The Wilms' tumor gene 1 (WT1) and leukemia -new insights and further complexity

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Wilms' tumor gene 1 (WT1) and leukemia -new insights and further complexity

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av

Emelie Svensson

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The Wilms´ tumor gene and leukemia - new insights and further complexity

Abstract

The Wilms tumor gene 1 (WT1) encodes a zinc-finger containing transcription factor which is highly expressed in immature hematopoietic progenitor cells. A high expression of WT1 and the presence of somatic mutations in acute leukemia indicate a role for WT1 in the pathogenesis of leukemia. The ojective of this thesis was to investigate the role of WT1 during human hematopoiesis and leukemia. To gain further insights in how wild type WT1 and mutant WT1 affect proliferation and differentiation of human hematopoietic progenitor cells, CD34+ progenitor cells from cord blood were transduced with wild type WT1 or with a mutant of WT1, lacking the entire zinc-finger region, thus incapable of binding DNA. In these experiments, WT1 but not mutant WT1 inhibited erythroid colony formation as well as erythroid differentiation in suspension cultures. Suprisingly, both WT1 and mutant WT1 were able to inhibit myeloid colony formation and stimulated myeloid differentiation of cells grown i suspension culture. These results suggest that the effects of WT1 are mediated by distinct molecular mechanisms that are both DNA-binding dependent and independent. To further characterize the DNA-binding dependent functions of WT1, a gene expression analysis of WT1-transduced CD34+ progenitor cells was performed to identify genes transcriptionally regulated by wild type WT1. We found that WT1 upregulated the expression of N-myc downstream regulated gene 2 (NDRG2) and downregulated interferon regulatory factor 8 (IRF-8). Using a bacterial II hybrid assay, we also identified cofilin 1 as a novel protein partner of WT1.

Our data also indicate that BCR/ABL1 induce expression of the WT1 gene via the phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway in the leukemic cell line K562. Given our finding that WT1 downregulated the expression of IRF-8, we propose that WT1 is a link between BCR/ABL1 and IRF-8. This provide a mechanistic explanation for BCR/ABL1 induced repression of IRF-8 and a general mechanism by which high expression of WT1 can contribute to leukemogenesis.

Key words: WT1, hematopoiesis, leukemia, NDRG2, cofilin 1, BCR/ABL1, IRF-8

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Wilms' tumor gene 1 (WT1) and leukemia -new insights and further complexity

Doctoral Thesis

by

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Division of Hematology and Transfusion medicine
Lund University, Sweden

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Utan tvivel är man inte klok

Tage Danielsson
Ur "Tankar från roten", 1974

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ABBREVIATIONS

aa	amino acids
AML	Acute myeloid leukemia
ALL	Acute lymphocytic leukemia
APL	Acute promyelocytic leukemia
BCR/ABL1	Breakpoint cluster region/Abelson murine leukemia viral homolog 1
BFU	Burst forming unit
BM	Bone marrow
CFU	Colony forming unit
CML	Chronic myeloid leukemia
DDS	Denys-Drash syndrome
FS	Fraiser syndrome
eGFP	Enhanced green fluorescence protein
HSC	Hematopoietic stem cell
IRES	Internal ribosomal entry site
IRF-8	Interferon regulatory factor 8
LCS	Leukemic stem cell
kbp	kilo base pair
kDa	kilo Dalton
MDS	Myelodysplastic syndrome
MRD	Minimal residual disease
NDRG	N-myc downstream regulated gene
WT1	Wilms' tumor gene 1

INTRODUCTION

Hematopoiesis and leukemia

Hematopoiesis is the process by which blood cells are produced in the bone marrow. A small population of so-called hematopoietic stem cells (HSC) in the bone marrow has the ability to self-renew and thus maintains hematopoiesis throughout life. As a result of asymmetric cell division, stem cells can differentiate into all the hematopoietic lineages. The endpoint of the hematopoietic lineages are terminally differentiated cell types with specialized functions, including leukocytes important for the immune system, erythrocytes functioning as oxygen transporters and thrombocytes critical for blood clotting and coagulation. The decision of the stem cell between self-renewal and differentiation involves a complex interplay between transcription factors and growth factors.

In leukemia, the differentiation process of HCS is disturbed. The production of normal, functional blood cells is repressed, leading to accumulation of immature leukemic cells in the bone marrow and blood. Patients with leukemia therefore suffer from loss of erythrocytes, leukocytes and thrombocytes, resulting in anemia, infection and bleeding disorders. Acute leukemia is rapidly fatal, if the disease is left untreated.

The aim of this thesis is to increase the understanding of the role of the transcription factor Wilms' tumor gene 1 (WT1) in normal hematopoiesis and leukemia.

BACKGROUND

The role of WT1 in normal development

Originally, WT1 was identified as a gene deleted or inactivated in a subset of patients with Wilms' tumor, a pediatric kidney cancer [1]. During embryogenesis, WT1 is necessary for the development of kidneys, gonads, spleen and mesothelial tissues as judged by severe malformations in transgenic mice with deleted WT1, which die in utero [2]. These transgenic mice lack kidneys, caused by a widespread apoptosis in the renal stem cells population, suggesting a role for WT1 in survival of these cells [3]. The effects of WT1 mutations in two human syndromes underscore the important role of WT1. Patients with Denys-Drash and Frasier syndromes, harbouring heterozygous WT1 mutations, are associated with severe renal failure, male-to-female sex reversal and a predisposition to development of Wilms' tumor.

Recently, it has been shown that WT1 also plays an important role in the development of the retina and olfactory system [4,5] as well as in spermatopoiesis [6]

WT1 in hematopoiesis and leukemia

Hematopoiesis

Hematopoiesis is the process by which all blood cells in the body are produced. Each day about 1 trillion blood cells are produced in the bone marrow [7]. All these blood cells are generated from a small population of so-called hematopoietic stem cells (HSC), which constitutes less than 0.1% of the nucleated cells in the bone marrow [8]. The HSCs must be capable of both self-renewal to maintain the HSC pool, but also to differentiate into all types of blood cells. The HSCs were first identified as a cell population capable of reconstitution of hematopoiesis in lethally radiated mice. These cells can not be recognized morphologically, but through phenotypical analysis of cell surface markers they can be highly enriched [9]. The multipotent HSC generate all the mature blood cells through successive differentiation into oligolineage progenitors including the common lymphocyte progenitors (CLPs) and common myeloid progenitors (CMP) (Figure 1). The CLPs generate T lymphocytes, B lymphocytes and NK killer cells, while CMPs generate granulocytic/monocytic progenitors (GMPs) and megakaryocytic/ erythrocytic progenitors (MEPs), which differentiate into granulocytes or monocytes/ macrophages and megakaryocytes or erythrocytes, respectively (Figure 1).

To produce an adequate number of mature cells, hematopoiesis has to be strictly regulated and the molecular mechanisms behind self-renewal and lineage commitment have been intensely studied. Previous studies have shown that intrinsic transcription factors, but also external signaling pathways mediated by regulatory cytokines, regulate these processes. According to the stochastic model, lineage commitment is a random event, where the expression of one lineage-specific transcription factor suddenly increase and initiate differentiation to a particular lineage. Cytokines are in this model only important for proliferation and survival of committed cells. In the instructive model, cytokines directly upregulate specific transcription factors driving lineage determination [10]. A more recent model combines the two models by suggesting that stem cells and progenitors express low levels of all lineage-specific transcription factors, which cross-antagonize each other. Some event, stochastic or environmental, increase the level of one lineage-specific

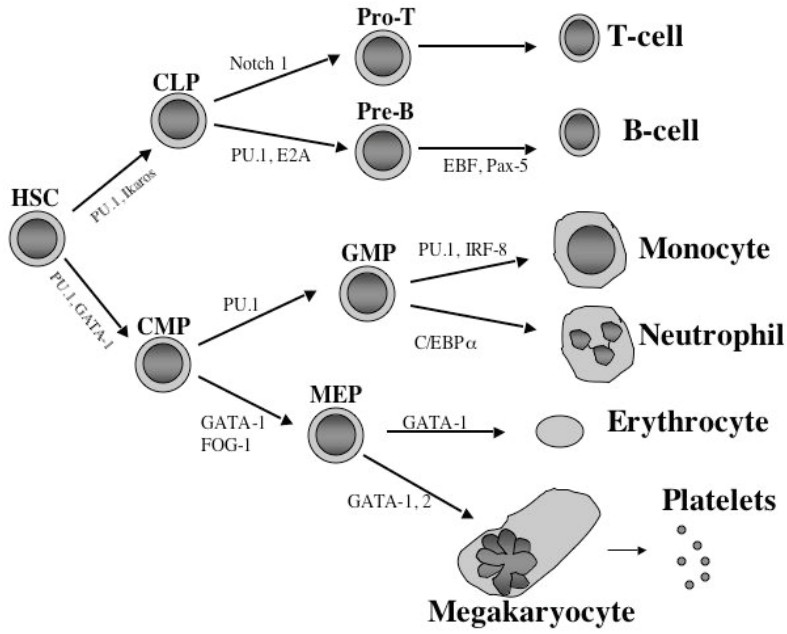

Figure 1. ***A simplistic picture of the hematopoietic stem cell tree and examples of transcription factors involved in lineage specific differentiation***. *Abbreviations used; HSC, hematopoietic stem cell; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocytic/monocytic progenitor; MEP, megakaryocytic/erythrocytic progenitor.*

transcription factor which then exceeds the other one and activates the target genes that characterize that lineage [7]. Positive auto-regulation feedback loops may further increase the levels of that specific transcription factor thereby promoting the differentiation process.

