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# WILMS' TUMOUR GENE 1 PROTEIN (WT1) – AN EFFECTOR IN LEUKEMOGENESIS?

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**LUND UNIVERSITY**  
Faculty of Medicine

**Thesis 2010**

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*”The journey of a thousand miles  
begins with one step”*

Lao Tzu (Chinese taoist), 600-531 BC



## LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals

- I. Svensson E\*, **Vidovic K\***, Lassen C, Richter J, Olofsson T, Fioretos T and Gullberg U. Deregulation of the Wilms' tumour gene 1 protein (WT1) by BCR/ABL1 mediates resistance to imatinib in human leukaemia cells. *Leukemia*, 21(12):2485-94, 2007 (\*equal contribution)
- II. **Vidovic K**, Svensson E, Nilsson B, Thuresson B, Olofsson T, Lennartsson A and Gullberg U. Wilms' tumour gene 1 (WT1) protein represses the expression of the tumour suppressor Interferon Regulatory Factor 8 in human hematopoietic progenitors and in leukemic cells. *Leukemia*, 24(5):992-1000, 2010
- III. Svensson E, **Vidovic K**, Olofsson T, Vallon-Christersson J, Borg A and Gullberg U. The Wilms' tumor gene 1 (WT1) induces expression of the N-myc downstream regulated gene 2 (NDRG2). *DNA Cell Biol.* 26:589-97, 2007
- IV. **Vidovic K**, Rosberg B, Olofsson T and Gullberg U. Leukemia – associated mutant WT1 protein promotes proliferation and erythroid differentiation of human hematopoietic progenitor cells. *Manuscript*

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## SELECTED ABBREVIATIONS

ABL1	Abelson murine leukemia viral oncogene homolog 1
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
BCR	Breakpoint cluster region
BLAST	Basic local alignment search tool
CD	Cluster of determination
ChIP	Chromatin immunoprecipitation
CML	Chronic myeloid leukemia
DAPI	4', 6-diamidino-2-phenylindole
FACS	Fluorescence activated cell sorting
GEO	Gene expression omnibus
GFP	Green fluorescence protein
G-CSF	Granulocyte colony-stimulating factor
HSC	Hematopoietic stem cell
IFN	Interferon
IRF8	Interferon regulatory factor 8
ISG15	Interferon stimulated gene of 15 kDa
mTOR	mammalian target of rapamycin
NCBI	National Center for Biotechnology Information
NDRG2	N-myc downstream regulated gene 2
PI3K	Phosphoinositide-3 kinase
RACE	Rapid amplification of cDNA ends
qPCR	quantitative polymerase chain reaction
STAT	Signal transducer and activator of transcription
WRE	WT1 response element
WT1	Wilms' tumour gene 1

## INTRODUCTION

The word leukemia originates from the Greek word *leukos*, meaning white and *haima* meaning blood. Patients suffering from leukemia often have high amounts of white blood cells sometimes as high as twenty-five times the normal value, because of accumulation of immature dysfunctional white blood cells in bone marrow and peripheral blood. These malignant blood cells may in some types of leukemia invade and enlarge organs and tissues as spleen, liver and lymph glands and patients may have symptoms of fatigue, sweating, growing thin, bleeding and infections.

The prevalence of newly diagnosed leukemias in Sweden is about 1150 cases per year, which represents 2.5% of the annual reported cancers in male and 2.0% of the reported cases in females. Leukemias are classified into myeloid or lymphoid depending on the differentiation status of the cells and they are further divided into chronic or acute leukemias. Acute leukemias, as the terminology implies, are quickly developed into deadly diseases if left untreated as compared to chronic leukemias, which have a more slow progress. Most chronic leukemias, however, finally enter a more progressive, acute phase.

The transition of a normal cell to a leukemic cell depends on genetic changes, leading to disturbed gene and protein function in the cell. Today, we have a large but yet incomplete knowledge about mutations, deletions, translocations and epigenetic changes involved in the development of leukemia. Importantly, obtained knowledge has in some cases led to novel ways to treat leukemia patients.

The Wilms' tumour gene 1 (WT1) is a gene that is frequently overexpressed in leukemia, and accordingly disturbed function of WT1 has been suggested to play a pathogenic role in the leukemic cell.

The aim of this thesis has been to increase our knowledge and understanding of the molecular mechanisms by which WT1 can be involved in leukemogenesis.

## BACKGROUND

### Wilms' tumour gene 1 (WT1)

#### *Historical Background*

Max Wilms (1867-1918) was a German surgeon who studied pediatric kidney tumours and a thorough review over his findings, *Die Mischgeschwülste der Niere*, was published in 1899. A certain kind of kidney cancer in children was named as Wilms' tumour. During operation of a French officer, an enemy prisoner of the World War I, Max Wilms was unfortunately infected with diphtheria and died in this disease. In 1990, almost 100 years after the publication of his monograph on the pathology of the childhood kidney tumours, two different research groups cloned the gene linked to a specific mutation, a deletion of chromosome region 11p13 found in 10% of the Wilms' tumour cases<sup>1,2</sup> and accordingly, the identified gene was named Wilms' tumour gene 1.



**Figure 1. Max Wilms (1867-1918)**

(Adopted from <http://pages.unibas.ch/alumni-medizin/wilms.jpg>)

#### *How WT1 is structured*

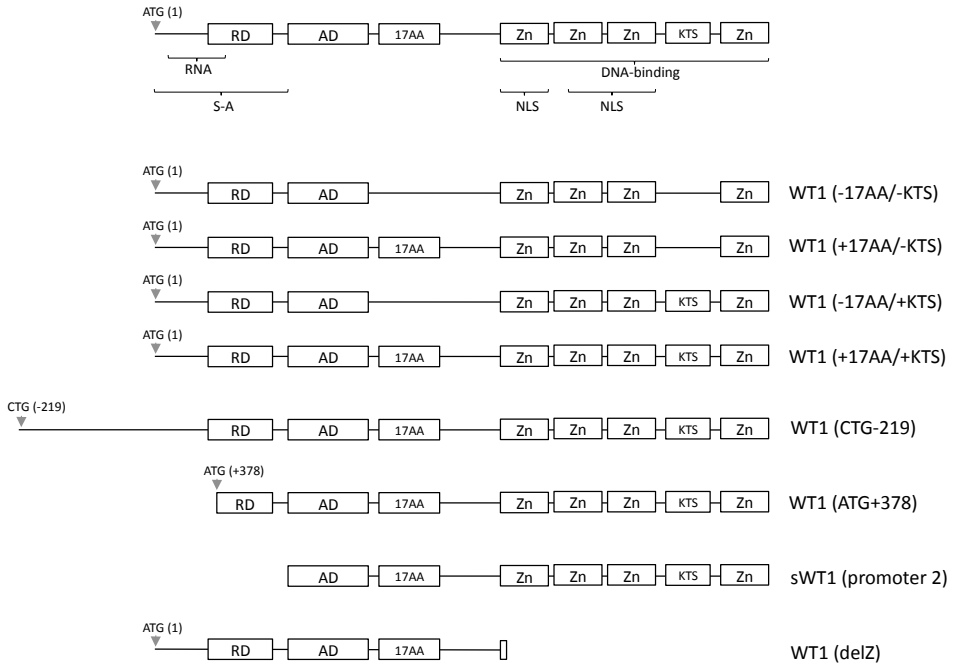
Wilms' tumour gene 1 (WT1) spans 50 kb genomic DNA and consists of 10 exons which encodes an mRNA transcript of about 3.2 kb. The mRNA translates into a 429 amino acid protein with a proline and glutamine rich amino terminus harbouring defined functional domains exerting transcriptional repression, activation, self-association, RNA-recognition and nuclear localization signals. The carboxyl terminal part of WT1 contains four Cys<sub>2</sub>His<sub>2</sub> zinc-fingers, encoded by exon 7-10, conferring specific DNA-binding. Due to

alternative splicing of the WT1 pre-mRNA there are four major isoforms: WT1 (-17AA/-KTS), WT1 (+17AA/-KTS), WT1 (-17AA/+KTS) and WT1 (+17AA/+KTS), ranging in size between 52-54 kDa (reviewed in Reddy and Licht, 1996)<sup>3</sup>.

The first alternative splicing event affects the entire exon 5 and leads to presence or absence of 17 amino acids (17AA). The second alternative splicing event generates an insertion/no insertion of three amino acids, lysine, threonine, serine (KTS), in the very end of exon nine, affecting the conformation of zinc-finger three and four in the WT1 protein. Additional functional WT1 protein isoforms are generated by RNA editing at codon 280, resulting in a replacement of leucine to proline<sup>4</sup>.

To further increase the complexity of WT1 activity and expression, there are also larger WT1 proteins, generated through a translation initiated at a CUG upstream of the initiator AUG<sup>5</sup>, and an evolutionary conserved internal translation initiation site at the second in-frame AUG of the WT1 mRNA with N-terminally truncated WT1 isoforms, ranging between 36-38 kDa, derived from this initiation site<sup>6</sup>.

A new isoform, termed sWT1 due to its small size (35-37 kDa), was identified a few years ago. A second promoter, located in intron one generates mRNA that codes for the sWT1 protein, which lacks the repression domain found in the N-terminus of the full-length protein. The sWT1 was reported to be overrepresented in leukemic samples and to confer oncogenic properties due to strong activation of certain WT1 target genes, as compared to full-length WT1<sup>7</sup>.



**Figure 2. Schematic picture over the structure and functional domains of WT1 protein (not drawn to scale).** Indicated in the figure are the repression domain (RD), activation domain (AD), RNA-binding domain (RNA), self-association domain (S-A), nuclear localization motifs (NLS) and the four zinc-fingers with DNA-binding capacity. The four major isoforms are shown, as well as isoforms generated by alternative translation start sites, and sWT1 generated by a second promoter. One mutant form of WT1, lacking the carboxyl terminal zinc-fingers is also shown. The numbers indicate the nucleotide position at the translation start site in the WT1 mRNAs. The translation start site for the major WT1 isoforms is defined as ATG(1).

### Regulation of the WT1 gene expression

The GC-rich WT1 promoter lacks a TATA box<sup>8,9</sup> and contains no CCAAT-motif<sup>10</sup>. The proximal promoter of the WT1 gene is transcriptionally active in several cell lines, independent of WT1 expression, and therefore tissue-specific expression of the WT1 gene must depend on additional regulatory elements<sup>10</sup>. The mechanisms for tissue-specific transcriptional regulation of WT1 are to a large extent unexplored.

