺ cells were grown in suspension culture with and without added erythropoietin as described in Materials and Methods. Cultures were carefully followed from day 0 until day 21 and diluted with fresh medium whenever needed to avoid overgrowth. Total fold expansion and viability of cells were determined. (A) Fold expansion of cells without added erythropoietin; (B) Percentage of viable cells without added erythropoietin; (C) Fold expansion of cells with erythropoietin; (D) Percentage of viable cells with erythropoietin; Mean values from four independent experiments, each in triplicate are shown, bars: \pm S.E.M. Statistical significant differences, determined by post-hoc analyses using the joint normal distribution of the estimates (“single-step” in multcomp) with all three pairwise comparisons at each time point, as described in Materials and Methods, are indicated (***) $p < 0.001$, (**) $p < 0.01$, (*) $p < 0.05$).

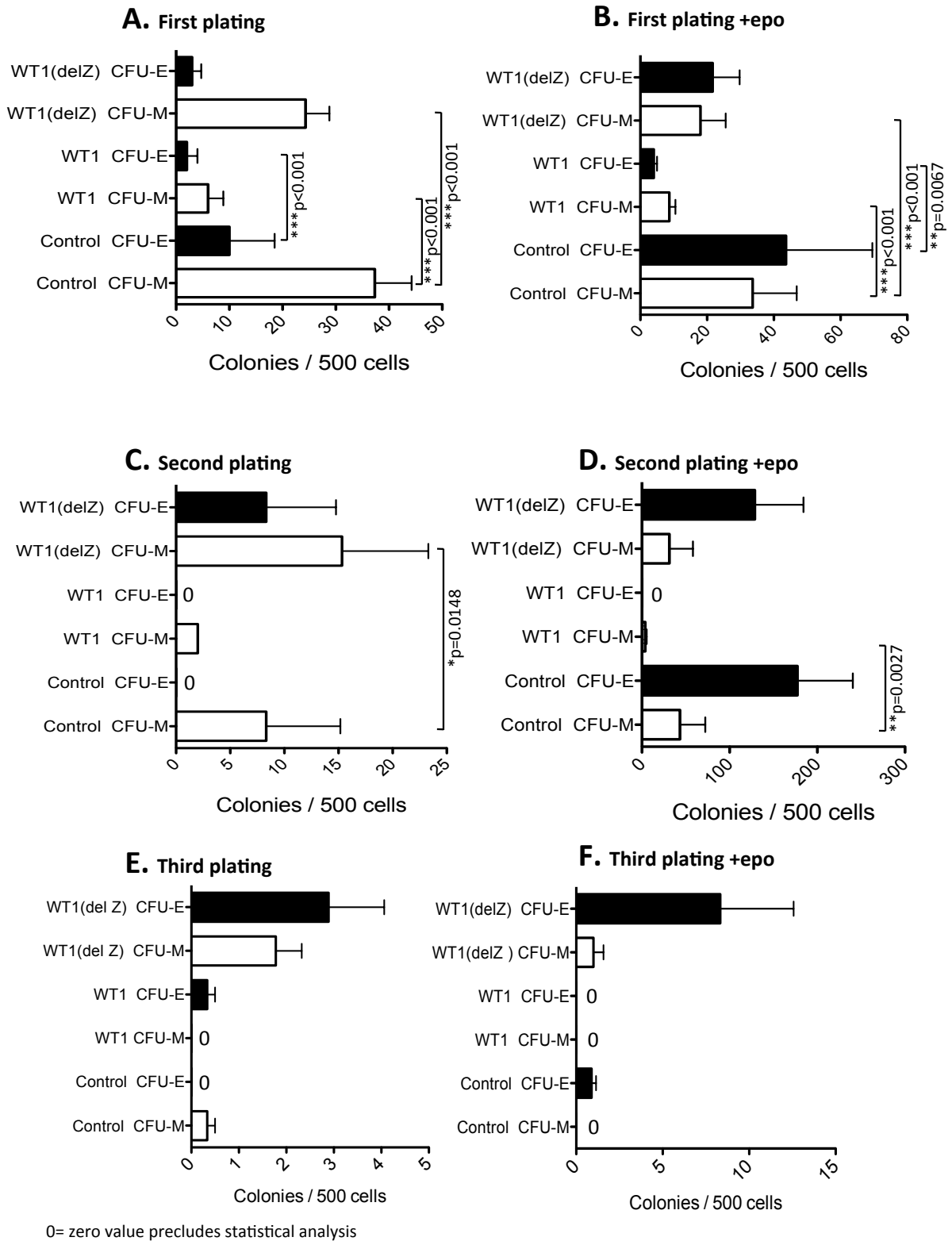


Figure 2: Effects on clonogenic growth after plating and replating. CD34⁺ cells were transduced with control vector, with WT1 or with WT1(delZ). Sorted GFP⁺ cells were plated in Methocult methylcellulose with and without erythropoietin as described in Material and