Whatever model that is correct for the initiation of differentiation, it is clear that transcription factors are indeed key regulators for lineage-specific differentiation. Specific combinations of transcription factors, rather than one master regulator promote lineage specific-differentiation. The transcription factors regulate the differentiation process via a number of mechanisms. One important mechanism is inhibition of alternative pathways through cross-antagonizing activities, exemplified by the PU.1-GATA-1 interaction (reviewed in [7]). PU.1 is absolutely required for granulocytic, monocytic and lymphocytic development, while GATA-1 is important for erythrocytic and megakaryocytic development (Figure 1). PU.1 inhibits GATA-

1 function by disrupting its ability to bind DNA. In contrast, GATA-1 inhibits PU.1 by preventing it from interacting with its essential co-activator c-Jun. Some studies have also shown that GATA-1, C/EBPα and PU.1 positively regulate their own expression via auto-regulation. Moreover, to induce terminal differentiation, the expression of additional lineage-specific target genes are activated by these transcriptional regulators. PU.1 has been shown to activate transcription of the GM-CSF, G-CSF and M-CSF growth-factor receptor genes that are expressed on myeloid cells, while GATA-1 activates the transcription of the erythropoietin receptor gene. Another transcription factor, which is interacting with PU.1 and is essential for monocytic development, is interferon regulatory factor 8 (IRF-8) [11]. IRF-8 was shown to potentiate monocyte differentiation while inhibiting granulocytic differentiation.

Thus, hematopoiesis is the process by which hematopoietic stem cells differentiate into all types of mature blood cells. This process is strictly regulated by transcription factors and regulatory cytokines to maintain hematopoiesis throughout life.

Acute leukemia

Leukemia is a malignant disease of hematopoietic tissues where acute and chronic forms exist. Acute myeloid leukemia (AML) is characterized by an increased number of immature hematopoietic cells of the myeloid origin, so called leukemic blasts, in the bone marrow and blood. These cells have a severe block in differentiation, but retain the ability of proliferation and survival. Chronic myeloid leukemia (CML) is on the other hand characterized by a massive expansion of myeloid cells with a more or less preserved differentiation. However, after additional genetic changes CML eventually develop into acute leukemia. In acute leukemia the normal hematopoiesis is suppressed by the leukemic blasts in the bone marrow, leading to a shortage of normal blood cells (Figure 2). Leukemia patients therefore suffer from anemia, and have an increased susceptibility to infections and bleeding.

Leukemia is a clonal disease which means that it originates from a single leukemic stem cell. A small pool of leukemic stem cells (LSC) supports the continued growth and propagation of the disease [12]. The origin of the LSC is controversial; it could be a transformed HSC with capacity of self-renewal or a progenitor cell that has acquired the ability to self-renew. Several transforming events causing deregulation of genes important in proliferation, differentiation and apoptosis are required for a cell to be fully transformed. These can be divided into two

categories: (1) mutations that confer proliferation and/or survival e.g. overexpression of c-myc which positively controls cell cycle, inactivation of tumor suppressors as p53 which negatively regulates cell cycle, or overexpression of antiapoptotic genes such as Bcl-2, and (2) mutations that impair differentiation. Chromosomal translocations, which are common in leukemia, are often examples of the latter. These kinds of translocations often involve transcription factors normally involved in lineage choice and differentiation, exemplified by PML/RARα in acute promyelocytic leukemia (APL) and AML1/ETO in acute myeloid leukemia (AML). The fusion proteins acquire novel dominant functions, blocking normal differentiation.

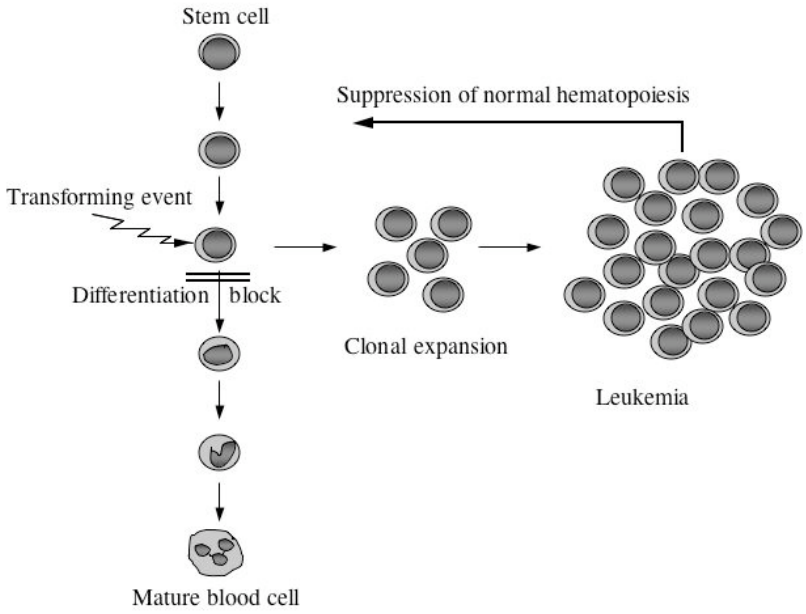

Figure 2. ***Simplified model of the development of acute leukemia***

Chronic myeloid leukemia (CML)

The chromosomal translocation between chromosome 9 and 22 fuses the gene encoding ABL1 to the BCR gene [13]. This translocation is present in more than 95 % of all CML-patients [14]. The normal ABL1 and BCR proteins are ubiquitously expressed. The ABL1 protein is a nonreceptor tyrosine kinase protein involved in signal transduction via integrins, in cell cycle regulation and in response to DNA damage, whereas the function of the BCR

protein is largely unknown. Fusion of the proteins enhances the tyrosine kinase activity of ABL1, which appears to be critical for transformation of target cells. One mechanism by which BCR/ABL1 contributes to the transformation process is by constitutive activation of the JAK/STAT, Ras/Raf/MEK/ERK and PI3K/Akt signal transduction pathways (reviewed in [13,15-17]). These pathways are normally activated by several hematopoietic growth factors and their receptors in a tightly controlled way to regulate cell proliferation and survival. Deregulation of these pathways contributes to malignant transformation. In the bone marrow, hematopoietic progenitor cells normally adhere to stroma cells and extracellular matrix via integrins, which functions as an important regulator of their proliferation, differentiation and apoptosis. CML is characterized by a massive expansion of myeloid cells and CML progenitor cells have been shown to have altered adhesion properties. Therefore, one could speculate that CML-cells escape growth-inhibiting signals due to decreased adhesion to stroma cells, resulting in an immature cell population able to expand and leave the bone marrow prematurely. Thus, BCR/ABL1-mediated effects in transformation seem to include activation of signaling pathways promoting cell proliferation and survival, as well as perturbed ability of the cells to interact with stroma and extracellular matrix.

The understanding of the molecular biology of CML has emerged from studies using CML cell lines, CML primary cells and animal models. The transforming potential of BCR/ABL1 has been shown both in vitro and in vivo. Expression of BCR/ABL1 in fibroblast cell lines induced anchorage-independent growth in soft agar and growth factor independence in growth factor dependent hematopoietic cell lines. Furthermore, retroviral transduction of BCR/ABL1 to murine bone marrow cells followed by transplantation to mice induced a chronic phase CML-like disease [13]. Human $CD34^+$ cells overexpressing BCR/ABL1 showed similar phenotypic features as CML $CD34^+$ cells, including altered adhesion, increased proliferation and delayed apoptosis. Thus, BCR/ABL1 is central for the conversion of the target cell into a CML-leukemic stem cell. However, the mechanisms underlying the progression of the chronic phase into the acute phase are not known, although additional genetic changes are most likely to be required. Deletions or inactivating mutations of tumor suppressor genes such as p53, pRB or p16 have been detected in leukemic cells from patients in blast crisis as well as overexpression of EVI-1 and c-myc. These findings support the notion that additional loss of tumor suppressors and/or gain of oncogene function is causing transformation into blast crisis.

The clinical importance of BCR/ABL1 in the pathogenesis of CML has called for a search of pharmacological inhibitors of the tyrosine kinase activity of ABL1. Today, CML patients are treated with imatinib mesylate, which inhibits the ABL1 tyrosine kinase activity and induces apoptosis in BCR/ABL1-positive cells, further emphasizing the important pathogenetic role of BCR/ABL1 in CML. However, not all BCR/ABL1-positive cells are eradicated and with time resistance to imatinib is often developed. It is therefore important to identify additional potential therapeutic targets in CML.

Expression of WT1 in normal hematopoiesis

Expression of WT1 in human cells of hematopoietic origin has suggested a role for WT1 in control of proliferation and/or differentiation of hematopoietic cells. Characterisation of its expression pattern indicate that WT1 is expressed in primitive immature cells; it is expressed in human $CD34^+$ bone marrow (BM) cells, but not in $CD34^-$ BM cells or in peripheral blood mononuclear cells [18-20]. Approximately 1% of the $CD34^+$ BM cells, which accounts for 1-4% of normal whole BM cell population, express WT1 [21]. *In vitro*, the WT1 expression in $CD34^+$ cells is rapidly downregulated upon differentiation [22]. These findings suggested a role for WT1 in early hematopoiesis. An ability of WT1 to enhance stem cell preservation and/or survival was proposed, as well as a prerequisite for downregulation of WT1 for differentiation. However, a biphasic expression pattern of WT1 during hematopoiesis has also been detected; WT1 was expressed in quiescent progenitor cells and then expressed in committed cells expressing surface markers for myeloid and B-cell differentiation [23].