The hematopoietic globin transcription factor 1, GATA-1, has been implied in the regulation of WT1 expression in hematopoietic cells<sup>11-13</sup>. In acute myeloid leukemias a correlation of PAX2 levels with WT1 expression has been observed<sup>12</sup>, which suggests PAX2 to be a WT1-activator in a subset of AMLs. PAX2 has previously been detected as a regulator of the WT1 promoter<sup>14,15</sup>. Pea3 (polyomavirus enhancer activator 3), a transcription factor co-expressed with WT1 in the developing kidney transactivates the WT1 promoter<sup>16</sup>. Other transcription factors reported to affect WT1 expression include Sp1<sup>17</sup>, WT1<sup>18</sup> (negative autoregulation), PAX8<sup>19</sup> and nuclear factor kappa B, NF- $\kappa$ B<sup>20</sup>.

A WT1 hematopoietic specific orientation-independent 3'-enhancer element activated by GATA-1 was identified in K562, HEL cells<sup>13</sup> and in acute leukemia samples, where a positive correlation between WT1 and GATA-1/GATA-2 mRNA levels was evident<sup>21</sup>. A second hematopoietic-specific enhancer sequence located in the region between the 3'-end of exon 2 and the beginning of intron 3 can be transactivated by GATA-1 and c-Myb. This intronic enhancer is more active in cells of myeloid lineage type, as compared to the 3'-enhancer, which is active in cells with erythroid phenotype<sup>11</sup>.

Transcription of WT1 is promoted by co-operation of p300, a histone acetyltransferase (HAT) and c-Myb, acting on the WT1 promoter and intronic enhancer<sup>22</sup>. A possible explanation for this regulation is acetylation of histones at these regions by p300, which facilitates transcription of the WT1 gene.

Gene regulation of WT1 through a WT1-antisense transcript complementary to sequences in exon 1 has been proposed to control the expression of WT1 in nephrogenesis<sup>18,23,24</sup>.

A transcriptional silencer, with requirement of Alu repeats, is located in the third intron of the WT1 gene and represses WT1 expression in cell lines of non-renal origin<sup>25</sup>.

In *paper I*, we investigated the effect of PI3K/Akt-signalling on WT1 mRNA-expression. In reporter experiments we detected a prominent repression of the WT1 promoter following PI3K inhibition. The greatest reduction was obtained with a vector construct containing promoter sequence and the 3'-enhancer element. Our data are consistent with involvement of GATA-1 in positive regulation of the WT1 promoter downstream of PI3K-signalling in leukemic cells.

### ***Post-translational modifications of WT1***

The WT1 protein has been proven to interact with the modifying E2-ligase, ubiquitin-conjugating enzyme 9 (Ubc9), now known as a SUMO-1 E2-ligase<sup>26</sup>. SUMO is an ubiquitin related protein, which is covalently attached to specific target proteins, resulting in alteration of their interaction with other proteins, altered localization and stability (reviewed in Wilkinson *et al.*, 2010)<sup>27</sup>. The SUMOylation and ubiquitination processes are biochemically similar and may in some cases function antagonistically, meaning that SUMOylation can counteract ubiquitination and thus prevent protein degradation. The aminoterminal part of WT1 has two consensus-binding sites for SUMO ( $\Psi$ -Lys-X), the lysine residues 73 and 177. Both sites were found to be SUMOylated, however with no functional consequences of SUMOylation identified. Also the nuclear speckled distribution of WT1 (+KTS) seems to be independent of the SUMOylation status of WT1<sup>28</sup>. Therefore, the functional consequences of SUMOylation (if any) remain to be revealed.

Phosphorylation at Ser-365 and Ser-393 in the C-terminal part of the WT1 protein by cAMP dependent protein kinase A (PKA) abolishes the DNA-binding ability, but the RNA binding function is maintained<sup>29,30</sup>. Retention of WT1 in the cytoplasm is detected after PKA-mediated enhanced phosphorylation of WT1<sup>31</sup>. This post-translational modification of WT1 therefore might be a way to modulate the function of WT1.

### ***The expression pattern and developmental role of WT1***

The ratio between +KTS and -KTS isoforms in normal fetus kidney tissues or in Wilms' tumours with no WT1 mutations is estimated to approximately 2:1<sup>32,33</sup>. The two protein isoforms have different distribution patterns in the nucleus with a more diffuse distribution pattern for the WT1 (-KTS) variants, as compared to the distinct speckled pattern for WT1 (+KTS) isoforms<sup>34</sup>. The WT1 (+KTS) variant is assumed to be involved in the pre-mRNA splicing machinery, since it is co-localized with small nucleoriboproteins in the poly (A)<sup>+</sup> fraction in cellular nuclear extracts<sup>34-37</sup>. The WT1 (+KTS) isoforms also mediate post-transcriptional regulation with enhanced translation from unspliced RNAs with a retained intron by promoting the association with polyribosomes<sup>38</sup>. *In vitro* studies confirm the expected preference for RNA-binding exerted by the WT1 (+KTS), as compared to the WT1 (-KTS) variants, which have a higher DNA binding affinity<sup>39</sup>, the latter confirmed in crystallization studies<sup>40</sup>.

The WT1 protein is a transcription factor with high nucleotide conservation among species. The WT1 (+KTS) and (-KTS) isoforms are present in species ranging from fish to mammals<sup>41-43</sup>. The ratio between the +KTS/-KTS isoforms is critical for proper gonadal and renal development and disturbance of this ratio causes intersex disorders<sup>44</sup>. Specific mutations within the zinc-finger region of the WT1 protein lead to dysregulated sexual and renal development as seen in Denys-Drash syndrome (DDS). A related condition is Frasier syndrome, which due to a mutation in the intron 9 alternative splice donor site, leads to loss of the WT1 (+KTS) isoform<sup>44,45</sup>.

The mammal-specific exon 5 (17AA) is present in mammals only, but seems not to be critical for normal development in mice<sup>46</sup>. The functional role of exon 5 is therefore unclear, but prostate apoptosis response factor 4 (par-4) interacts with the +17AA domain, which mediates conversion of the exon 5 domain into a transcriptional activation domain, possibly affecting cell survival and proliferation<sup>47</sup>. Constitutive expression of WT1 (+17AA) isoforms inhibited apoptosis in a leukemic cell line treated with cytotoxic agents<sup>48</sup>, which further indicates a survival mechanism conferred by these splice isoforms, at least in some cellular contexts.

Studies of the mRNA expression pattern of WT1 during development in the human, the rat and the mouse suggest WT1 to be a tissue-specific gene with spatial and temporal variation in expression levels during development, restricted to the urogenital system, spleen, heart, liver, thymus, mesenchymal structures and central nervous system (CNS) (reviewed in Scharnhorst *et al.* (2001); reviewed in Lee and Haber (2001)<sup>49,50</sup>. In addition, other studies implies that certain WT1 splice variants are predominantly expressed and have essential functions during development of the ganglion cells in the retina and the olfactory epithelium<sup>51,52</sup>.

The essential role for WT1 proteins in development is evident in WT1 knockout mice with both alleles inactivated, showing intrauterine death and a median survival time of less than 14 days *in utero*. The WT1<sup>-/-</sup> mice suffer from abnormalities of the heart and mesothelial structures and a complete failure of the kidney, gonads and spleen organogenesis<sup>53,54</sup>. The WT1<sup>-/-</sup> embryos show a higher degree of programmed cell death, as compared to normal embryos. Most likely, the failure of proper organogenesis depends, at least partially, on enhanced apoptosis.

In the adult, WT1 expression is limited to very few tissues, including podocytes in the kidney<sup>55</sup>, differentiating Sertoli cells in the testis<sup>56</sup> and to granulosa and

epithelial cells of the ovary<sup>49</sup>. WT1 protein expression is enhanced in early pregnancy during differentiation of human endometrial stromal cells into decidual cells<sup>57</sup>.

In adult hematopoiesis WT1 is only expressed in a small fraction of the CD34<sup>+</sup> progenitor cells<sup>58-61</sup>, with a rapid decline during differentiation, suggesting a role for WT1 in regulation of early hematopoietic progenitor cells. In the literature only one study report on detectable WT1 expression in mature blood cells<sup>62</sup>.

### ***WT1 and differentiation***

Besides preventing apoptosis during development, there are indications that WT1 directs differentiation of the developing cells. One example of this is during nerve cell development, where the WT1 protein levels decrease in response to differentiation induced nerve growth factor (NGF) expression. Reduced WT1 mediates less activation of its target gene the epidermal growth factor receptor (EGF-R), leading to lowered mitogenic EGF response, promoting differentiation rather than proliferation. This may indicate that WT1 is involved in neuronal differentiation<sup>63</sup>.

Another example is the insulin-like growth factor 1 receptor (IGF1-R), which is directly controlled at transcriptional level by the WT1 protein. Mutated or absent WT1 protein resulting in persistent IGF-1R expression may contribute to the development of Wilms' tumour, due to disturbed differentiation of the metanephrogenic blastema into renal epithelium<sup>64</sup>.

The effect of ectopic WT1 expression in differentiating human CD34<sup>+</sup> cells, extracted from umbilical cord blood, appears to be stage-specific in hematopoiesis, since quiescence is enhanced in primitive cells, but cellular differentiation in lineage-committed precursors into the myelo-monocytic lineage seems to be stimulated<sup>62,65</sup>. Thus, WT1 can potentiate differentiation in several contexts, and one mechanism may be to restrict cell division, which favours maturation of the cells.

### ***WT1 and cell cycle control***

WT1 is reported to both enhance and stall the cell cycle progression. For example, when WT1 is over-expressed at early to mid-G1 phase in mouse NIH 3T3 cells, the cell cycle is blocked in the S-phase, and one potential explanation for the blocked cell cycle progression is the ability of WT1 to alter expression

of genes encoding cyclins and cyclin dependent kinases (CDKs)<sup>66</sup>, and/or through transcription of p21<sup>cip1</sup><sup>67</sup>. WT1 directly represses transcription of the cyclin E gene<sup>68</sup>.

WT1 may also have positive effects on cell cycle control: in breast cancer cells expressing the HER2/neu oncogene, high levels of WT1 protein stimulates S-phase progression and cell proliferation<sup>69</sup>.

### ***WT1 and apoptosis***

There are reports of both negative and positive effects of WT1 on apoptosis. The way how WT1 mediates an anti-apoptotic function is unclear, but several suggestions have been proposed including transcriptional control of cell-surface receptors, such as the insulin-like growth factor receptor (IGF-1R)<sup>64,70</sup> and the epidermal growth factor receptor (EGF-R)<sup>63,71</sup>, which both are connected to survival signals and modulation of apoptosis regulating genes like bcl-2<sup>72</sup>, bak<sup>73</sup> and A1/BFL1<sup>74</sup>.