Methods. Myeloid (unfilled bars) including CFU-GM, CFU-M and CFU-G, and erythroid (filled bars), including BFU-E and CFU-E, colonies were scored after 10 days, after which cells were resuspended and replated into new methyl cellulose. After 10 days of culture colonies were again scored, and cells were once again replated into new methylcellulose. Determined number of colonies are shown. (A) First plating without erythropoietin; (B) First plating with erythropoietin; (C) Second plating without erythropoietin; (D) Second plating with erythropoietin; (E) Third plating without erythropoietin; (F) Third plating with erythropoietin. Mean values from three independent experiments, each in triplicates, are shown, bars: \pm S.E.M. Statistical significant differences between log-odds, generated by a generalized linear mixed model for the binomial response, as described in Materials and Methods, are indicated. In panel C, D, E and F: when value was zero, statistical analysis was precluded.

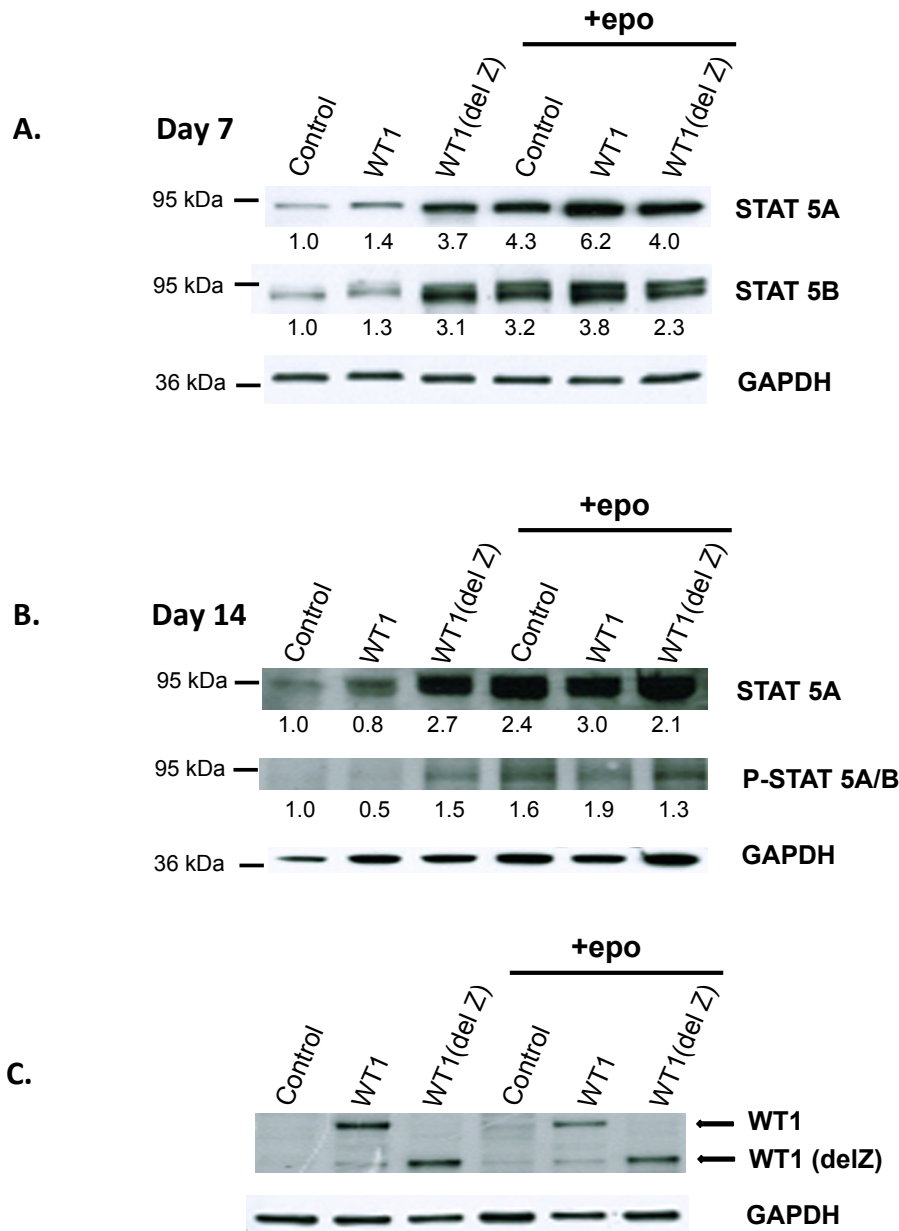


Figure 3: Effects on the expression of STAT5 protein. (a) Transduced and sorted CD34⁺ cells (control, WT1 and WT1(delZ)) were analysed for the expression of STAT5 protein after (A) 7 and (B) 14 days in culture with or without