Results from murine transgenic knockout models do not strongly support a role for WT1 in hematopoiesis. WT1 is expressed in murine embryonic liver and yolk sac at day E12.5, which is the tissue for active hematopoiesis at that time [19]. Although WT1-null mice die *in utero* before mature hematopoiesis is achieved, these mice show no hematological defects [3]. However, one study using embryonic stem cells lacking WT1, demonstrate that although WT1 is not absolutely essential for hematopoiesis, it leads to functional defects in growth potential. Cells lacking WT1 reconstitute the hematopoiesis of an irradiated mouse, but not in competition with normal HSC [24]. One could therefore speculate that WT1 activates genes that confer a competitive advantage to HSC, leading to increased proliferation and/or survival. However, a more recent study showed no reduced in vitro colony-forming ability or reconstitution of hematopoiesis in irradiated mice by WT1-null cells [25]. It is possible that this discrepancy can be explained by differences between the mouse-strains used.

Nevertheless, one must be aware of the differences between mice and man. WT1 is expressed in almost all human leukemias but not in mouse leukemias, and $WT1^{+/-}$ knockout mice are not predisposed to Wilms tumor, which is the case for children with inactivation of one WT1 allele.

Expression of WT1 in leukemia

The WT1 protein is highly expressed in the majority of patients with myelodysplastic (MDS) syndromes, acute myeloid (AML) and acute lymphoid (ALL) leukemia, chronic myeloid leukemia (CML) and leukemic cell lines [26-30]. It has been a matter of debate whether WT1 is indeed overexpressed in leukemic cells, as compared to normal progenitors. However, a recent report using single cell analysis of WT1 expression show convincingly that WT1-expression is normally restricted to a small subset of hematopoietic progenitor cells and that the expression levels per cell among these progenitors are quite similar to those in leukemic cells [21]. Thus, the level of WT1 expression seems to correlate to immaturity rather than malignant phenotype and the leukemic cells represent an expansion of those early progenitor cells expressing WT1.

No correlation between WT1 expression and age, FAB type or karyotype at diagnosis was found in AML-patients [26,31], with the exception of the more differentiated AML subtypes M4 and M5 with lower WT1 expression [26,30]. This is consistent with the finding that expression of WT1 was downregulated during differentiation of the leukemic cell lines K562 and HL60 [32,33]. Moreover, expression of WT1 mRNA was significantly lower in ALL cells, as well as M5 AML, as compared to other acute leukemias [34]. However, in another investigation, no striking differences were found between different FAB subtypes [35]. Some groups found a clear correlation between low WT1 levels and complete remission, disease-free survival and overall survival for acute leukemia patients [28,36-38], which was not confirmed by others [31,39].

WT1 as a marker for minimal residual disease (MRD)

In view of the finding that the majority of patients with acute leukemia express WT1, it has been suggested that WT1 expression might be used as a marker for the minimal residual disease (MRD) in treated leukemia patients, even in the absence of a tumor-specific DNA marker e.g. BCR/ABL1. Several groups have addressed this question using different methods, which have produced conflicting results [36,40-42]. Moreover, since WT1 is also expressed

in a subset of normal stem/progenitor cells, it might be difficult to detect small blast cell population in the bone marrow. New studies, however, using the sensitive real time RT-PCR method to examine WT1 expression after treatment showed an early detection of relapse [34,38], indicating that this might be a good method for both short- and long-term monitoring of leukemia patients.

WT1 as a target for immunotherapy in acute leukemia

Since WT1 is expressed in most acute leukemias, WT1 protein was suggested to be useful as a tumor antigen suitable for immunotherapy. The aim is to elaborate a WT1 vaccine eliciting a WT1-specific immune response, including generation of WT1-specific cytotoxic T-cells (CTL) and antibodies, to eradicate leukemic cells expressing WT1. However, there are two potential problems with this type of therapy; (1) since WT1 normally is expressed in some tissues, it may be impossible to elicit an immune response to the protein and (2) an effective immune response could result in destruction of tissues normally expressing WT1.

Nevertheless, mouse models have shown promising results of WT1 as a tumor antigen for immunotherapy, where immunization with WT1-peptides or WT1 DNA generated WT1-specific CTLs which lysed leukemic cells but not cells normally expressing WT1 [43-46]. The detection of WT1-specific antibodies in leukemia patients has also proven that WT1 is immunogenic in vivo [47] and WT1-expressing leukemic cells were also killed by human WT1-specific CTLs generated in vitro [48,49]. Based on these results clinical trials with WT1-peptide based vaccination have been initiated, which so far seem very promising [50].

Effects of WT1 in leukemia

A potential role for WT1 in leukemia was initially suggested based on the observations that WT1 is expressed in almost all acute leukemias and downregulated during induced differentiation of both normal hematopoietic progenitor cells as well as leukemic cell lines [22,32,33]. Several studies have since then strengthened the hypothesis that WT1 promotes an undifferentiated phenotype and interferes with differentiation. High levels of WT1(+KTS) (one of four WT1 isoforms, as described below) in murine hematopoietic bone marrow or the murine myeloid progenitor cell line 32D cl3 was shown to inhibit differentiation, but promote proliferation in response to G-CSF [51,52]. Moreover, constitutive expression of WT1(-KTS) and WT1(+KTS) in the human leukemic cell lines K562 or HL60 arrested TPA-induced differentiation [53,54] and blocked part of the differentiation program in leukemic U937 cells [55]. Repression of WT1 expression, using methods such as antisense oligonucleotides in

K562 and MM6 cells or siRNA in K562, Kasumi-1, MV 4-11 and NB-4 cells, as well as in cells from CML patients, resulted in inhibited proliferation and induced apoptosis [56,57], lending further support for a pro-leukemic role of WT1. Consistent with an oncogenic function of WT1, it was recently shown that when bone marrow cells overexpressing WT1 were transduced with AML1/ETO, acute myeloid leukemia was rapidly induced in transplanted mice [58]. Neither WT1 nor AML1/ETO were able to induce leukemia alone, consistent with the idea that several genetic alterations of genes involved in proliferation and differentiation are required for the development of leukemia. On the molecular level, WT1 has also been shown to regulate genes that promotes proliferation and counteracts apoptosis e.g. c-myc and Bcl-2 [59,60]. Thus, these studies are all consistent with an oncogenic function of WT1 promoting proliferation and survival and blocking differentiation.

However, other studies suggest that WT1 promotes differentiation and decreases survival. Smith *et al*, showed that stable expression of WT1(+KTS) induced monocytic differentiation in the murine myeloblastic leukemia cell line, M1, followed by terminal macrophage differentiation and apoptosis after addition of leukemia inhibitory factor (LIF) [61]. Establishment of clones expressing the WT1(-KTS) isoforms was not possible to achieve, pointing to even stronger growth suppression effects by these isoforms. These data were corroborated by results from two other groups showing G1 arrest and apoptosis in M1 cells induced by WT1(-KTS) [62], and G-CSF induced differentiation promoted by WT1(-KTS) in the murine myeloid progenitor cell line 32D cl3 [63].

Recent reports from retroviral overexpression of WT1 in primary hematopoietic progenitors and leukemic cell lines also support a role for WT1 as a tumor suppressor rather than an oncogene. Retroviral transduction of WT1(-KTS) in cord blood progenitor cells induces cellular quiescence of early progenitors and myelo-monocytic differentiation of later progenitors [23,64,65]. The molecular mechanism(s) by which WT1 induces this phenotype is unknown, but WT1 has been shown to upregulate the expression of antiproliferative proteins such as p21 [66]. Clearly, there are conflicting results concerning the role of WT1 in proliferation and differentiation, which possibly reflects different experimental conditions or dependence on the cellular context.

Mutations of WT1 in leukemia

WT1 mutations have been found in about 15% of cases of acute myeloid leukemia (AML), 20% of biphenotypic leukemia, but mutations are rare in acute lymphoblastic leukemia (ALL) [67-69]. In AML, the presence of WT1 mutations is correlated to low response to chemotherapy, poor prognosis and low survival rate. WT1 mutations in leukemia are often heterozygous with one wild-type allele remaining. The mutations are often small insertions or missense mutations resulting in either point mutations in the zinc-finger domain or in truncated WT1 protein, lacking most of the zinc-fingers, both types with reduced DNA-binding ability (Figure 3) [67,69]. Zinc-finger deficient WT1 retains, however, the ability to interact with certain proteins, including WT1 itself, through binding to the aminoterminal. Therefore WT1 mutants could contribute to leukemogenesis by exerting a dominant negative effect on remaining wild type WT1. The functional properties of mutated WT1 are, however, not experimentally studied, with the exception of one study with murine embryonic stem (ES) cells harboring WT1 truncated at zinc-finger 3 (heterozygous, homozygous) showing delayed, but not abolished hematopoiesis [70].