Further, WT1 has an ability to inhibit p53-mediated apoptosis, induced by irradiation through binding, stabilization and functional inactivation of the p53 protein<sup>75</sup>. A recent study underscores the anti-apoptotic activities of WT1, since proteolysis of WT1 by the serine protease HtrA2/Omi, mostly found in mitochondria but also a small fraction in the nucleus, generated increased apoptosis<sup>76</sup>.

The finding that ectopic WT1 in leukemic cell lines inhibits apoptosis induced by genotoxic agents<sup>48,72</sup>, as well as our finding in **paper I**, that K562 cells overexpressing WT1 have increased resistance to imatinib induced apoptosis<sup>77</sup> are in agreement with an anti-apoptotic function for WT1. Additional evidence for anti-apoptotic properties include the observations that elimination of WT1 in WT1<sup>-/-</sup> mice or treatment of some cells with WT1 antisense oligonucleotides or WT1 siRNA, leads to enhanced apoptosis<sup>48,78,79</sup>.

There are, however, also indications of a pro-apoptotic function for WT1, since forced expression of WT1 in a number of cell lines as Hep2B, U2OS and Saos-2 contributes to apoptosis<sup>71,80</sup>.

Taken together, available data support the complexity of the role and function of the WT1 protein in the regulation of differentiation, cell division and apoptosis mechanism. Some data support the notion of WT1 as a tumor suppressor, with an ability to rescue cells from transforming events, while other

results indicate a role for WT1 as a protein with a potential to keep the cells alive and proliferating, thus increasing the risk of malignant transformation.

### ***Target genes of WT1***

WT1 is a transcription factor that promotes gene activation or repression depending on cellular and promoter context<sup>81</sup>. WT1 shares extensive homology with the early growth response gene 1 (EGR1) and recognizes the same DNA binding consensus sequence<sup>82,83</sup>. Other identified DNA-binding consensus sequences for WT1 include TC-rich sequences, often found in promoter sequences from growth-related genes, and a 5'-GCGTGGGAGT-3' sequence. The latter is classified as a high affinity-binding site, termed WTE<sup>84</sup>. WT1, mainly the WT1 (-KTS) isoforms are reported to repress or activate a large number of genes. Data are from promoter-reporter assays and/or from studies of endogenous gene transcription levels. The target genes may be classified into 6 groups depending on their function in the cell:

- ***Growth factors*** as insulin-like growth factor II (IGF-2), erythropoietin and amphiregulin (important for kidney development) are activated<sup>70,85,86</sup> but connective tissue growth factor (CTGF) and platelet-derived growth factor A (PDGF-A) are repressed by WT1<sup>87,88</sup>. Colony-stimulating factor-1 (CSF-1), a multifunctional protein that affects proliferation and differentiation of myeloid progenitors, is also repressed by WT1<sup>89</sup>.
- ***Growth factor receptors*** as insulin-like growth factor 1 receptor (IGF-I-R), estrogen receptor- $\alpha$  (ERA) and epidermal growth factor receptor (EGF-R) are repressed by WT1<sup>90,91,71</sup>. The erythropoietin receptor is activated by WT1<sup>92</sup>.
- ***Cell cycle regulating proteins*** as c-myc, p21<sup>CIP1</sup> and retinoblastoma suppressor associated protein 46 (RbAp46) are activated<sup>93,94,67</sup>, but cyclin E and ornithine decarboxylase (ODC) are shown to be repressed by WT1<sup>68,95</sup>.
- ***Apoptosis regulating proteins*** upregulated by WT1 are the anti-apoptosis regulating bcl-2<sup>72</sup>, c-myc<sup>93,96</sup> and A1/BFL1

proteins<sup>74</sup>, the latter mediates resistance to chemotherapy in high-risk leukemias. The proto-oncogene JunB is also repressed by WT1<sup>97</sup>. The pro-apoptotic regulating protein, Bak, is also upregulated by WT1<sup>73</sup>.

- ***Proteins important for development*** as Dax-1, SRY and the anti-Müllerian hormone receptor 2 (Amhr2)<sup>98989898</sup>, which are important for sex-determination in mammals and are all activated by WT1<sup>99,100,98</sup>. Sprouty 1, a regulator of a tyrosine kinase receptor and nestin, an intermediate protein, are both involved in kidney development and they are activated by WT1<sup>101,102</sup>. Another target gene, Pou4f2, seems to be activated by WT1 simultaneously during kidney and retina formation<sup>103</sup>. A taurine gene, TauT, also linked to renal development is a direct target gene, activated by WT1<sup>104</sup>. A newly identified target gene, WT1-induced inhibitor of Dishevelled (WID), is co-expressed with WT1 in podocytes in the maturing kidney. Upregulation of WID by WT1 leads to a protein interaction between WID and Dishevelled, which negatively regulates the WNT/β-catenin pathway<sup>105</sup>. WT1 also regulates coronary vessel formation through activation of the TrkB neurotrophin receptor<sup>106</sup>.
- ***Other target genes*** are the vitamin D receptor (VDR), E-cadherin and Syndecan-1, all transcriptionally activated by WT1<sup>107,108,109</sup> and the last two mentioned genes are involved in epithelial differentiation and tumour invasion. WT1 has been shown to exert repression on human telomerase reverse transcriptase (hTERT)<sup>110</sup> and activation of the podocyte specific proteins, nephrine<sup>111</sup> and podocalyxin<sup>112</sup>.

In conclusion, WT1 mediates transcriptional regulation of various genes involved in divergent processes as growth, cell cycle control, development and apoptosis. Different reports with positive and negative effects of WT1 on target genes may seem conflicting. However, WT1-mediated suppression or activation of target genes is most likely explained by different cellular environment and co-operating oncogenes. Thus, it is possible that expression of each target gene of WT1 may be either positively, or negatively regulated by WT1, depending on cellular context.

In **paper II** we report the novel finding of WT1 to exert repression of the myeloid tumor suppressor gene interferon regulatory factor 8 (IRF8)<sup>113</sup> and in **paper III** we show that the WT1 protein acts as a transcriptional activator of the N-myc downstream regulated gene 2, NDRG2<sup>114</sup>.

### ***Protein partners of WT1***

Several interacting protein partners to WT1 have been identified by use of yeast two-hybrid assays, co-immunoprecipitations or GST pull-down assays. Interactions with the amino terminus, the zinc-finger containing carboxyl terminus or in some cases with both parts of the WT1 protein have been reported (**Table I**). The functional importance of these interactions is in many cases unclear, but it is interesting that WT1 lacking the entire DNA-binding zinc-finger C-terminal part still retains some cellular effects, indicating functions mediated through the N-terminal part of the WT1 protein, independent of DNA-binding<sup>115</sup> and as shown in **paper IV**.

Some of the genes transcriptionally regulated by WT1 express proteins that function as protein partners of WT1 with ability to enhance or prevent the transcriptional activity for WT1 in feed back loops. An example of this is the transcription factor SRY, involved in sex-determination and transcriptionally activated by WT1. The SRY protein is together with the SF-1 (steroidogenic factor 1) protein also an interacting partner with the WT1 protein, controlling the sexual differentiation process<sup>116,117</sup>. Another important protein synergy is the direct interaction between WT1 and the co-activator CREB-binding protein (CBP)<sup>118</sup>, which facilitates and modulates the transactivation of WT1 and may also contribute to the protein interaction/stability between WT1 and the tumor suppressor p53<sup>75,119</sup>.

An example of how protein hetero-complexes may alter the function of the individual proteins in the complex is the trimeric “adenovirus E1B 55K-WT1-p53” protein complex, sequestered in the perinuclear cytoplasmic body and with hindered transactivational abilities of both WT1 and p53<sup>120</sup>.

So far only one protein partner, prostate apoptosis response factor 4 (Par-4) has been identified to interact with the 17 amino acids in the N-terminal part of WT1 (+KTS). Par-4 functions as a transcriptional co-activator when bound to the 17 AA domain of WT1<sup>47</sup>, but it also interacts with the zinc-fingers in the C-terminal part of WT1 leading to decrease of transcriptional activation by WT1<sup>121,122</sup>.

Obviously, the reported opposing effects by WT1 on target genes and cellular functions may result from the presence of distinct protein partners in various cellular contexts.

***Table I. A selection of protein partners for WT1***

<b>Interacting protein</b>	<b>WT1 interacting domain</b>	<b>Function/consequences</b>
BASP1	residues 71-101, SD	transcriptional co-suppressor
HtrA2	residues 71-101, SD	serine protease/apoptosis-dependent cleavage of WT1
hUBC9	85-179 aa	SUMO-1 E2-conjugating enzyme/SUMOylation of WT1
Hsp70	1-180 aa	decreased proliferation
PAX2	1-466 aa	joint co-expression in renal development/unknown
STAT3	1-281 (TAD)	promotes cell proliferation
SF1	N-terminus	co-activates MIS expression with WT1 (-KTS)/regulation of sexual differentiation
WT1	1-180 aa	WT1 protein self-association /dominant negative effect with WT1 mutations?
Par-4	+17 AA Zn-fingers	involved in apoptosis/co-activator with WT1 (+17AA) and lowered transcriptional activity with Zn-finger interaction
BMZF2	Zn-fingers	TF regulating expression in fetal tissues/represses WT1 activation
CBP	Zn-fingers	transcriptional cofactor/enhances WT1 transcriptional activity
Ciao 1	Zn-fingers	WD40 protein /inhibition of WT1 transactivation, no influence on WT1 repression

Interacting protein	WT1 interacting domain	Function/ consequences
E1B55K	Zn-fingers	adenovirus protein/WT1 induced cell death is prohibited
HCMV-1E2	Zn-fingers	human cytomegalo virus protein/WT1 inhibits 1E2 transactivation ability
p53	Zn-fingers	tumor suppressor/p53 stabilization and inhibited apoptosis, inhibited WT1 activation
p73	Zn-fingers	cell cycle regulator, involved in apoptosis/inhibited transcriptional activity for p73 and WT1 (no DNA-binding)
SRY	Zn-fingers	regulator of sex determination/synergistic transcriptional activation
U2AF65	Zn-fingers	splicing factor/WT1 (+KTS) a component in pre-mRNA splicing
WTAP	Zn-fingers	Undetermined/WT1-interacting protein with unknown significance

**Abbreviations and references used but not clarified in Table I:**

*BASP1*<sup>123</sup> - Brain acid soluble protein 1, *HtrA2*<sup>76</sup> - High temperature requirement protein A2, *hUBC9*<sup>26,28</sup> - human Ubiquitin-conjugating enzyme 9, *Hsp 70*<sup>124</sup> - Heat shock protein 70, *PAX2*<sup>125</sup> - Paired box 2, *STAT3*<sup>126</sup> - Signal transducer and activator of transcription 3, *SF1*<sup>117</sup> - Steroidogenic factor 1, *WT1*<sup>127</sup> - Wilms' tumour gene 1, *Par-4*<sup>47,122</sup> - Prostate apoptosis response factor 4, *BMZF2*<sup>128</sup> - Bone marrow zinc finger 2, *CBP*<sup>118</sup> - CREB binding protein, *Ciao 1*<sup>129</sup> - Cytosolic iron-sulfur protein assembly 1 homolog, *E1B55K*<sup>120</sup>, *HCMV-1E2*<sup>130</sup>, *p53*<sup>75,119</sup>, *p73*<sup>131</sup>, *SRY*<sup>116</sup> - Sex-determining region of the Y chromosome, *U2AF65*<sup>132</sup>, *WTAP*<sup>133</sup> - WT1 associating protein.