erythropoietin as indicated. Amount of STAT5A, STAT5B and phospho-STAT5A/B proteins was analysed by Western blotting. (C): Western blot of WT1 and WT1(delZ) protein is shown as control for transgene expression. The faint bands in the WT1 lanes, running at the same molecular mass as WT1(delZ) are probably the result of some degradation of WT1 in the cells. GAPDH is used as equal loading control. Quantification was performed by densitometry and expression value of STAT5 was normalized that of corresponding GAPDH. Normalized values of STAT5, relative to the value of control, set to 1, are shown.

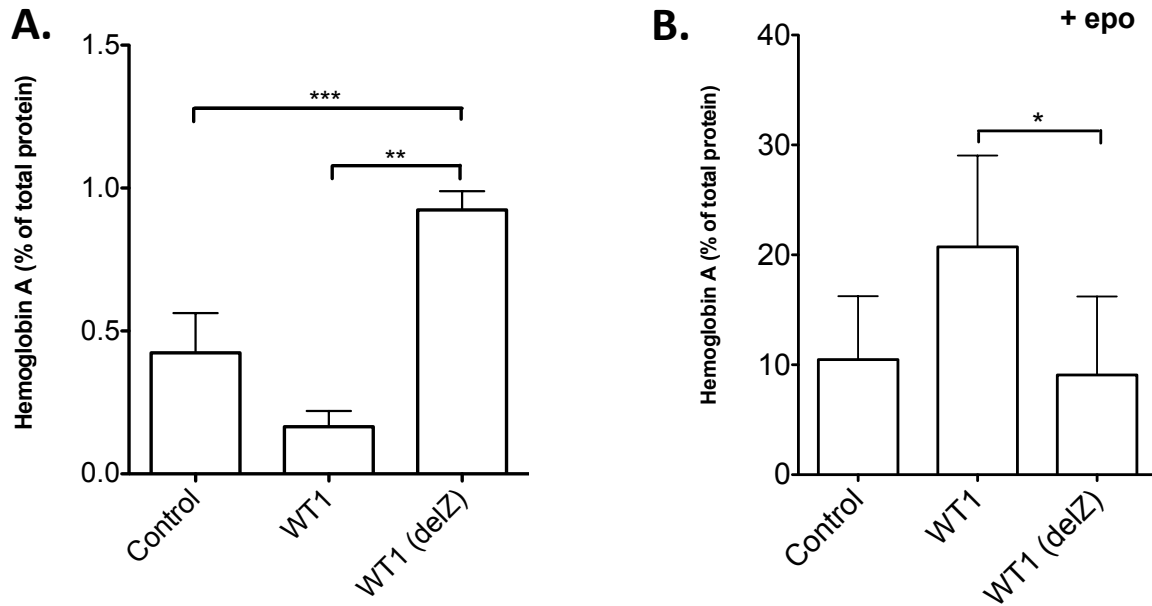


Figure 4: Effects on the amount of hemoglobin. Transduced and sorted CD34⁺ cells (control, WT1 and WT1(delZ)) were analysed for the amount of hemoglobin A (HbA) after 14 days in culture (A) without or (B) with added erythropoietin. Mean value of HbA (% of total cell protein content) is shown. Mean values from three independent experiments performed in triplicates, bars: \pm S.E.M. Statistical significant differences, determined by a quasipoisson-model with log-link and log (total protein) as offset, as described in Material and Methods, are indicated (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