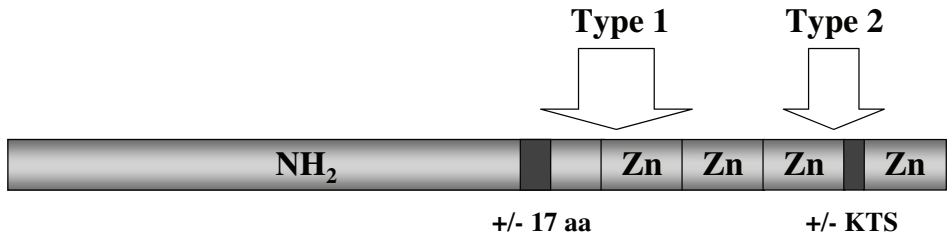

Figure 3. ***Two types of WT1 mutations found in leukemia****. Type 1 mutations are represented by framshift mutations producing a truncated WT1 protein, while type 2 mutations are point mutations resulting in amino acid substitutions, affecting DNA-binding.*

Structural and functional properties of WT1

The WT1 gene, mRNA and protein

The human WT1 gene is localized at chromosome 11p13 and spans about 50 kb. It encodes a mRNA about 3 kb long which contains 10 exons [71,72]. WT1 protein has many properties that are typical of transcription factors. It contains transactivation and repression domains, nuclear localization signals and four C-terminal Cys_2His_2 zinc-fingers mediating DNA-

binding. The N-terminal also contains an oligomerization domain and a RNA recognition motif (Figure 4).

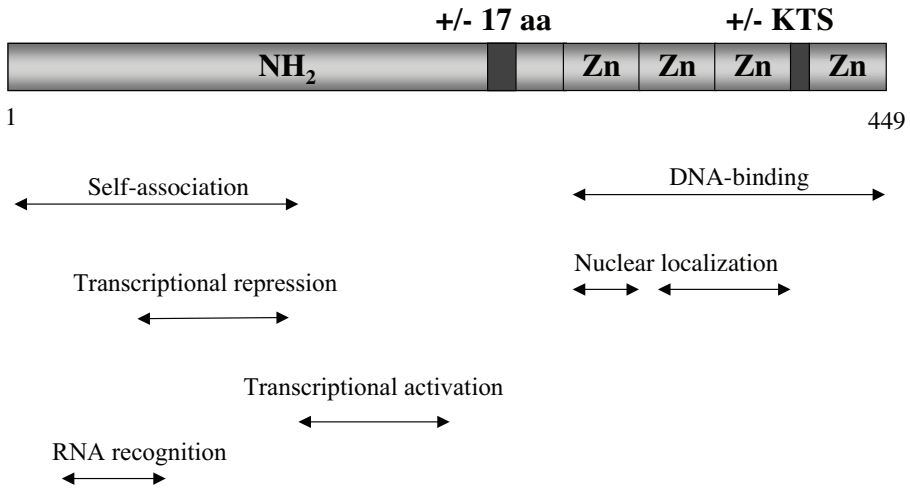

Figure 4. ***Schematic picture of the WT1 protein and its functional domains.***

Differential splicing of the WT1 mRNA transcript produce four major variants. The first splicing includes or excludes exon five, coding for 17 amino acids, between the transactivation domain and the zinc-fingers [73]. This sequence is conserved in all studied mammalian species [74]. The second alternative splice includes or excludes 3 amino acids, lysine, threonine and serine (KTS), between zinc finger 3 and 4 [73]. The importance of this alternative splice is underscored by its conservation in all vertebrates [75,76]. The major WT1 isoforms are designated -/- (lacks both inserts), +/- (contains the 17 aa but lacks the KTS insert), -/+ (lacks the 17 aa but contains the KTS insert) and +/+ (contains both the 17 aa and KTS insert) (Figure 4). The WT1 splicing pattern is similar in normal hematopoietic cells and leukemic cells with a relatively higher level of exon 5-containing variants of WT1 as compared to KTS containing WT1-variants [35,77]. Neither the regulation of the splicing pattern nor the significance of the different levels of each isoform is known. Depending on the different splicing inserts, proteins with molecular masses between 52-54 kDa are produced [78].

Transcriptional regulation of the WT1 gene expression

Expression of the WT1 gene is developmentally regulated and restricted to specific cell types. What regulatory factors that control the expression of WT1 are largely still unknown.

The WT1 promoter is a member of the GC-rich, TATA-less and CCAAT-less class of RNA polymerase II promoters and contains binding sites for the regulatory factors PAX2, PAX8, SP1 and WT1 itself [79-85]. However, when studying the transcriptional activity, the promoter was active in all cell lines investigated, indicating that other regulatory elements outside the promoter region are needed for the tissue-specific expression of WT1 [86]. A hematopoietic specific enhancer located in the 3' end of the gene, 50 kbp downstream of the promoter, containing two GATA sites was also identified. This 3' enhancer was highly active in the erythroleukemia cell lines K562 and HEL, consistent with an erythroid specific function. Subsequently, another hematopoietic specific enhancer in the third intron was identified [87]. This enhancer was transactivated by GATA-1 and c-myb in hematopoietic cell lines, especially those of myeloid origin. Thus, so far two hematopoietic specific enhancer sequences have been identified but further investigations are needed to completely reveal the mechanisms controlling WT1 expression.

Post-translational modifications of the WT1 protein

Post-translational modifications such as phosphorylation, sumoylation, ubiquitination and acetylation are mechanisms that can regulate the activity state, stability and subcellular localization of proteins. Recently, two sumoylation sites in the N-terminal domain of WT1 (Lys-73 and Lys-177) were identified [88]. Although both sites were indeed sumoylated in vivo, no effects on WT1(-KTS) mediated transcriptional activation of the target genes amphiregulin or podocalyxin could be detected. Moreover, the nuclear localization of WT1 was not affected by direct sumoylation conjugation. The functional consequence of sumoylation, if any, is therefore presently unknown. Two phosphorylation sites have been identified in zinc-finger 2 and 3 (Ser-365 and Ser-393) of WT1. When phosphorylated, the DNA-binding of WT1 was inhibited followed by a decreased transcriptional repression activity, and some cytoplasmic retention of WT1 protein was detected [89,90]. Thus, some data indicate that phosphorylation of WT1 modulate transcriptional activity and nuclear localization of WT1.

Different splicing – different function?

Some experimental evidence indicate that the WT1(+KTS) and WT1(–KTS) variants have different functions; while the WT1(–KTS) isoform is proposed to have a role in transcriptional regulation, a role for WT1(+KTS) in pre-mRNA splicing has been suggested. It has been shown that WT1(+KTS) binds DNA with less affinity than WT1(–KTS) but mRNA with higher affinity than WT1(-KTS) [91]. Moreover, WT1(+KTS) isoforms colocalize with different proteins involved in mRNA-splicing [92-94] and interact with the splicing factor U2AF65 [95]. The different isoforms also localize to different subnuclear compartments; the WT1(–KTS) form is diffusely located throughout the nucleus, as other transcription factors e.g. Sp1, while the WT1(+KTS) form is expressed in a speckled pattern within the nucleus [96]. In humans, the importance of WT1(+KTS) is underscored by the developmental defects in kidneys and gonads as well as male-to-female sex reversal in patients with Fraiser syndrome (FS) [97]. In FS the expression of WT1(+KTS) is decreased as a result of a germline mutation in the exon 9-donor consensus site.

As mentioned above, exon 5 encoding the 17 amino acid insert is unique to mammals. The exact function of exon 5 is presently unknown. In transgenic mice, an exon 5 knockout model showed no functional requirement for this insert since the mice developed normally [74]. However, it is possible that the phenotypic effects of this insert are subtler and thus escaped detection. Recently, two groups showed that siRNA to WT1(+17aa) but not WT1(–17aa) induced apoptosis in human leukemic cell lines, suggesting an antiapoptotic role of 17 aa [98,99]. In support of this notion, constitutive expression of the WT1(+17aa) isoforms in K562 inhibited apoptosis induced by etoposide and doxorubicin [98] and expression of WT1(+17 aa/-KTS) in 293 cells rescued them from UV-induced cell death in contrast in WT1(-17aa/-KTS) [100]. Interestingly, exon 5 has been reported to interact with the prostate response protein 4 (Par4) involved in apoptosis [100]. Thus, it is possible that 17aa confers a survival mechanism of WT1 by interacting with Par-4 or other proteins.

WT1 target genes

As a transcription factor, WT1 was first characterized as a transcriptional repressor of genes containing the GC-rich EGR1-binding sequence in transient transfection studies. Later on, however, both transcriptional activation and repression of reporter constructs have been reported, depending upon the number of binding sites within the promoter, cellular context and choice of expression vector [101]. Target genes regulated by WT1 include genes involved

in proliferation and cell cycle regulation, growth factor receptors, apoptosis, development of the genitourinary system and hematopoietic specific genes. Most often, these target genes are most efficiently transactivated by the WT1(–KTS) isoform of WT1 (Table I).