**WT1- an oncogene?**

Taken together, WT1 is a transcription factor with a multitude of isoforms, which some are evolutionary conserved, like the WT1 (+KTS) and (-KTS) isoforms, while the splice isoforms with exon 5, WT1 (+ 17AA) is mammal

specific. WT1 expression increases and declines during development of a wide range of tissues, first studied in the kidney formation (reviewed in Reddy and Licht, 1996)<sup>3</sup>. Transcription factors linked to urogenital-, retina- and coronary vessel formations are transcriptionally regulated by WT1, further indicating a role for WT1 in normal development, but what role may WT1 have in carcinogenesis?

WT1 has been shown to regulate the cell cycle; introduction of cell cycle block in the S-phase through inhibition of the activity of cyclin/CDK complexes<sup>66</sup> and activation of p21<sup>CIP1</sup><sup>67</sup>, but also to enhance cell cycle progression in breast carcinoma cells, where high WT1 levels was induced by the HER2/neu oncogene<sup>69</sup>. Furthermore, down-modulation of WT1 was required for proliferative arrest and subsequent differentiation in breast cancer cells treated with progestins (synthetic analog of progesterone) with reduction of S-phase and cyclin D expression, while overexpression of WT1 in the cancer cells partly attenuated the progestin-mediated growth arrest<sup>134</sup>. These data may indicate that high levels of WT1 in breast cancer cell lines contribute to the pro-proliferative effect, and consequently suggest WT1 as a potential breast cancer oncogene.

STAT3, one of the protein partners for WT1 has impact on cell proliferation. WT1 enhanced activated STAT3 transcriptional activity through protein-interaction in cell lines, which was confirmed in primary Wilms' tumours<sup>126</sup>. The accelerated cell proliferation may contribute to tumourigenesis.

WT1 also exerts transcriptional regulation of growth factors (IGF-2, erythropoietin, amphiregulin, PDGF-A, CTGF, CSF-1) and growth factor receptors (IGF-1-R, EGFR) affecting cellular proliferation and differentiation. Inhibited apoptosis via activation of the WT1 target genes bcl-2, c-myc, JunB and A1/BFL1, and through p53 stabilization inactivation in the WT1/p53-protein complex, implies WT1-regulated cell survival.

Therefore, although WT1 was originally defined as a tumour suppressor gene in Wilms' tumour, WT1 may also positively affect carcinogenesis. This is supported by the accumulation of reports of adult cancers expressing high amounts of WT1, mainly in the cytoplasm. WT1 is found in tumour cells from a wide range of cancers, including renal cell carcinoma<sup>135</sup>, malignant melanoma<sup>136</sup>, ovarian cancer<sup>137</sup>, breast cancer<sup>138</sup>, lung cancer<sup>139</sup>, mesothelioma<sup>140</sup>, desmoid tumors<sup>141</sup>, brain tumors<sup>142</sup> and also in leukemia<sup>143,144</sup>. High levels of WT1 in these tissues, normally not expressing WT1, indicate an oncogene function. The altered cellular localization for WT1, from nucleus to cytoplasm, may reflect the oncogene potential. Promising results are achieved in patients

suffering from lung-, breast cancer or leukemia, when treated with WT1-specific cytotoxic T-cells, indicating WT1 as a tumour-specific target<sup>145</sup>. Importantly, mutations in the WT1 gene are rarely detected, arguing against loss of tumour suppressor function of WT1 in cancer.

## Hematopoiesis

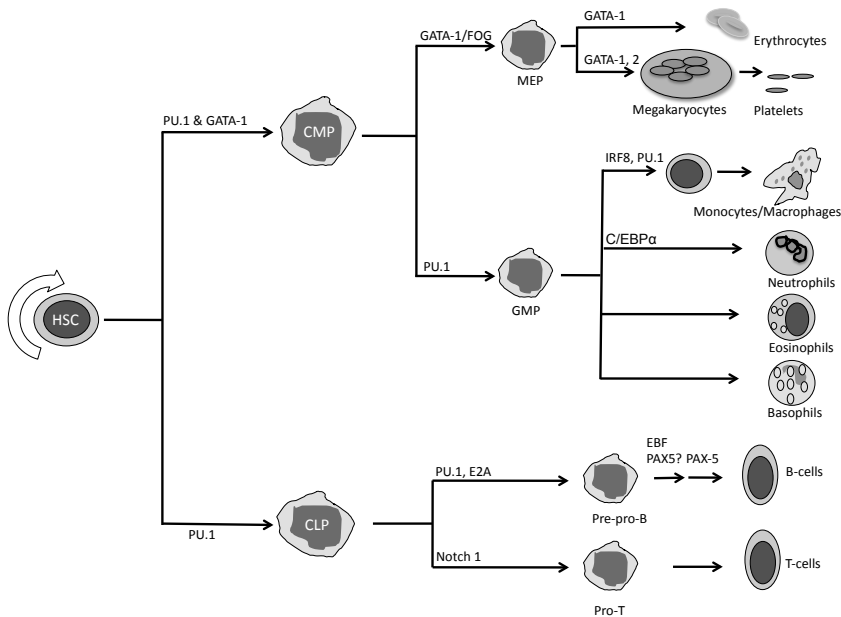
Hematopoiesis is the process of production of all blood cell, originating from a few hematopoietic stem cells (HSCs), which occurs in the bone marrow (BM) (reviewed in Zhu and Emerson, 2002; Kondo *et al.*, 2003)<sup>146,147</sup>. The HSCs with multipotent and “self-renewing” capacity, important for maintenance of the stem cell pool, yield committed progenitor cells through symmetric or asymmetric divisions. The important choice between self-renewal and differentiation of the slow cycling HSCs is controlled by both intrinsic and extrinsic regulation in the bone marrow microenvironment. Under normal conditions, the pool of HSC must remain roughly the same, with self-renewing in about half of the HSC divisions.

The genes that promote HSC self-renewal are to a large extent unexplored, but developmental regulators as Wnts<sup>148</sup>, Notch<sup>149,150</sup> and Sonic hedgehog (Shh)<sup>151</sup> have been shown to promote expansion of HSCs *in vitro*. Interestingly, WT1 has been reported to affect Wnt-signalling, but the relevance of this remains to be shown<sup>152,153</sup>.

The complex regulation of gene transcription throughout hematopoiesis is dependent on co-operation of transcription factors, cytokines and the microenvironment, the location of the HSCs (reviewed in Sigvardsson, 2009; Eliasson and Jönsson, 2010)<sup>154,155</sup>. Committed progenitor cells are directed to differentiate along the hematopoietic differentiation program to common myeloid progenitors (CMPs), further differentiating to granulocyte/monocyte progenitors (GMPs) or megakaryocyte/erythrocyte progenitors (MEPs) or the other main branch, the common lymphoid progenitors (CLPs). The CLPs generates T- and B-lymphocytes, whereas GMPs differentiate into neutrophils, basophils, eosinophils and monocytes/macrophages and MEPs give rise to erythrocytes and megakaryocytes/thrombocytes (fig 3).

Co-expression of GATA-1 and PU.1 directs HSCs to CMPs and elevated PU.1 levels lead to GMPs, whilst, dominant GATA-1 expression leads to differentiation of MEPs to erythrocytes and megakaryocytes. Excessed expression of PU.1 is required for differentiation to CLPs and differentiation to monocytes is induced by interaction of the myeloid transcription factor, IRF8,

and PU.1 (fig 3). IRF8 potentiates macrophage differentiation of myeloid progenitor cells and inhibits granulocytic differentiation<sup>156</sup>.



**Figure 3. A simplified picture of hematopoiesis.** HSCs with "self-renewing" and multipotent capacity are the origin of all differentiated blood cells. Key hematopoietic transcription factors relevant for the development of the lineages are indicated. Abbreviations used: HSC-hematopoietic stem cell, CMP-common myeloid progenitor, CLP-common lymphoid progenitor, MEP-megakaryocyte/erythrocyte progenitor, GMP- granulocyte/monocyte progenitor. (Modified from Zhu and Emerson, 2002)<sup>146</sup>.

## WT1 and hematopoiesis

### WT1 in hematopoiesis

In human hematopoiesis few of the multipotent progenitor cells (1.2% with equal distribution CD34<sup>+</sup> CD38<sup>+</sup>/CD34<sup>+</sup> CD38<sup>-</sup>) in the bone marrow express WT1 and during differentiation the expression is rapidly down modulated<sup>58-61</sup>. In a murine model, the highest WT1 levels were detected in megakaryocyte/erythrocyte progenitors (MEPs)<sup>157</sup>. In the terminally differentiated blood cells, WT1 seems to be below detection limit, although one paper report WT1 mRNA expression in monocytes, granulocytes and B-lymphocytes<sup>62</sup>.

WT1 is not essential for reconstitution of hematopoiesis, at least not in mice<sup>158</sup>, since embryonal stem cells (ES) devoid of WT1 can give rise to all types of mature blood cells. Neither was WT1 detected in long-term hematopoietic stem cells (LT-HSC) and in less than 1% of the multipotent progenitor cell in a transgenic mouse model with a GFP-knock-in allele, WT1<sup>GFP/+</sup>, at the WT1 gene locus<sup>157</sup>.

However, even though WT1 does not seem to be a prerequisite for hematopoiesis, an impact of WT1 is nevertheless evident, since murine WT1<sup>-/-</sup> ES compete poorly with normal ES in a competitive reconstitution assay<sup>159</sup>. In addition, increased numbers of BFU-E, CFU-GM and CFU-GEMM in the bone marrow cells overexpressing WT1 point to a positive impact on progenitor cell expansion mediated by WT1 in these cells<sup>160</sup>.

In human hematopoietic CD34<sup>+</sup> progenitor cells forced expression of WT1 lead to inhibited proliferation<sup>65,115</sup>, also confirmed in **paper IV**, but no alteration in cell cycle phase distribution was observed<sup>65</sup>. The effect of ectopic WT1 expression in differentiating human CD34<sup>+</sup> cells appears to be stage-specific; while quiescence is enhanced in primitive cells, cellular differentiation in lineage-committed precursors into the myeloid-monocytic hematopoietic lineage is stimulated<sup>62,65</sup>.