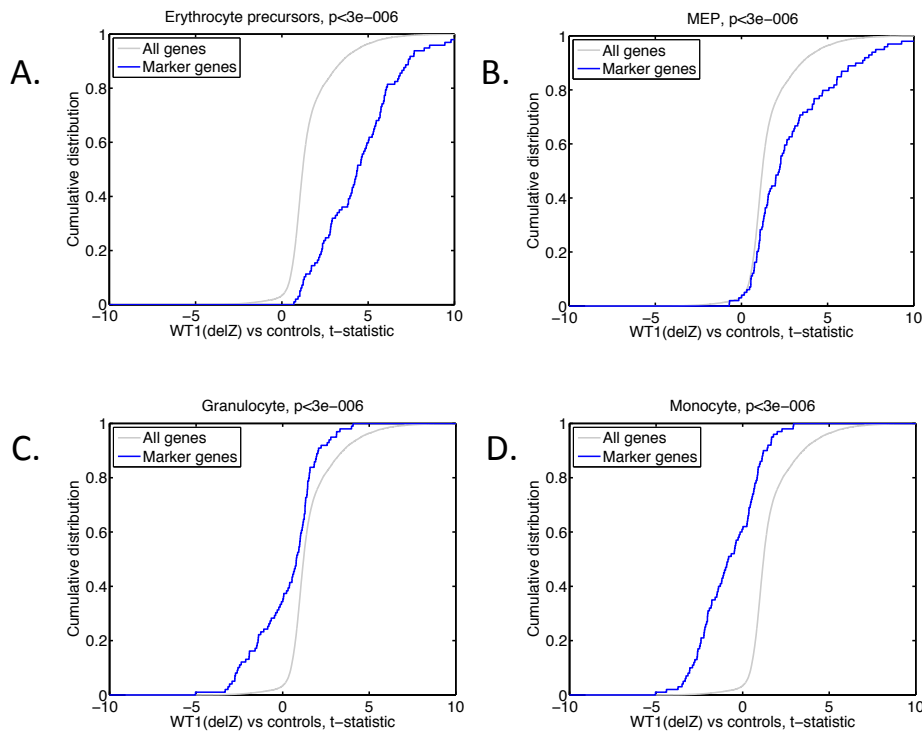


Figure 5: Effects of WT1(delZ) transduction on global gene expression and differentiation.

Transduced and sorted CD34⁺ cells (control and WT1(delZ)) were subjected to genome wide expression profiling after 14 days in culture without added erythropoietin. Distribution plots for the gene scores are computed from the collected microarray data as moderated t-statistics for WT1(delZ) transfected cells vs controls [35]. The blue curves indicate the cumulative distribution of the resulting gene scores for sets of genes that are highly expressed in specific genome. In essence, a right-shift indicates that the set of marker genes is enriched among up-regulated genes; a left-shift indicates that the set of marker genes is enriched among down-regulated genes. Enrichment of (A) erythroid marker genes and (B) megakaryocyte erythrocyte progenitors (MEP) marker genes among up-regulated genes, and (C) enrichment of granulocyte and (D) monocyte marker genes among down-regulated genes is shown. For complete analysis of 16 marker gene sets and further experimental information and details, see Supplemental Material.

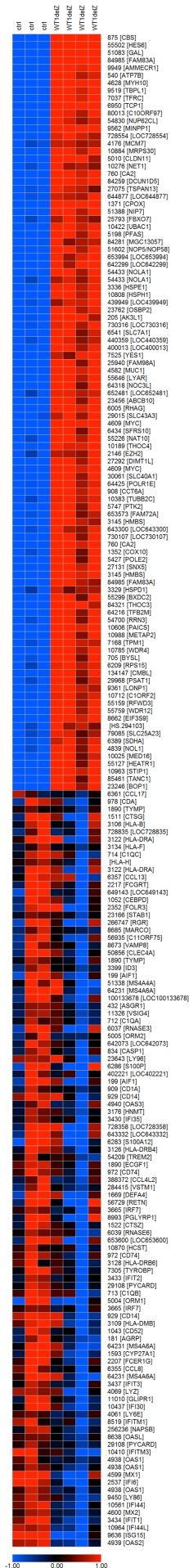


Figure 6. Highest differential gene expression. Transduced and sorted CD34⁺ cells (control and WT1(delZ)) were subjected to genome wide expression profiling after 14 days in culture without added erythropoietin. A heatmap of the 100 probe-sets with the highest differential gene expression (control vs WT1(delZ) cells) is shown.