Table I. Genes regulated by WT1 at the endogenous level

Target gene	Regulation of transcript	References
Growth factor receptors		
Epidermal growth factor receptor	Repression	[102]
Insulin growth factor I receptor	Repression	[103]
Growth factors		
Amphiregulin	Activation	[104]
Erythropoietin	Activation	[105]
Insulin-like growth factor II	Activation	[106]
Connective tissue growth factor	Repression	[107]
Transcription factor genes		
Dax-1	Activation	[108]
SRY	Activation	[109]
Pou4f2	Activation	[110]
Apoptosis regulators		
A1/Bfl-1	Activation	[111]
Bak	Activation	[112]
Bcl-2	Activation	[60]
Cell cycle regulators		
Cyclin E	Repression	[113]
c-myc	Activation	[59]
Ornithine decarboxylase	Repression	[114]
P21	Activation	[66]
RbAp46	Activation	[115]
Others		
E-cadherin	Activation	[116]
Syndecan-1	Activation	[117]
Nephrin	Activation	[118]
Podocalyxin	Activation	[119]
Sprouty1	Activation	[120]
TauT	Activation	[121]
TERT	Repression	[122]
Vitamine D receptor	Activation	[123,124]

WT1 interacting proteins

Several protein partners binding to WT1 have been identified using yeast two-hybrid assays and coimmunoprecipitation experiments (Table II). Many of these proteins are also transcription factors and/or modulate the activity of WT1. The identification of protein partners may reveal novel information on how WT1 is involved in cellular proliferation and differentiation. WT1 interact with a number of proteins that modulate the transcriptional regulation exerted by WT1, exemplified by Par-4 and CBP. Par-4 (prostate apoptosis response 4) is upregulated during apoptosis in prostate cancer cells [125]. It interacts with the zinc-finger of WT1 and acts as a transcriptional repressor. As mentioned above, Par-4 has also been shown to interact with the 17 aa of WT1 and thereby rescue UV-treated cells from apoptosis [100]. Another interesting cofactor is CBP (CREB binding protein), which also binds to the zinc-fingers of WT1. CBP works as a coactivator for WT1 and transcriptional activation is hereby enhanced [126]. It has been suggested that CBP is mediating binding between WT1 and p53. The interaction is mediated by the zinc-fingers of WT1 and results in modulation of their transactivational activities and protein stability [137]. WT1 has also been reported to bind the p53 homologues p63 and p73. Since most acute leukemias harbour wild type p53 and p73 [151], it is tempting to speculate that the poor response to chemotherapy in leukemias with mutated WT1 is related to perturbation of p53/p73-function.

Also the molecular chaperone heat shock protein 70 (Hsp70) interacts with WT1 [127]. The interaction between the N-terminal part of WT1 and Hsp70 induce expression of Hsp70, and is also important for the induction of p21 and G1 arrest, as well as for inhibition of colony formation of osteosarcoma cells. In other cases, it is rather WT1, which functions as a transcriptional cofactor. WT1 has been shown to interact with SRY (sex-determining region) and SF-1 (steroidogenic factor 1) to activate genes involved in sex-determining processes [128,129].

Hence, apart from direct regulation of target genes by zinc-finger dependent binding to DNA, several effects of WT1 are probably mediated by direct interactions of WT1 with other proteins.

Table II. WT1 binding proteins

Protein	WT1-interacting domain	Remarks	References
WT1	N-terminus	Oligomerization important for dominant negative function of WT1 mutants?	[130]
Hsp70	N-terminus	Promotes growth inhibition of WT1	[127]
UBC9	N-terminus	Involved in sumoylation of WT1	[131]
SF1	N-terminus	Promotes WT1(-KTS) regulation of MIS expression	[129]
BASP1	N-terminus	WT1 TA⇓	[132]
Pax-2	N-terminus	Consequence of interaction is unknown	[133]
Par-4	17 aa	WT1(+17aa) TA⇑; Rescues cells from UV-induced apoptosis	[100]
	Zn-fingers	WT1 TA⇓; WT1 TR⇑	[125]
U2AF65	Zn-fingers	Suggested to have a role in pre-mRNA splicing	[95]
WTAP	Zn-fingers	Unknown function	[134]
Ciao1	Zn-fingers	WT1 TA⇓; no effect on TR	[135]
BMZF2	Zn-fingers	WT1 TA⇓	[136]
p53	Zn-fingers	Stabilization of p53 and inhibition of p53-mediated apoptosis; TA of WT1 is inhibited	[137,138]
p63	Not determined	Consequence of interaction is unknown	[139]
p73	Zn-fingers	p73 inhibits DNA-binding and TA by WT1; WT1 inhibits TA by p73	[139]
CBP	Zn-fingers	WT1 TA⇑	[126]
SRY	Zn-fingers	WT1 and SRY act synergistically to activate transcription	[128]
E1B55K	Zn-fingers	Inhibits WT-mediated cell death	[140]
HCMV-1E2	Zn-fingers	Consequence of interaction is unknown	[141]

The abbreviations used; TA, transcriptional activation; TR, transcriptional repression; Zn-fingers, Zinc fingers; WT1, Wilms tumor gene1; Hsp70, Heat shock protein 70; Par-4, Prostate apoptosis respone 4; UBC9, Ubiquitin-conjugating enzyme 9; SF-1, Steroidogenic factor 1; BASP1, Brain acid soluble protein 1; WTAP, WT1 associating protein; BMZF2, Bone marrow zinc finger 2; CBP, CREB binding factor; HCMV, Human cytomegalovirus.

THE PRESENT INVESTIGATIONS

AIMS OF THIS THESIS

The aim of my work was to investigate the role of the transcription factor Wilms' tumor gene 1 (WT1) in normal hematopoiesis and leukemia. This task was addressed by:

- Overexpression of wild type WT1 and a zinc-finger deleted form of WT1 in human hematopoietic progenitor cells to study and compare effects on proliferation and differentiation

- Investigating the molecular mechanisms for WT1-mediated effects by searching for novel WT1 target genes and WT1 interacting proteins

EXPERIMENTAL CONSIDERATIONS

Retroviral transduction and overexpression

An efficient way of introducing a foreign gene into cells is retroviral transduction. In paper I-III, retroviral transduction of WT1 or a control vector (MIG) into leukemic cell lines and hematopoietic progenitor cells was used. The viral envelope used in these experiments was RD114 which interacts with a neutral amino acid transporter (RDR), highly expressed on hematopoietic progenitor cells and leukemic cell lines [142]. The virus contained a bicistronic vector containing cDNA encoding WT1 linked by an internal ribosomal entry site (IRES) to a cDNA encoding the enhanced green fluorescent protein (eGFP). The virus was preloaded on retronectin-coated plates to facilitate subsequent the receptor-mediated uptake into the cells. Since virus only relocates to the nucleus during mitosis, the cells have to be cycling to allow insertion of the gene into the genome. Therefore, the $CD34^+$ cells were stimulated into cell cycling with stem cell factor (SCF), Flt3-ligand and thrombopoietin (TPO) during 48 hours prior to transduction. After 48 hours of transduction, the cells were subjected to sorting of the GFP^+ cells. Thus, no geneticin-selection of transfected cells to establish clones stably expressing the gene was needed, excluding the possibility of selection of cells resistant to negative effects of WT1. The transduction efficiencies obtained were between 50-80% and comparable for MIG and WT1 transduced cells, indicating that similar $CD34^+$ populations were transduced.

In colony forming assays, the expected cloning efficiency of about 15% was obtained in MIG-transduced cells, indicating that the transduction procedure in itself was not toxic to the cells.

Analysis by real time RT-PCR revealed a robust expression of WT1 as compared to MIG-transduced cells. However, the expression of WT1 was clearly increased as compared to the physiological levels in the leukemic cell lines HL60 or K562. This could be a potential experimental problem leading to non-physiological effects.

Oligonucleotide Array

Gene expression analysis was used in paper II and III, to screen for novel target genes of WT1. Single-stranded DNA complementary for a specific target sequence was spotted on a glass slide, referred to as an oligonucleotide array. In our case, about 18 000 genes were present on the array. Two-color array was used, which means that RNA from WT1-transduced cells and RNA from MIG-transduced cells were labeled with two different fluorophores and hybridized simultaneously to the array. The fluorescence of each fluorophore in the same spot was measured and compared to each other. Two biological replicates were analyzed and differences in gene expression evaluated. There is a number of factors e.g. array-to-array variability, statistical analysis of the array data, false negative and positive signals, which affect the results. Therefore, in our case the array was just used as a screening method to find potential target genes, up- or downregulated by WT1, which was further validated by additional methods such as real time RT-PCR.

Human hematopoietic colony-forming cell assay

Human hematopoietic colony-forming cell assays are developed to evaluate the proliferation and differentiation of hematopoietic progenitor cells in vitro. In the CFU-GM/BFU-E assay, cells are cultured in a semi-solid matrix (methylcellulose) supplemented with nutrients and cytokines allowing the formation of colonies of the erythrocyte, monocyte-macrophage, and granulocyte lineages. The colonies were evaluated approximately after 14 days depending on morphological criterias. Erythroid colonies included BFU-E and CFU-E, clearly identified as hemoglobinized cells and colony morphology, while myeloid colonies included CFU-GM, CFU-M and CFU-G. In paper I, $CD34^+$ cells were retrovirally transduced for 48 hours and seeded into methylcellulose after sorting of the GFP^+ cells. In paper II, $CD34^+$ was transfected by electroporation and seeded into methylcellulose after 24 hours. The clonogenicity for the control cells was in paper I about 15% and in paper II about 3%, indicating that the two

methods transduced different cell populations. Thus, more progenitor cells were transduced retrovirally as compared to transfection by electroporation, resulting in a higher number of colonies.

Real time RT-PCR

To analyze gene expression, real time RT-PCR was performed in paper I-III. Real time RT-PCR is a very sensitive method where small amounts of mRNA can be quantified. This method is based on the detection and quantification of a dye-labeled probe (TaqMan probe). RNA was extracted and converted into complementary DNA (cDNA) during reverse transcription (RT). The TaqMan probe and primers are designed to hybridize specifically to a complementary sequence. If the probe anneals to its target sequence, which is amplified during PCR, the reporter dye starts to emit fluorescence, which increases in each cycle. Unlike conventional PCR methods detecting the final amount of amplified product, the PCR product is quantified after each round of amplification based on the amount of fluorescence produced. The amplification can be followed in real time during the exponential phase allowing accurate quantification of gene expression in the starting material. An internal control is used to exclude that the differences in mRNA expression is solely a result of unequal loading and the target amount is normalized to the internal control in each reaction. A gene that is to be used as an internal control should not change significantly in expression during different experimental conditions.