Taken together, available data indicate that WT1 is not critical for hematopoiesis, but that WT1 nevertheless may influence the proliferation, viability and/or differentiation of hematopoietic cells.

### **WT1 expression in primary leukemia**

In leukemic cells the normal maturation is stalled through a differentiation block, which gives rise to clonal expansion of immature leukemic blasts accumulating in bone marrow and blood. This leads to suppression of normal hematopoiesis.

WT1 is highly expressed in acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL), in myelodysplastic syndromes (MDS), chronic myeloid leukemia in blast crisis and in leukemic cell lines<sup>161</sup>. Eighty percent of the lymphoid leukemias and 90% of the myeloid leukemias, both adult- and childhood, have elevated WT1 expression<sup>143,144</sup>. The majority of AML patients express WT1 at diagnosis with frequencies varying from 73-93%<sup>162</sup> with a significant dominance of the WT1 (+17AA) isoform<sup>163</sup> and increased amount of WT1 at relapse. When combining four different studies, 66% of ALL cases

expressed WT1 at diagnosis<sup>164</sup>, while the prevalence of WT1 in the subgroup adult acute T-lymphoblast leukemia was even lower, approximately 55%<sup>165</sup>.

Some reports show no significant correlation between WT1 expression and different myeloid leukemia FAB subtypes<sup>163</sup>, while there are other reports indicating higher WT1 expression in M3 AML<sup>166</sup>, harbouring the PML/RAR $\alpha$  fusion protein, and less WT1 transcripts in M5 AML<sup>166-168</sup>. Depending on analysis method, the WT1 expression in leukemic blast cells have been quantified to be similar<sup>61,169</sup> or more than ten times as high as detected in the WT1 expressing CD34<sup>+</sup> progenitor cells<sup>170</sup>.

Thus, a majority of leukemias express high levels of WT1 with no strong correlation to phenotype, suggesting that WT1 may positively affect proliferation and/or viability of the leukemic cells, rather than interfere with specific differentiation mechanisms.

### ***Expression levels of WT1 correlate to poor clinical prognosis***

In acute leukemias increased WT1 levels have prognostic significance and are associated to poor prognosis<sup>143,171,172</sup>. In adult MDS and AML, WT1 mRNA levels in bone marrow at diagnosis may be used as a predictor of clinical outcome, since several studies show a positive correlation between high WT1 expression and a worse long-term outcome<sup>167,173,174</sup>, although not confirmed in all studies<sup>175</sup>. In pediatric ALL the prevalence of WT1 overexpression is lower than in AML or in adult AML and ALL. In fact, in childhood ALL the WT1 expression levels are very variable and both abnormally high and very low WT1 expression is associated with increased risk of relapse<sup>176</sup>.

During and after treatment, quantification of WT1 mRNA in peripheral blood (PB) is used as a measurement of minimal residual disease (MRD) in myeloid leukemias and in myelodysplastic syndrome<sup>177-180</sup>. Persistent high WT1 level is regarded as a prognostic predictor of poor outcome with a significant lower complete remission rate and worse overall survival<sup>167,181</sup>.

Moreover, WT1 levels rise as progression occurs in acute leukemias and myelodysplastic syndromes and at relapse of the disease after clinical remission<sup>181-184</sup>. Altogether, the positive correlation between WT1 expression and poor clinical outcome further support the notion of WT1 as an oncogene in leukemia.

### ***Immunotherapy targeting of WT1***

Higher titer of WT1 antibodies was detected in sera from patients suffering from AML, ALL, CML and MDS, as compared to control samples, which suggests an immune response with elevated WT1 as antigen<sup>185</sup>. Promising results with regression of the leukemic disease have been obtained in human vaccine trials in AML patients. WT1 peptides was used to generate CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) targeting the elevated WT1 protein<sup>145,186,187</sup>. The use of autologous T-cells response directed against the "self"-leukemia antigen, in this case WT1, resembles the graft-versus-leukemia (GVL) effect, which is the purpose of the therapeutic WT1 vaccines<sup>161</sup>.

### ***Effects of WT1-modulation in leukemic cell lines and animal models***

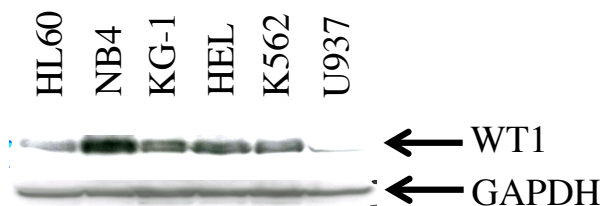
Down-regulation of WT1 gene expression in leukemic cell lines induces apoptosis<sup>78,188,189</sup>, indicating that WT1 is important for viability of leukemic cells. Moreover, constitutive expression of WT1 inhibits apoptosis in K562 leukemic cell line treated with etoposide and doxorubicin<sup>48</sup> (apoptosis inducing agents), further supporting that WT1 may antagonize cell death. Our own demonstration that WT1 confers resistance to imatinib in K562 cells (**paper I**) is consistent with this notion.

32D cl 3 cells, an IL-3 dependent murine myeloid cell line, ceased to differentiate in response to G-CSF administration when overexpressing WT1 and continued to proliferate due to constitutively active STAT3<sup>190</sup>. Also, WT1 negative monoblastic U937 cells had impaired differentiation responses to all-*trans*-retinoic acid (ATRA) and vitamin D3, when overexpressing WT1<sup>191</sup>, which suggests a WT1-induced phenotype resembling the differentiation block found in acute leukemia. The level of WT1 mRNA rapidly declined when K562, HL60 and NB4 cells were induced to differentiate in culture, independent on differentiation pathway<sup>192-195</sup>. This suggests that down-modulation of WT1 is a prerequisite for differentiation of leukemic cells.

In transgenic mice co-operation of WT1 and AML1-ETO protein (fusion protein, common in human AML) rapidly induces an acute myeloid leukemia, while induction with AML-ETO alone only generates a disturbed myelopoiesis<sup>160</sup>. A myeloproliferative-like disease develops in mice induced with WT1 alone<sup>160</sup>. This suggests that overexpression of WT1 together with other oncogenic events may play a co-operative role in leukemogenesis.

Murine bone marrow cells with induced constitutive expression of WT1 (+17 AA/+KTS) have inhibited differentiation and enhanced proliferation of myeloid progenitor cells in response to G-CSF<sup>196</sup>, indicating an alteration of this pathway in these cells, supporting the oncogenic role mediated by WT1 in hematopoietic cells.

Thus, a large amount of clinical and preclinical data indicate that WT1 functions like an oncogene in leukemogenesis, but the molecular mechanisms contributing to the elevated WT1 levels in leukemia and how WT1 perturbs normal cellular regulation are still to a large extent unknown.



**Figure 4. A Western blot showing screening of WT1 protein levels in different leukemic cell lines:** HL60 (human promyelocytic leukemia), NB4 (APL, M3 AML, with PML/RAR $\alpha$ ), KG-1 (human FAB-M7), HEL (human erythroleukemia), K562 (human erythroid blast crisis of CML, with BCR/ABL1) and U937 (human monoblastic cells). All cell lines with exception of U937 express the WT1 protein in various amounts. Detection of GAPDH protein is used as equal loading control. Antibodies used in the analysis; WT1(C-19), sc-192 and GAPDH (6C5), sc-32233, both from Santa Cruz Biotechnology.

### **WT1 mutations in leukemia**

Not only high WT1 expression, but also mutations in the WT1 gene are found in leukemias. The first report of WT1 mutations, associated with development of AML, was published more than fifteen years ago by King-Underwood and co-workers<sup>197</sup>. They found WT1 mutations in 15% of AML cases and in one biphenotypic leukemia and also noted that the patients with WT1 mutations were more refractory to chemotherapy with worse disease-free and overall survival rate<sup>198</sup>.

Since then there is a growing amount of data on WT1 mutations detected in various leukemias. Large cohorts of cytogenetically normal (CN) AML cases confirm the frequency of about 10% mutated WT1 in adult AML<sup>199-201</sup>. WT1

mutations are in general not found in conjunction with cytogenetic abnormalities such as t(15;17)(q22;q12), t(8;21)(q22;q22), inv(16)(p13q22) or t(16;16)(p13;q22)<sup>202</sup>, which have a relatively good prognosis. Other molecular markers with established prognostic significance, as mutations in NPM1 (nucleophosmin 1), CEBP $\alpha$  and MLL (myeloid/lymphoid or mixed-lineage leukemia) more or less frequently coincide with WT1 mutations<sup>200</sup>, but so far the synergistic impact is not thoroughly investigated.

Analysis of almost 2500 patients provide evidence of aggressive forms of the AML disease with increased risk of relapse and death associated with WT1 mutation (reviewed in Owen *et al.*, 2010)<sup>162</sup>. WT1 mutations in adult CN-AML appears as an independent unfavourable prognostic predictor, regarding inferior cytogenetic remission rate and higher frequency of resistance to chemotherapy.

In 58% of CN-AML patients, WT1 mutations and FLT3 with internal tandem duplication (FLT3-ITD) was concurrent with a possible worse clinical outcome<sup>162,203</sup>. In all childhood AML 12% harbour WT1 mutations, but the incidence in pediatric CN-AML is as high as 22%<sup>204</sup>, with elevated expression levels of mutated WT1 as compared to wild-type cases<sup>205</sup>.

WT1 mutations found in AML are mostly heterozygous, with a remaining WT1 wild-type allele. They are missense mutations, deletions and insertions, resulting in truncated WT1 protein<sup>201,204</sup> with loss of DNA-binding. The mutational “hotspots” are in exon 7 and 9, but rare mutations are also reported in exon 1, 2, 3 and 8<sup>198,203,204</sup>. Frameshift mutations in exon 7 are the most predominant<sup>162</sup> (fig 5). They generate a truncated WT1 protein lacking the four DNA-binding zinc-fingers and with loss of the nuclear localization signal (NLS). These WT1-mutants have been suggested to function in a dominant-negative way, if sequestered in the cytoplasm with remained self-association domains leading to retention of the wild-type WT1 in the cytoplasm. Sequestration of wild-type WT1 in cytoplasm by truncated WT1 could however not be confirmed, at least not in 293T/17 cells<sup>115</sup>. Heterozygous mutations could also lead to haploinsufficiency of WT1 function.