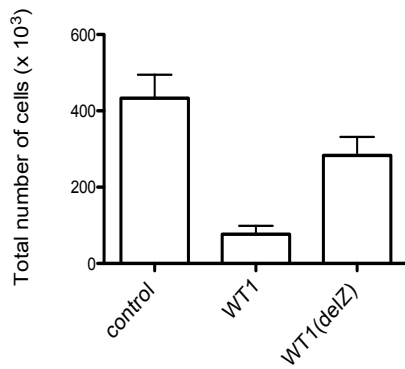
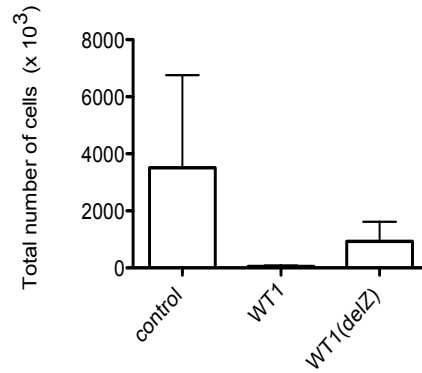
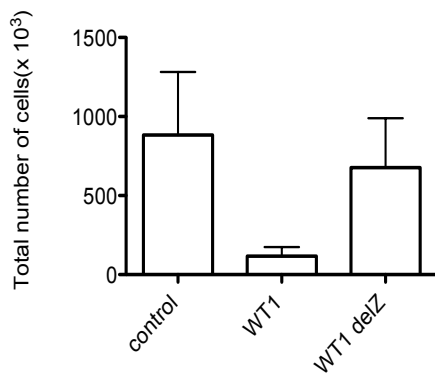
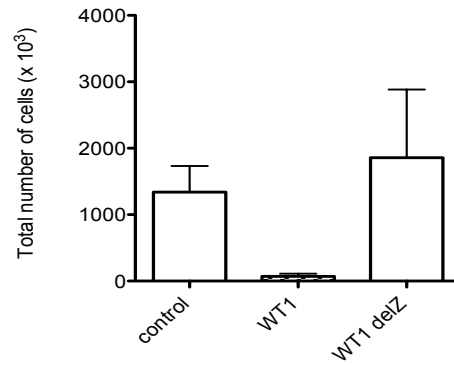
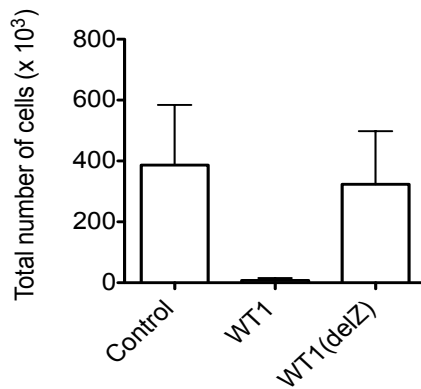
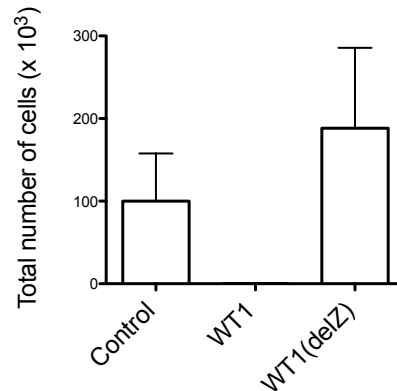
A. First plating**B. First plating +epo****C. Second plating****D. Second plating +epo****E. Third plating****F. Third plating +epo**

Figure S1. CD34⁺ cells were transduced with control vector, with WT1 or with WT1(delZ). Sorted GFP⁺ cells were plated in Methocult methylcellulose with and without erythropoietin as described in Materials and Methods. After 10 days of culture, cells were resuspended and the total number of cells in the culture was determined. (A) After first plating without erythropoietin; (B) After first plating with erythropoietin; (C) After second plating without erythropoietin; (D) After second plating with erythropoietin; (E) After third plating without erythropoietin; (F) After third plating with erythropoietin. Mean values from three independent experiments, each in triplicates, are shown, bars: \pm S.E.M.