Luciferase assay

To investigate the transcriptional effects of WT1 on the NDRG2 and IRF-8 promoters, a dual-luciferase reporter system was used in paper II and III. The promoter sequences were cloned into the pGL3Basic reporter vector upstream of the firefly luciferase gene. Adherent cells were transiently transfected with the expression plasmid, the firefly reporter and a vector expressing renilla luciferase. The firefly luciferase activity was measured by adding substrate, which resulted in a luminescence signal, after which the reaction was quenched, and a substrate for the renilla luciferase was added. The renilla vector was used as a transfection efficiency control, and the firefly luciferase values were normalized to those of the renilla luciferase.

However, a number of factors have to be considered when evaluating results from luciferase assays: both expression- and reporter-plasmids are overexpressed, critical transcription factors may be present in limited quanitities, the vectors are not integrated into the genome and are

therefore not in the correct chromosomal environment. Thus, it is also important to investigate the regulation of the endogenous gene expression by other methods such as real time RT-PCR.

Bacteriomatch II Two-Hybrid assay

The Bacteriomatch II Two-Hybrid assay was used in paper IV to identify proteins interacting with the N-terminal part of WT1. The N-terminal part of WT1 was cloned into a bait vector and interactions with proteins expressed from target vectors were investigated. In our case, a cDNA library from K562 cells was used. One limitation with this system is how the cDNA library is constructed. First-strand cDNA synthesis begins with a poly(dT) primer that binds to the poly(A) tail in the 3' end of the mRNA and the reverse transcription is initiated. After second-strand synthesis, the product is ligated into the target vector. In this manner, the cDNA is sometimes ligated out of reading frame and proteins not naturally occurring are translated. One might also consider that this is a bacterial and not a mammalian system. Therefore, interactions between nuclear proteins might escape detection, since the proteins are not in their proper milieu and important cofactors might be missing.

Coimmunoprecipitation (Co-IP) and Glutathione S-Transferase (GST) pull down assay

Co-IP and GST-pull down assay are two methods for investigating protein-protein interactions. These methods were used in paper IV. In Co-IP, interacting proteins are immunoprecipitated with an antibody recognizing one of the proteins, and thereafter detected by an antibody directed against the other protein in Western Blotting. Usually two plasmids expressing the proteins of interest are transiently transfected to adherent cells and Co-IP is then performed. However, in this way, protein levels are very high, possibly resulting in interactions not seen at more physiological protein levels. Mixing of the nuclear and cytosolic fractions may also result in not naturally occurring interactions. In a GST-pull down assay, the protein of interest is GST-tagged and bound to a matrix in a column. Nuclear extract or in vitro translated protein is applied to the column to allow complex formation. Protein complexes are eluted and analyzed by Western Blotting technique. If one or both proteins bind non-specifically to the sepharose in Co-IP or the GST-matrix in the column, false positive results will be obtained. Postitive and negative controls are therefore of great importance in these types of studies.

RESULTS AND GENERAL DISCUSSION

How does WT1 affect proliferation and differentiation of hematopoietic progenitor cells?

During the past 15 years a large amount of effort has been made to identify the functional role of WT1 in hematopoiesis. Although murine WT1 knockout models have told us a lot about the importance of WT1 in the development of kidneys and gonads, the functions of WT1 in hematopoiesis is still poorly understood. Moreover, as discussed below, some data indicate that conclusions from mouse models may not always be completely relevant for the human situation.

WT1 is expressed in murine embryonic hematopoietic tissues, adult BM and blood, indicating a possible role of WT1 in murine hematopoiesis [19]. There are no obvious hematological defects in heterozygous WT1-knockout mice and homozygous WT-null mice die early in utero, excluding analysis of adult hematopoiesis [3]. In some mouse models, where WT1 deficient cells are transplanted to irradiated mice, WT1 is not critical for hematopoiesis [25]. However, in other mouse models where WT1-null fetal liver cells were transplanted, WT1-null cells could not reconstitute hematopoiesis in competition with its normal counterparts, suggesting functional defects in growth potential of WT1-null cells [24]. Interestingly, similar phenotypes with competition defects are seen in hematopoietic cells deficient of cell-signaling molecules or their receptors e.g. Pbx-1, c-mpl and flk2/flt3 [143-145], suggesting an involvement of WT1 in hematopoietic signaling pathways important for proliferation and/or survival. WT1 overexpression in bone marrow in transgenic mice also resulted in an expansion of the hematopoietic progenitor pool, further strengthening a role of WT1 in proliferation and expansion of immature blood cells [58].

A role for WT1 in human hematopoietic cells was suggested based on the initial observations that WT1 is expressed in $CD34^+$ hematopoietic progenitor cells, but not in $CD34^-$ BM cells or in peripheral blood mononuclear cells [18-20]. Upon differentiation, WT1 expression in $CD34^+$ cells is rapidly downregulated [146]. To study the function of WT1 during normal hematopoiesis, $CD34^+$ human hematopoietic progenitor cells were retrovirally transduced with WT1 and cultured in methylcellulose and liquid cultures to study effects on proliferation and differentiation (Paper I, [23,64]). The effects of WT1 were dual; WT1 induced quiescence of early progenitor cells and myelo-monocytic differentiation in more mature hematopoietic progenitor cells. These effects were associated with an upregulation of p21 and induced

growth arrest. P21 is suggested to be a direct WT1 target gene [66] and is a key molecule in cell cycle regulation. In hematopoiesis, the expression of p21 is suggested to be important for quiescence and self-renewal capacity of hematopoietic stem cells (HCS); in p21-null mice, the number of HCS in quiescence was reduced and serial transplantations of these cells resulted in earlier exhaustion of the HCS population compared to their normal counterparts [147]. Therefore, upregulation of p21 could provide a mechanistic explanation for the WT1-mediated effects. However, in $CD34^+$ progenitors, p21 could not alone induce differentiation, suggesting that WT1 activates additional genes or interacts with proteins to mediate differentiation. If WT1 is indeed able to physiologically induce quiescence via p21 or other mechanisms, this raises the possibility that WT1 could be an important factor for the maintenance of the leukemic stem cell pool. However, others and we have not been able to confirm an upregulation of p21 by WT1, questioning p21 as a direct target gene (Paper I, [113]).

Results from human and mouse models of WT1 function do not seem to be completely compatible with each other. In human progenitor cells, WT1 induces quiescence while WT1 seems to be important for sustained or even enhanced proliferation in mouse models. One explanation might be that different cell populations are studied. In the mouse models it is the function of hematopoietic stem/progenitor cells that are studied, while more mature progenitor cells are studied in the human models. It might be that the effects are different depending on the maturation state of the cell.

The role of WT1 in leukemia

There is an ongoing debate whether WT1 is functioning as a tumor suppressor gene or oncogene in leukemia. Based on the observations that WT1 is highly expressed in almost all acute leukemias and that growth of WT1-expressing leukemic cells was suppressed by WT1 antisense oligomers, WT1 was initially suggested to be an oncogene. Several groups have also shown that WT1 interferes with differentiation of leukemic cell lines, supporting the idea of WT1 as an oncogene in leukemia [51-55].

However, the recent reports where WT1 was retrovirally transduced to $CD34^+$ cells, which induced quiescence and differentiation in hematopoietic cells argues against an oncogenic role of WT1 (Paper I, [23,64]). A tumor suppressor function, rather than oncogenic effects, is also in consistence with the obvious suppressor function of WT1 in Wilms' tumor. If WT1 is acting as a tumor suppressor also in leukemia, one might speculate that WT1 mutations confer

loss of function and thus are involved in leukemogenesis and that the leukemic cells expressing wild type WT1 have rendered other mutations making them resistant to the action of WT1. In both sporadic Wilms' tumor and leukemia the incidence of WT1 mutations is about 10-15% [67-69,148]. In Wilms' tumor, most WT1 mutations follow the pattern of the classical Knudson 2-hit hypothesis for tumor-suppressor gene function, meaning that it is homozygously inactivated or deleted, leading to development of the tumors. In leukemia, however, WT1 mutations are most often heterozygous with one remaining wild-type allele. However, it is possible that the mutated WT1 protein could exert dominant negative effects on the remaining wild type protein leading to "homozygous" inactivation of WT1.

However, one could speculate on a number of alternative ways that mutated WT1 may contribute to leukemogenesis. Besides exerting a dominant negative effect on remaining wild type WT1, mutant WT1 could sequester cofactors essential for WT1 function, acquire quite novel oncogenic properties unrelated to the function of wild type WT1 or exert the same functions as wild type WT1 (Figure 5).