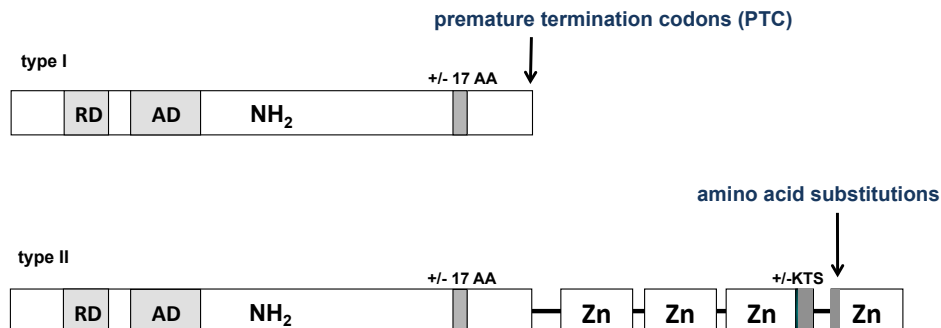
Other recurrent type of mutations in AML is point mutations in exon 9, conferring amino acid substitutions at similar positions as in Denys Drash syndrome (DDS)<sup>116</sup>, where the substitutions confer impaired DNA-binding. Frameshift mutations are less frequent than the point mutations in exon 9. These may lead to a WT1 protein deficient of zinc-finger 3 and 4.

The few reported homozygous WT1 mutations in AML are biallelic, through loss of heterozygosity due to somatic acquired uniparental disomy<sup>206</sup>, leading to removal of the remaining wild-type allele.

The incidence of WT1 mutations in T-ALL is 12% in adult and 13% in pediatric cases at diagnosis. Most of the detected T-ALL mutations are similar to those observed in AML, resulting in truncation of the zinc-finger domain of WT1. In adult- and childhood T-ALL WT1 mutations do not confer adverse prognosis<sup>207</sup>. Regarding WT1 mutations in pediatric B-ALL, no data are available in the literature.

Taken together, haploinsufficiency of WT1 as a result of WT1 mutations contributing to leukemic disease may seem in consistency with WT1 as a tumour suppressor. But, the high wild-type WT1 levels at diagnosis in most leukemias used as an independent prognostic factor for poor outcome, rather support an oncogenic function mediated by WT1. Furthermore, cell line and animal models strongly indicate an oncogenic property of wild-type WT1 in leukemia. If WT1 then is an oncogene, mutated WT1 may therefore show gain-of-function, being more oncogenic as compared with wild-type WT1. The co-existence of WT1-mutants and FLT3-ITD in CN-AML patients may possibly indicate a synergistic effect in leukemogenesis.

In **paper IV**, we show that ectopic expression of the WT1-mutant, WT1(delZ), devoid of zinc-fingers confers enhanced and prolonged proliferation of CD34<sup>+</sup> cells and induces erythroid differentiation. This may indicate new oncogenic properties through altered cell signalling cascades mediated through the WT1-mutant, regardless of DNA-binding.



**Figure 5.** A simplistic picture of the two main types of WT1 mutations found in leukemia (not drawn to scale). The type I mutations cluster in exon 7 and are frameshift mutations introducing a premature termination codon (PTC) which generates truncated WT1 protein lacking the four zinc-fingers and as a consequence loss of DNA-binding capacity. Type II mutations affecting exon nine are most frequently point mutations resulting in amino acid substitutions leading to impaired/changed DNA-binding. RD-repression domain, AD-activation domain.

## THE PRESENT INVESTIGATION

### AIM

The aim of this thesis has been to increase the understanding of the molecular mechanisms by which WT1 can be involved in normal hematopoiesis and leukemogenesis.

## RESULTS AND GENERAL DISCUSSION

### WT1 functions as an oncogene in leukemia

High WT1 levels detected at diagnosis in adult AML and MDS are associated with a poor clinical outcome and may be used as a prognostic predictor. Remaining excessive WT1 transcripts during and after treatment of the patients, increased levels at relapse and raised WT1 levels during disease progression indicate an impact on differentiation, proliferation and/or cell survival mediated by WT1 (reviewed in Owen *et al.*, 2009; Algar, 2002)<sup>162,208</sup>.

A negative effect of WT1 on differentiation is indicated also by results from leukemic cell line models, in which WT1 transcripts rapidly decrease during induced differentiation<sup>193-195</sup>. Moreover, forced WT1 expression in leukemic cell lines can inhibit the differentiation response<sup>134,191,192</sup>.

The pro-survival function of WT1 is confirmed by data from leukemic cell lines, in which down-modulation of WT1 leads to apoptosis<sup>48,78,79</sup>. Whereas, constitutive overexpression of WT1 in the leukemic K562 cell line confers resistance to etoposide and doxorubicin induced apoptosis<sup>48</sup> and also to increased resistance to imatinib induced cell death, as shown by us in **paper I**. A number of anti-apoptotic genes; bcl-2, c-myc, JunB and A1/BFL1 have been reported as target genes for WT1<sup>72,93,96,97,74</sup>. Among these we could not detect any effects on bcl-2 (**paper I**). Among other apoptosis-related genes associated to imatinib-resistance, we could also not detect any effect on pro-apoptotic Bim, anti-apoptotic bcl-X<sub>L</sub> (a bcl-2 homologue) or Survivin by WT1 (unpublished). The mechanism, by which WT1 confers resistance to imatinib, therefore remains unclear.

Murine BM cells lacking WT1 fail to compete with normal BM cells in a competitive repopulation transplantation assay. Furthermore, in a transgenic mouse model, WT1 transduced BM transplanted to mice generates a myeloproliferative disease. Both observations support the notion of positive effects on proliferation. Mice receiving a transplant of AML1-ETO transduced BM alone show abnormal myelopoiesis, with undifferentiated myeloid cells, but no leukemia. WT1 in conjunction with AML1-ETO, however, rapidly induces an AML-like disease, when co-expressed in the transgenic mice<sup>160</sup>, further demonstrating the oncogenic potential for WT1 *in vivo*.

Put together, a growing amount of experimental data argue for that WT1 can influence differentiation, apoptosis and proliferation in leukemic cells and thus that WT1 has an effector role in leukemogenesis.

### ***Molecular mechanisms for overexpression of WT1 in leukemia***

As aforementioned, cancers of various kinds as kidney cancer<sup>135</sup>, skin cancer<sup>136</sup>, ovarian cancer<sup>137</sup>, breast cancer<sup>138</sup>, mesothelioma<sup>140</sup>, desmoid tumors<sup>141</sup>, lung cancer<sup>139</sup> and brain tumors<sup>142</sup> overexpress wild-type WT1. Also a variety of hematological malignancies as AML, ALL, MDS and CML at blast crisis show high levels of unmutated WT1 at diagnosis, with increasing levels when transition to a more aggressive leukemic disease occurs (reviewed in Miwa *et al.*, 1992; Miyagi *et al.*, 1993)<sup>143,144</sup>. This suggests an important role for WT1 in

carcinogenesis and leukemogenesis. The overexpression of WT1 in cancer and leukemia occurs at transcriptional level. Consistently, reduced mRNA level through WT1-target specific antisense oligomers or siRNAs confers growth inhibition and may induce apoptosis, both in cell lines and in primary leukemic samples<sup>48,78,79</sup>.

What is the molecular mechanism behind overexpression of WT1 mRNA in leukemia? The mechanisms regulating transcription of WT1 to restricted organs are yet quite unknown. Regulation of transcription from the WT1 gene is complex: a proximal promoter lacking tissue specificity and tissue-specific enhancer elements control it. During kidney formation endogenous WT1-antisense expression and a transcriptional silencer, restrict promoter activity to nephronal cells<sup>16,18,23-25</sup>. Pea3, PAX2, PAX8, Sp1 and WT1 itself are all identified transcription factors mainly linked to development in the fetal kidney<sup>15-19,125</sup>.

The large numbers of different leukemias overexpressing WT1, however, indicate a common mechanism, driving transcription of the WT1 gene in these cells. GATA-1 and the proto-oncoprotein c-Myb have been shown to transactivate a myeloid associated enhancer element in the WT1 gene in *in vitro* studies<sup>11</sup> and in AML a correlation between increased PAX2 and WT1 transcripts have been reported<sup>12</sup>, with a possible PAX2 activation of the WT1 gene.

Cilloni and colleagues reported that inhibition of BCR/ABL1 tyrosine kinase activity generated lowered amounts of WT1 transcripts in Philadelphia chromosome (Ph) positive cell lines and CML patient samples<sup>209</sup>. A correlation between BCR/ABL1 activity and WT1 was confirmed in another study<sup>210</sup>, although one recent report showed low overall correlation of WT1 and BCR/ABL1 transcripts in primary leukemias<sup>211</sup>.

Ph-positive cells harbour the translocation between the ABL1 gene on chromosome 9 and the BCR (breakpoint cluster) gene on chromosome 22, t(9;22)(q34;q11). Depending on the breakpoint in the BCR-gene, the translocation results in a fusion protein (BCR/ABL1) of 210 kDa detected in 90% of CMLs or a protein of 190 kDa, restricted to Ph-positive ALLs (reviewed in Wong and Witte, 2004)<sup>212</sup>. In BCR/ABL1 the ABL tyrosine kinase is constitutively active and mediates oncogenic signalling. BCR/ABL1 can promote cell proliferation and survival via SHP2 affecting RAS/ERK (which may also be activated by BCR phosphorylation) and phosphatidylinositol 3-kinase (PI3K)-Akt cell signalling pathways. The activated pathways lead to

activation of downstream targets as JAK/STATs, Cyclin D, c-myc and NFκB (reviewed in Sattler and Salgia, 1997; Ren, 2005)<sup>213,214</sup>. Further, we have shown that forced expression of BCR/ABL1 kinase in human progenitor cells represses IRF8 transcription (**paper II**), via a hitherto unknown mechanism, as discussed below.

Imatinib mesylate (imatinib) is a potent inhibitor of the ABL kinase activity in the BCR/ABL1 fusion protein. Imatinib binds to an ATP-site in the activation domain of the kinase, inhibiting target substrate binding and further activation. More than 80% of the CML patients achieve a complete cytogenetic response when treated with imatinib in the chronic phase of the CML<sup>213</sup>. Excessive WT1 levels are not reported in the chronic phase. However, with time progression often occurs from the chronic phase of the CML into blast crisis, and as earlier mentioned, this progression of the disease is correlated to raised WT1 expression levels.

The finding that BCR/ABL1 tyrosine kinase confers raised WT1 levels made us investigate possible involved pathways. In **paper I**, we confirmed the down-modulation of WT1 expression as a result of imatinib treatment in CML cell lines. Through inactivation of potential target proteins in cell signalling cascades downstream BCR/ABL1 tyrosine kinase, we identified the PI3K/Akt pathway as a main mediator of WT1 transcription. PI3K is a heterodimer consisting of a p85 regulatory and a p110 catalytic subunit. The p110 interacts with protein tyrosine kinases and tyrosine-phosphorylated proteins. We also found that the regulation of WT1 through PI3K/Akt pathway occurs at the transcriptional level and is not due to alteration in mRNA stability mediated by PI3K. Transduction of human progenitor cells with ectopic expression of BCR/ABL1 induced raised WT1 mRNA and protein, further confirming the link between BCR/ABL1 and WT1.