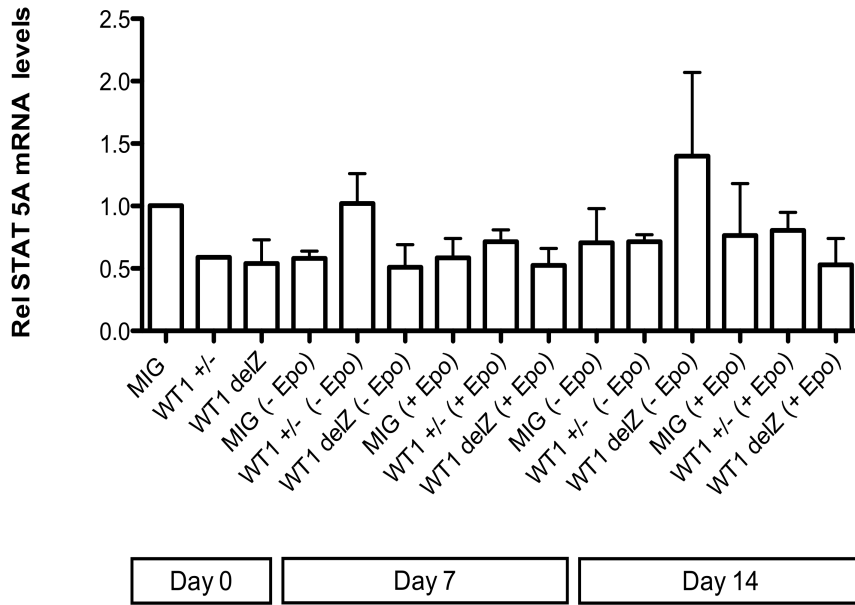


Figure S2: Effects on the expression of STAT5 mRNA. In transduced and sorted CD34+ cells the amount of STAT5A mRNA was determined by RT-qPCR as described in Material and Methods. Expression level in control cells at day 0 is set to 1. Mean values, bars \pm S.E.M.

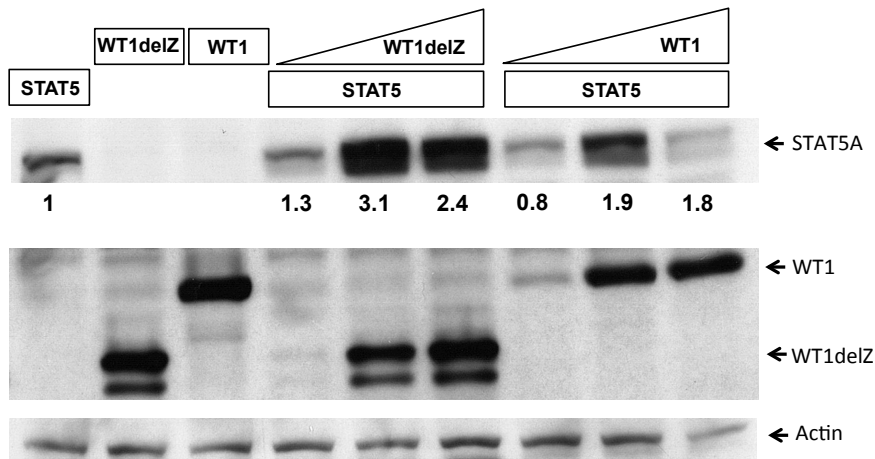


Figure S3: Cotransfection of WT1 and STAT5. 293T/17 cells were transfected with STAT5A and increasing amounts of WT1 or WT1(delZ) as indicated. Forty-eight hours post-transfection the amount of STAT5A, WT1 and WT1(delZ) was determined by Western Blot analysis. Quantification was performed by densitometry and values were normalised to those of actin. Relative amount of STAT5 is shown, with amount in control cells set to 1.

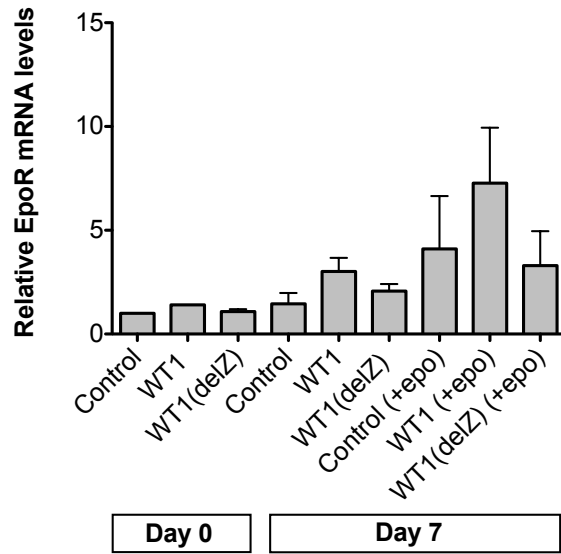


Figure S4: Effects on the expression of erythropoietin receptor. Transduced and sorted CD34⁺ cells (control, WT1 and WT1(delZ)) cells were analysed for expression of the erythropoietin receptor (EpoR) mRNA by qPCR after 7 days in culture with or without added erythropoietin, as described in Materials and Methods. Expression level in control cells at day 0 is set to 1. Mean values, bars: \pm S.E.M.