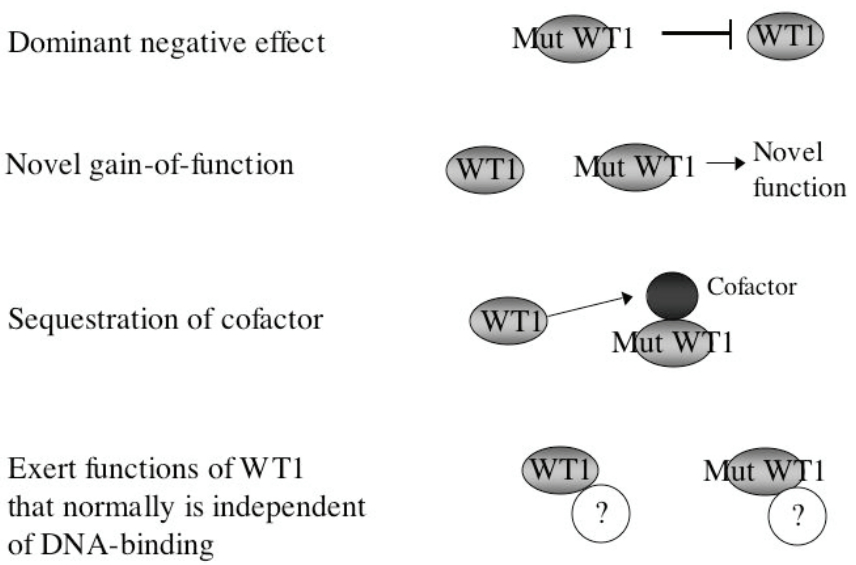

Figure 5. ***Possible effects of mutant WT1 protein***

In paper I, we were interested in investigating the effects of a zinc-finger deleted WT1, WT1(delZ), representing a WT1 mutant protein without DNA-binding ability, during human hematopoiesis in vitro. We used a highly effective retroviral system to transduce $CD34^+$ cells from cord blood with either wild type protein WT1(+/-) or zinc-finger deleted WT1(delZ). The transduced cells were seeded into methylcellulose to study the effects of WT1 on clonogenic capacity of progenitor cells and also grown in uni-lineage suspension cultures with growth factors supporting granulocytic or erythroid differentiation, to investigate the effects on proliferation, viability and differentiation. WT1(+/-), but not WT1(delZ), inhibited erythroid colony formation as well as erythroid differentiation in suspension cultures. However, our finding that both WT1(+/-) and WT1(delZ) were able to inhibit myeloid colony formation and to stimulate myeloid differentiation of cells grown in suspension culture was a surprising finding. These results suggest that cellular effects of WT1 are mediated by distinct molecular mechanisms; the effects on myeloid progenitors are independent of DNA-binding, suggesting protein-protein interaction with the N-terminal domain of WT1, while the erythroid effects were dependent of WT1 as a DNA-binding transcriptional regulator.

Patients with the Denys-Drash syndrome (DDS) have heterozygous germline WT1 point mutations in the zinc-finger region, disrupting the DNA-binding ability of WT1 [149]. These mutations results in genitourinary defects and predisposes to Wilms' tumor. If WT1 mutations confer an increased risk for developing leukemia one might think that patients with DDS would develop leukemia at higher frequency than normal. Although the incidence of DDS is low, making a modest increase in the risk of developing leukemia difficult to detect, DDS patients do not seem to have hematological defects or to develop leukemia, which would argue against a strong leukomogenic effect of DDS-associated mutations.

WT1 binding proteins

Several proteins interacting with WT1 have been identified (Table II). Even though most reported partners of WT1 interact with the zinc-fingers, there are a few proteins interacting via the N-terminal of WT1, including WT1 itself. The first 182 amino acids of WT1 contain a oligomerization domain. Although high levels of ectopic expression are required to demonstrate self-association *in vivo*, this is a potential mechanism for naturally occurring WT1 mutants. For instance, WT1 mutations that impair the DNA-binding domain but leave the self-association domain intact, may act in a dominant negative manner to inhibit the functions of wild type WT1. It is tempting to speculate that a dominant negative effect of

WT1(delZ) explains the increased number of erythroid colonies from WT1(delZ)-transduced cells (Paper I).

As also shown in paper I, the zinc-finger deleted WT1(delZ) mediated the same effects as WT1 on myeloid progenitor cells, indicating that WT1(delZ) by interaction with some WT1-interacting proteins can exert functions in myeloid cells. Therefore, we tried in paper IV, by use of a bacterial II hybrid assay, to identify proteins interacting with the N-terminal part of WT1. Cofilin 1 was one WT1(delZ)-interacting protein that was found. Cofilin 1 is reported to be involved in apoptosis [150]. Upon apoptosis induction, cofilin 1 is translocated from the cytoplasm to the mitochondria, where it can enhance the release of cytochrome C. Since WT1(delZ) accumulate in the cytoplasm (Paper I), we hypothesized that an interaction between WT1 and cofilin 1 would inhibit the apoptosis-inducing effect of cofilin 1. This could contribute to the leukemogenic process by rendering the cells more resistant to apoptosis. However, although WT1(delZ) and cofilin 1 did bind each other, no functional effects of this interaction were detected during apoptosis induced by etoposide (our unpublished data). These results are consistent with the finding that the antiapoptotic effect of WT1 in etoposide-induced K562 cells was dependent of the zinc-fingers [98]. Furthermore, cofilin 1 did not significantly interfere with transcriptional regulation of two known WT1 target genes (Paper IV), arguing against a WT1-modulating function by cofilin 1.

Target genes in leukemia and hematopoiesis

Several potential WT1 target genes have been identified (Table I). A number of them are involved in the development of the genitourinary system, whereas some are involved in more general mechanisms such as proliferation and apoptosis. A few of them could possibly be involved in the WT1-mediated effects in $CD34^+$ and leukemic cells.

Some target genes fit nicely into the model of WT1 as a tumor suppressor. The ability of WT1 to induce cellular quiescence in early progenitor cells and promote differentiation of later progenitors could be explained by the ability of WT1 to induce expression of p21 and reduce expression of Cyclin E, two proteins involved in regulation of G1 arrest [113] and to upregulate the Vitamin D receptor [123,124]. Binding of Vitamin D to its receptor has been shown to induce differentiation of leukemic cell lines. However, WT1 is also involved in upregulation of c-myc, which promote proliferation, and the antiapoptotic Bcl-2 gene, which supports the idea of WT1 as a protein with oncogenic functions [59,60].

Thus, transcriptional effects of WT1 are indeed complex and the WT1-regulation of target genes seems to be cell type specific. Therefore, in an attempt to identify novel WT1 target

genes in $CD34^+$ cells, a gene expression array was performed (Paper II and III). Out of several potential WT1 target genes, two were selected for further characterization. N-myc downstream regulated gene 2 (NDRG2) was shown to be upregulated by WT1 according to the array and real time RT-PCR analysis (Paper II). NDRG2 is expressed weakly in $CD34^+$ progenitor cells but not in peripheral blood pointing to a possible coexpression of WT1 and NDRG2 (Paper II, [152]). However, we could not prove NDRG2 to be a direct target gene of WT1 since WT1 was not able to activate transcription of NDRG2 in promoter studies. Thus, although expression of WT1 clearly resulted in increased endogenous NDRG2 gene expression in vivo, the mechanism is indirect, or exerted by more distant regulatory elements in the NDRG2 gene, not present in the promoter investigated. In our experiments, we could not demonstrate that NDRG2 is responsibe for mediating the WT1 effects seen in $CD34^+$ cells. NDRG2 affected neither the colony forming ability in methylcellulose nor the differentiation of $CD34^+$ cells in suspension cultures. The second potential WT1 target gene identified was interferon regulatory factor 8 (IRF-8), which was negatively regulated by WT1 (Paper III).

Is WT1 a link between BCR/ABL1 and IRF-8 in CML?

In paper III, WT1 is suggested to contribute to leukemogenesis in BCR/ABL1-positive CML cells by downregulating interferon regulatory factor 8 (IRF-8). The BCR/ABL1 protein functions as a tyrosine kinase, that constitutively activate the JAK/STAT, Ras/Raf/MEK/ERK and PI3K/Akt signal transduction pathways to regulate cell proliferation and survival [13,14]. BCR/ABL1 has also been shown to positively regulate WT1 gene expression: WT1 expression was reduced in CML cells treated with the tyrosine kinase inhibitor imatinib mesylate [153]. Both BCR/ABL1 and WT1 proteins are expressed in the majority of CML-patients in blast crisis [14,153]. BCR/ABL1 mediated activation of the JAK/STAT or Ras/Raf/MEK/ERK signaling pathways did not seem to influence WT1 mRNA levels. However, we found that WT1 mRNA levels was reduced during imatinib as well as during PI3K and Akt inhibitor treatment, suggesting that the BCR/ABL1 effect on WT1 is mediated via the PI3K/Akt signaling pathway. This finding is supported by Tuna *et al*, who reported that the tyrosine kinase receptor HER2/neu regulated WT1 expression via Akt in breast cancer cell lines [154]. One could speculate that the activation of WT1 expression is regulated by different kinds of tyrosine kinase and tyrosine kinase receptors. This might be a common mechanism in cancer cells that could contribute to the malignant phenotype. WT1 is indeed expressed in a number of nonhematopoietic malignancies [155-157].

How is then the WT1 mRNA levels influenced by BCR/ABL1 and PI3K/Akt? Increased mRNA levels could be a result of transcriptional activation of the WT1 gene, or of increased mRNA stability. Our results clearly indicate that WT1 mRNA stability is not increased by BCR/ABL1, since inhibition of the PI3K signaling pathway did not decrease the half-life of WT1 mRNA. A WT1 promoter construct, also containing two hematopoietic specific enhancer regions, was active in K562 cells. The promoter activity was strongly decreased after addition of the PI3K inhibitor, suggesting that the expression of WT1 mRNA is regulated at the transcriptional level via the PI3K signaling pathway. The WT1 promoter alone was only weakly active, indicating that the two enhancer regions are important for the transcriptional activity. Both enhancer regions contain GATA-1 sites. Interestingly, it was recently suggested that erythropoietin phosphorylates and thereby activates GATA-1 via the PI3K signaling pathway [158]. We therefore hypothesize that the mechanism for increased WT1 expression in CML is PI3K-mediated phosphorylation and activation of GATA-1. Further, it is tempting to speculate that activated GATA-1 can contribute to neutrophil differentiation disturbances (Figure 6).