Thus, tyrosine kinase signalling from BCR/ABL1 via PI3K/Akt pathway is an obvious mechanism responsible for WT1 overexpression in CML. However, BCR/ABL1 only exists in CML and other mechanisms therefore must be operation in other forms of leukemia. Could it be that several distinct tyrosine kinases serve as the general activating mechanism of WT1 transcription in leukemia?

Her2/neu (erbB2) oncogene is a transmembrane glycoprotein with intrinsic tyrosine kinase activity that have been proven to enhance the expression of WT1 in breast cancer cells through Akt activation<sup>69</sup>. Akt phosphorylation leads to increased proliferation and anti-apoptosis, possibly through WT1

transcriptional activation of cyclin D and bcl-2. Although not in leukemic cells, these observations support the notion of oncogenic tyrosine kinase signalling as a general mechanism for WT1 overexpression.

Also, as aforementioned, FLT3-ITD and WT1 mutations coincide in a subset of CN-AMLs (58%) and this may indicate a synergistic oncogenic potential in leukemic cells. Is there a correlation also between wild-type WT1 and FLT3-ITD? The constitutively active tyrosine kinase of FLT3-ITD is present in a large fraction of AML and this led us to investigate if FLT3-ITD tyrosine kinase activity also affects WT1 levels in leukemic cells.

FLT3 (Fms-like tyrosine kinase 3) is a cytokine receptor, which is expressed at 70-100% in AML and to a lesser extent in ALL. The FLT3-ITD oncogene with its constitutively activating internal tandem duplication mutation of the receptor tyrosine kinase confers an unfavourable prognosis in AML. The prevalence of FLT3-ITD in AML is around 20%, and the other main mutation found in FLT3, the D835 mutation also conferring constitutive activity, is found in 7% of the patients. FLT3-ITD is suggested to be involved in the pathogenesis of AML, possibly promoting survival through phosphorylation of BAD. The FLT3-ITD-receptor is activated via dimerization and it potentiates the MEK/ERK and PI3K/Akt pathway (reviewed in Gilliland and Griffin, 2002; Meshinchi and Appelbaum, 2009)<sup>215,216</sup>.

We used the FLT3-ITD expressing MV4-11 cell line (AML) as an experimental model for leukemic cells dependent on the constitutive tyrosine kinase activity and HL60 cells as negative control cells expressing FLT3 wild-type. The FLT3 inhibitor Tyrphostin AG1296 (6,7-Dimethoxy-3-phenylquinoxaline) was used to inhibit the tyrosine kinase of the FLT3-ITD mutation. Initial control experiments were performed to evaluate the potency of the AG1296 compound and to control the used cell lines, regarding responsiveness to the kinase inhibitor and toxicity. AG1296 is not entirely specific for the FLT3 ligand receptor, but also inhibits the PDGF receptor and stem cell factor receptor (c-kit), which is important to notice.

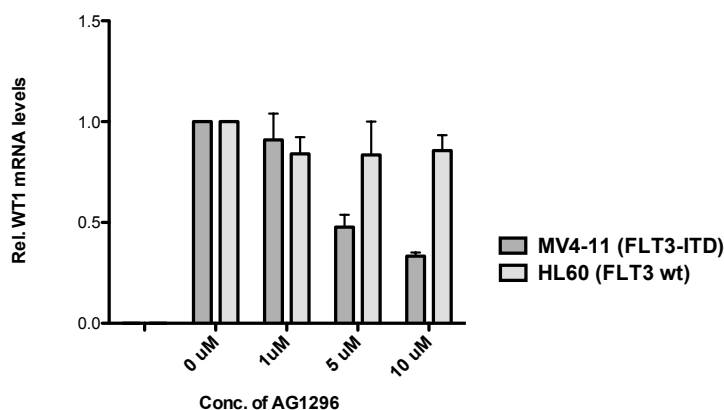
We found that inhibition of FLT3-ITD activity in MV4-11 cells results in reduced levels of WT1 mRNA and protein, but not in control cells (HL60 cells) (unpublished data, Fig 6). This suggests that FLT3-ITD tyrosine kinase activity drives transcriptional activation of WT1 in leukemic cells. To evaluate the importance of WT1 expression for the leukemic phenotype, we generated MV4-11 cells with forced WT1 expression. Interestingly, these cells were more resistant to AG1296 induced anti-proliferative effects, as compared to MV4-11

control cells (unpublished data, Table II). Viability was always above 90%, indicating that toxic effects of the kinase inhibitor were not present in this cellular model. The results indicate that oncogenic FLT3-ITD drives overexpression of WT1 in leukemic cells, and that WT1 contributes to the leukemic phenotype, in a manner similar to what is observed with BCR/ABL1 positive cells (**paper I**).

In **paper I** we analysed transcription from the WT1 gene using K562 cells transfected with WT1 promoter reporter constructs, with the WT1 promoter alone or in conjunction with the hematopoietic-specific 3' enhancer (erythroid) and/or intron 3 enhancer (myeloid) elements. The reporters were activated by BCR/ABL1-PI3K/Akt signalling, as judged by inhibition of promoter activity by a PI3K inhibitor. Inhibition was most prominent in the presence of enhancer elements. GATA-1 sites are present in both enhancer sequences, suggesting GATA-1 as a possible transacting factor responsible for the elevated WT1 levels in the BCR/ABL1, PI3K/Akt cell signalling axis.

In line with this hypothesis, GATA-1, an essential transcription factor for erythropoiesis, is phosphorylated and activated via the PI3K/Akt pathway. Post-translational modification of GATA-1, phosphorylation via the PI3K/Akt signalling pathway, promoted GATA-1 transcriptional activity in erythroid cell maturation in co-operation with activated Akt<sup>217</sup>. In **paper I** the WT1 mRNA levels induced in progenitor cells with forced BCR/ABL1 expression did not correlate to the GATA-1 levels, which continued to raise when the WT1 transcripts were declining. Further, no correlation between GATA-1 mRNA and WT1 mRNA are found in studies of AML cases with high WT1 levels<sup>12,166</sup>, nor in MDS or ALL<sup>166</sup>. This might indicate that post-transcriptional regulation of GATA-1 is important for WT1 expression, but the precise role of GATA-1 in WT1 expression in leukemic cells remains to be determined.

In conclusion, tyrosine kinase activity of BCR/ABL1 in CML, FLT3-ITD in AML and Her2/neu in breast cancers and possibly other tyrosine kinases in several forms of tumours, may be one of the common upstream regulatory molecular mechanisms for increased WT1 levels in neoplasms. Transcription factors as GATA-1, c-myb and PAX2 have been suggested to be mediators of transactivation of the WT1 promoter. However, given their tissue-specific expression, they are unlikely the overall mechanism for WT1 expression in all malignant cells. Therefore, other transcription factors driving WT1 expression in malignant cells probably remain to be identified.



**Figure 6.** *WT1 mRNA levels are decreased after 6 hours induction with the FLT3 inhibitor Tyrphostin AG1296 (6,7-Dimethoxy-3-phenylquinoxaline) in FLT3-ITD positive MV4-11 cells but not in HL60 control cells. Various concentrations; 1 μM, 5 μM and 10 μM of AG1296 was used on the MV4-11 (FLT3-ITD) and HL60 (FLT3 wt) cells and real-time qPCR analysis was performed in triplicates. GAPDH mRNA values were used as calibrator. Relative values are shown, mean value ± SEM.*

**Table II.**

	Rel. Proliferation (Cell titer assay)	Proliferation (Total number of cells)	Viability (Trypan blue exclusion)
<b>MV4-11/MIG</b> Control	100%	1.40 x 10 <sup>6</sup> /ml	95%
<b>MV4-11/MIG</b> 10 μM AG1296	84%	0.75 x 10 <sup>6</sup> /ml	92%
<b>MV4-11/WT1</b> Control	100%	1.30 x 10 <sup>6</sup> /ml	90%
<b>MV4-11/WT1</b> 10 μM AG1296	106%	1.25 x 10 <sup>6</sup> /ml	91%

**Table II.** *MV4-11 cells overexpressing WT1 were cultured with or without 10 μM AG1296 for 48 hours after which determination of the total number of viable cells was performed using CellTiter96®AQ<sub>ueous</sub> One Solution Cell Proliferation Assay, cell counting and trypan blue exclusion. As shown in the table, the MV4-11/WT1 cell culture was more resistant to the decreased proliferation induced by AG1296 than MV4-11/MIG (empty vector). Mean values from one experiment performed in triplicates are shown.*

### ***What are the molecular consequences of overexpressed WT1?***

As outlined above, clinical and experimental data strongly indicate that WT1 is important for the leukemic phenotype. WT1 has a multitude of identified potential target genes with proposed oncogenic ability. WT1 represses Cyclin E<sup>68</sup>, a key regulator of the cell cycle that activates cdk2 leading to Rb phosphorylation and progression from G<sub>1</sub> into S-phase. A majority of AMLs have overexpressed cyclin E<sup>218</sup>, which might seem inconsistent with a WT1-mediated repression. However, it is possible that when binding to the cyclin E promoter, WT1 might have repressing or activating effects depending on cellular context. Furthermore, 10% of adult AMLs express high levels of mutated WT1 that might have dominant negative actions<sup>199-201</sup>, possibly inhibiting a WT1-mediated repression of the cyclin E promoter in AML with mutated WT1.

In cancers where aberrant high WT1 expression is detected, the WT1 target gene bcl-2<sup>72</sup> is upregulated, suggesting that bcl-2 mediates increased viability downstream of WT1. In consistence with this, other studies show that WT1 overexpression is associated with chemotherapy resistance, due to repression of the pro-apoptotic gene Bak and induction of bcl-2<sup>48</sup>, suggesting an oncogenic cell survival advantage of WT1 expressing cells. However, in **paper I** we could not confirm upregulation of bcl-2 in association with WT1-mediated resistance to imatinib in CML-cells.

WT1 is suggested to be able to repress JunB<sup>97</sup> in *in vitro* studies, thus promoting survival. Interestingly, in AML JunB expression is down-modulated in HSC<sup>219</sup>, raising the question if this is partly due to WT1 repression of the JunB gene? In breast cancer cells overexpression of WT1 induces upregulation of the proto-oncogene c-myc<sup>93</sup> and also repression of the estrogen receptor-A (ERA)<sup>91</sup> leading to positive effects on survival. Transcriptional activation of the anti-apoptotic A1/BFL1 gene by WT1 conferred resistance to chemotherapy in a cancer cell line and a correlation between A1/BFL1 and WT1 was also identified in primary AML samples<sup>74</sup>, suggesting a co-operative role in drug resistant leukemias.