We also found that the gene expression of interferon regulatory factor 8 (IRF-8) was reduced by WT1. WT1 repressed the IRF-8 promoter activity as well, pointing to a direct WT1-mediated effect on the proximal promoter. Interestingly, several findings indicate an important function of IRF-8 in the pathogenesis of CML [11]. Downregulation of IRF-8 was shown in CML cell lines as well as in cells from patients with CML. Furthermore, homozygous, but also heterozygous, deletion of IRF-8 in transgenic mice results in a CML-like disease, suggesting a haploinsufficiency. This might imply that also a modest reduction in IRF-8 levels could result in deregulation of signaling pathways controlled by IRF-8. Moreover, forced expression of IRF-8 was shown to counteract BCR/ABL1-induced leukemia in mice, pointing to a potential role of IRF-8 as a tumor suppressor. The tumor suppressor function of IRF-8 may, at least in part, be explained by the ability of IRF-8 to upregulate the tumor suppressor $p15^{Ink4b}$ and downregulate the antiapoptotic protein Bcl-2 and c-myc [159-161].

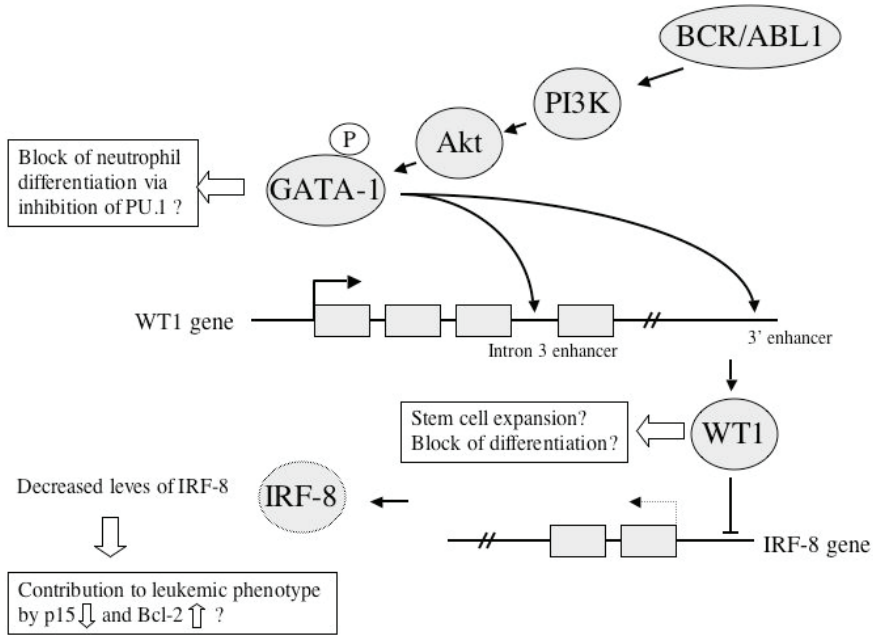

Figure 6. ***Hypothetical schematic picture of BCR/ABL1-WT1-IRF8-axis in CML***

These observations combined with our findings led us to propose that WT1 may be a connection between BCR/ABL1 and IRF-8. It is therefore possible that the WT1-mediated downregulation of IRF-8 contributes to the leukemogenic effect of BCR/ABL1 in CML cells.

WT1 – friend or foe in leukemia?

Taken all available information into account, it is difficult to form a clear picture about the function of WT1 in hematopoiesis and in leukemia. As was shown by several groups, WT1 induces quiescence in human $CD34^+$ progenitor cells, which might be a potential mechanism for extended survival of these cells. For the leukemic stem cells this could be an advantage, since high WT1 expression could contribute to the maintenance of the leukemic stem cells pool. One might also speculate that the high expression of WT1 and enhanced survival could provide a favorable milieu for these cells, even though WT1 might not be oncogenic in itself. The proliferation and survival drive exerted by WT1 might allow cells to be more resistant to the toxic effect of common leukemia-associated fusionproteins such as AML1/ETO or

BCR/ABL1, thus allowing transformation. Indeed, it was recently shown that WT1 and AML1/ETO together induced leukemia.

As discussed above, in recent years the knowledge on the functional role of WT1 has increased. The results in this thesis have contributed to new insights about possible physiological and pathophysiological roles. Novel molecular mechanisms upstream and downstream of WT1 have been identified and phenotypic consequences have been characterized. However, it is becoming increasingly apparent that the cellular context and cooperating factors have dramatic effects on WT1. Thus, although several achievements, the complexity regarding this enigmatic protein has not decreased.

SAMMANFATTNING PÅ SVENSKA

Hematopoes är den process under vilken alla röda och vita blodceller i kroppen bildas. Denna process sker kontinuerligt i benmärgen och förser kroppen med blodceller under hela livet. I benmärgen finns en viss typ av omogna celler sk stamceller som alla olika typer av blodceller bildas ifrån. Genom att stamceller gradvis mognar så bildas mogna blodceller som transporteras ut i blodet för att utföra sina sysslor. Mogna blodceller har mycket specialiserade funktioner. De röda blodcellerna transporterar syre. De vita blodcellerna är viktiga för kroppens immunförsvar. Trombocyterna är livsviktiga för blodets förmåga att koagulera. För att det alltid ska finnas tillräckligt många blodceller i blodet så är utmognadsprocessen mycket väl reglerad. Den styrs främst av proteiner som kallas transkriptionsfaktorer. Transkriptionsfaktorerna reglerar vilka gener som skall aktiveras för att en viss typ av cell ska mogna ut.

Vid akut leukemi kan de hematopoetiska stamcellerna i benmärgen ej mogna ut på normalt sätt utan befinner sig i en så kallad utmognadssblockad. Detta leder till att man får en ansamling av omogna blodceller i benmärgen som trycker ner den normala blodbildningen. Till följd av denna brist på normala blodceller får man bla blodbrist och ett försvagat immunsystem. Det uppkomna tillståndet, akut leukemi, måste behandlas för att man inte ska dö.

Mognadsblockaden är ofta en följd av förändringar i transkriptionsfaktorernas funktion. En transkriptionsfaktor som tros ha betydelse vid utveckling av leukemi är Wilms' tumör gen 1 (WT1). I den normala hematopoesen uttrycks WT1 i tidiga stamceller men nedregleras i samband med utmognad av celler. Detta skulle kunna betyda att WT1 måste nedregleras för att utmognad av celler ska kunna ske. I nästan alla leukemier är WT1 mycket högt uttryckt, vilket skulle kunna förhindra utmognad. Man har även sett ett samband mellan höga WT1 nivåer och sämre prognos. Dessutom är WT1 muterat i 15% av fallen. Detta skulle kunna innebära att ett felaktigt protein bildas vilket kan bidra till utmognadsblockaden genom att proteinet får nya felaktiga funktioner eller bara inte kan göra samma uppgifter som det vanliga WT1.

Trots mycket forskning om WT1s funktion i leukemiutveckling, så vet man fortfarande väldigt lite. Detta beror bla på att man har fått fram delvis olika resultat i de olika undersökningarna. I vissa försök har WT1 hindrat utmognad av celler men i andra fall inte. Jag har arbetat med att försöka klarlägga vilken funktion WT1 har vid normal blodbildning

och leukemi, vilket i framtiden förhoppningsvis skulle kunna leda fram till nya terapistrategier vid behandling av leukemi.

För att studera effekterna av WT1 och muterat WT1 protein i normal blodbildning så överuttrycktes dessa proteiner i stamceller. Det muterade WT1 protein som användes representerar en naturligt förekommande mutation som hittats i leukemipatienter. Denna mutation medför att WT1 proteinet saknar den del som kan bilda till DNA och på detta sätt aktivera andra gener. I dessa försök visade det sig att både WT1 och det muterade WT1, kunde minska tillväxten och stimulera utmognaden av de vita blodcellerna. Eftersom det muterade WT1 ej kan binda till DNA och aktivera gener, så tyder detta på att WT1 och muterat WT1 kan binda till andra proteiner som ger dessa effekter. De röda blodcellernas tillväxt minskades dock bara av WT1 och ej av muterat WT1 dvs dessa effekter verkar vara beroende av att WT1 kan binda till DNA för att aktivera vissa gener. Eftersom WT1s effekter verkar vara beroende av att dels kunna aktivera gener men också att binda till andra proteiner så var nästa steg att just försöka identifiera gener som WT1 reglerar samt leta efter proteiner som WT1 binder till. Jag hittade dels ett nytt protein, cofilin 1, som WT1 interagerade med och en gen, N-myc downstream regulated gene 2, som aktiverades av WT1. Jag har dock inte kunnat fastställa betydelsen av dessa fynd.
WT1 visade sig även minska genuttrycket av interferon regulatory factor 8 (IRF-8). Andra undersökningar har visat att om IRF-8 inte uttryckts i musstamceller, så utvecklar dessa möss kronisk myeloid leukemi (KML). BCR/ABL1 är ett annat protein som har visat sig vara betydelsefullt för utvecklingen av KML. Både tidigare och egna resultat visar att BCR/ABL1 även kan reglera WT1-uttrycket så att detta är högt i leukemicellerna. Dessa fynd tyckte jag var mycket intressanta och jag tror att det finns ett samband mellan dessa proteiner. Det skulle kunna vara så att BCR/ABL1 gör så att ett högt WT1 uttryck finns i leukemicellerna och WT1 trycker i sin tur ner uttrycket av IRF-8. Jag tror att detta samband bidrar till utvecklandet av leukemi och hoppas att dessa fynd i framtiden kan bidra till utveckling av nya behandlingsstrategier för leukemi.

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