In conclusion, there are a number of potential target genes of WT1 that might contribute to the oncogenic effect of WT1 in leukemia, but the exact underlying molecular mechanisms mediated by WT1 in leukemogenesis, the effector role for WT1, still needs to be made clear.

### ***WT1 down-modulates IRF8***

In **paper II**, we show that the WT1 protein directly represses transcription of the interferon regulatory factor 8 gene.

Interferon regulatory factor 8 (IRF8), also designated interferon consensus sequence binding protein (ICSBP), belongs to the interferon response factor family with IRF1 to IRF10 identified so far. IRFs contain a conserved N-terminal DNA-binding domain (DBD) that recognizes DNA sequences with IFN-stimulated response elements (ISREs), and a C-terminal IRF association domain (IAD). IRF8 binds to ISRE when interacting with other IRF proteins, like IRF1 and IRF2 and also to various Ets/IRF composite elements, when interacting with the Ets family transcription factor, PU.1 (reviewed in Ozato *et al.*, 2007)<sup>220</sup>. Phosphorylation of specific tyrosine residues is essential for efficient protein-protein interaction. The IRF8 promoter contains a GAS-site activated by Interferon- $\gamma$  (IFN- $\gamma$ ) that binds STAT1<sup>221</sup> and a NF $\kappa$ B-site induced by lipopolysaccharide (LPS)<sup>222</sup>. In addition to IFN- $\gamma$ , also IFN- $\alpha$  activates the IRF8 promoter<sup>223</sup>.

IRF8 is predominantly expressed in hematopoietic cells. The stem cell population and early progenitor cells express IRF8 and it is also detected in cells of the immune system with high levels in macrophages, mature B-cells and dendritic cells (DCs). The expression is low in granulocytes and resting T-cells (reviewed in Tamura *et al.* 2008)<sup>224</sup>. A recent report implies IRF8 to be critical for development of eosinophils<sup>225</sup>. IRF8 and IRF1 co-operatively activate expression of interleukin 12 (IL-12), important for T-cells response, and mice lacking IRF8 are susceptible to intracellular pathogens, due to defect IL-12 production supporting the role of IRF8 in the immune system (reviewed in Tamura *et al.*, 2008)<sup>224</sup>. Most interestingly, IRF8 deficient mice also develop a myeloproliferative CML-like disorder<sup>226</sup>. IRF8 regulates myeloid cell development; macrophage differentiation is stimulated, while granulocyte differentiation is inhibited, in both cases cell growth is decreased (reviewed in Tamura and Ozato, 2002)<sup>156</sup>. A negative effect of IRF8 on proliferation is consistent with the effects on transcriptional activation of neurofibromin 1, which in turn inactivates Ras<sup>227,228</sup>. IRF8 functions as a positive regulator of apoptosis in leukemic U937 cells<sup>229</sup>. Most human myeloid leukemias have reduced or no IRF8 transcripts.

Given (i) the high expression of WT1 and low expression of IRF8 in most leukemias, (ii) the tumour suppressor like activity of IRF8, and (iii) the report of

BCR/ABL1-mediated down-modulation of IRF8 expression in murine cells<sup>231</sup>, led us to speculate that WT1 might be a general repressor of IRF8 in leukemia. In **paper II** we present evidence for this: we report a negative correlation between WT1 and IRF8 mRNA levels in primary AML and CML samples and show that WT1 represses mRNA and protein levels of IRF8 in hematopoietic progenitor cells.

By what mechanism does WT1 repress IRF8? We found no proof of WT1 induced silencing of IRF8 through promoted methylation of the IRF8 promoter (**paper II**). Bisulfite sequencing was made in a CpG-rich region of the IRF8 promoter, which is methylated in some cancers<sup>232,233</sup>. WT1 binds directly to a WT1-binding site in the IRF8 promoter, showed as modest transcriptional repression of IRF8 activity in a reporter assay, and convincingly demonstrated by chromatin-immunoprecipitation (ChIP) analysis. We cannot exclude, however, that also other regions of the IRF8 gene might be involved in the response to WT1. Repression of IRF8 by WT1 is probably not part of a general WT1 associated down-modulation of interferon-responsive genes, since IRF8 transcripts were specifically decreased when also IRF1, IRF4 and ISG15 mRNA levels were quantified. In contrast to IRF8 the other tested genes showed even raised transcript levels in response to WT1. As expected, the WT1 mutant, WT1(delZ) with no DNA-binding capacity, had no influence on the analysed interferon-responsive gene transcripts, consistent with promoter binding and transcriptional regulation by full-length WT1.

To evaluate whether high WT1 levels are associated with low IRF8 expression in clinical samples, we compiled a compendium of four leukemia microarray data sets from primary AML and CML samples<sup>234-237</sup> and found a relatively strong anti-correlation between WT1 and IRF8 mRNA levels supporting that WT1 acts as a repressor of IRF8 in leukemia. This led us to investigate whether down-modulation of WT1 is a prerequisite for interferon-induced upregulation of IRF8. However, IFN- $\gamma$  induction of IRF8 mRNA in a CML cell line (K562) conferred no alteration of WT1 transcript (data not shown), which made us conclude that IFN- $\gamma$  induced IRF8 is not dependent on changed WT1 expression, at least not in these cells.

In a murine model, forced expression of BCR/ABL1 led to a CML-like disease with significantly lowered IRF8 protein<sup>231</sup>, and importantly, the induced leukemic phenotype could be counteracted by ectopic expression of IRF8, both *in vivo* and *in vitro*<sup>231,238,239</sup>. The underlying mechanism by which IRF8 was repressed by BCR/ABL1 was not identified. In our BCR/ABL1 transduced

human progenitor cells, WT1 expression was increased (**paper I**) and IRF8 levels were decreased (**paper II**). WT1 expression may also be induced by other tyrosine kinases like leukemic FLT3-ITD (Fig 6).

Given the findings by us and others, we propose that WT1 is the missing link in the BCR/ABL1-IRF8 axis.

What are the consequences of repressed IRF8? IRF8 is implied to be a myeloid tumour suppressor, evidenced by several observations; (i) a high percentage of human AML and CML lack IRF8 transcripts<sup>230</sup>, (ii) both homozygous and heterozygous elimination of IRF8 generate a myeloproliferative disorder to varying degree, pointing to a tumour suppressor gene showing haploinsufficiency<sup>226</sup>, (iii) in the IRF8<sup>-/-</sup> mice, the hematological disturbance, a CML-like disease, can be counteracted by forced expression of IRF8<sup>231,238,239</sup>, (iv) IRF8 deficient mice develop acute leukemias in collaboration with NUP98-TOP1 or AML1-ETO fusion genes, with oncogenic signalling from SHP2 tyrosine phosphatase, with forced expression of Meis1/Meis3<sup>240</sup> or with neurofibromin 1 (NF1) haploinsufficiency<sup>241</sup>. Therefore, a WT1 mediated repression of IRF8 is potentially a general mechanism by which WT1 confers leukemogenic effects in several forms of leukemia.

In myeloid cells IRF8 negatively regulates c-myc through induction of B-lymphocyte-induced maturation protein-1 (Blimp-1) and mitogenic Ets (METS) and directly stimulates expression of inhibitor of cyclin-dependent kinase p15<sup>Ink4b</sup> (reviewed in Tamura and Ozato, 2002)<sup>156</sup>, leading to inhibition of cell division. In myeloid cells, genes associated to granulocyte differentiation such as G-CSF receptor, C/EBP $\alpha$  and C/EBP $\epsilon$  are down-modulated after IRF8 transduction, whereas, Egr-1 a transcription factor linked to differentiation of macrophages is strongly induced by IRF8<sup>156</sup>. This reflects a role of IRF8 in lineage selection. IRF8 modulates survival of myeloid cells through repression of the anti-apoptosis associated proteins bcl-X<sub>L</sub> and bcl-2.

Further, the PTPN13 gene that is repressed by IRF8, encodes a Fas-associated phosphatase 1 (Fap-1) that negatively effects Fas phosphorylation and Fas-mediated apoptosis. In complex with IRF2 and PU.1, IRF8 activates NF1 expression leading to suppressed Ras signalling, that decreases proliferative response to cytokines and prevents proliferation of myeloid cells (reviewed in Tamura *et al.*, 2008)<sup>224</sup>.

A functional tumour suppressor role for IRF8 in other malignancies than leukemia is also implied, since IRF8 is epigenetically silenced in a multitude of carcinomas. Methylation of the IRF8 promoter leading to disruption of IFN- $\gamma$

stimulation of IRF8, was found in nasopharyngeal, esophageal, breast, cervical, lung and colorectal cancers, while forced expression of IRF8 inhibited carcinoma cell clonogenicity<sup>242</sup>.

### ***Effects of WT1 on expression of the NDRG2 gene***

To screen for additional potential WT1-target genes in leukemic cells with high WT1 levels, we performed an oligonucleotide array analysis on total-RNA from sorted WT1-transduced CD34<sup>+</sup> human progenitor cells and empty vector transduced control cells. The used vector contains an internal ribosomal entry site (IRES) and the sequence for enhanced green fluorescent protein (eGFP) is expressed bicistronically with the cloned WT1 cDNA. The transduced CD34<sup>+</sup> cells were sorted for GFP<sup>+</sup>, since GFP is used as a marker for cells expressing the gene of interest. The generated probe-signals in control cells and WT1-transduced cells were compared. Candidate mRNAs that showed altered expression in the WT1-transduced progenitor cells, as compared to control cells, were further analysed by real-time qPCR, to confirm or reject new potential WT1-target genes. Among the genes confirmed as upregulated by WT1, we chose the N-myc downstream regulated gene 2 (NDRG2) for further investigation (**paper III**).

NDRG2 is a candidate tumour suppressor gene that belongs to the N-myc downregulated gene family NDRG1 to NDRG4, and despite the name NDRG2, it is not negatively regulated by N-myc<sup>243</sup>. NDRG2 is a cytosolic protein, widely expressed, especially in brain where it has a putative role in neuronal differentiation<sup>244</sup> and upregulation of NDRG2 is associated with Alzheimer's disease<sup>245</sup>. NDRG2 is also expressed in heart, skeletal muscle and kidney<sup>246,247</sup>. Induced expression of the NDRG2 protein in dendritic cells (DC) during differentiation<sup>248</sup> and specific expression of NDRG2 in macrophages, in contrast to neutrophil specific expression of NDRG1, implies a distinct role for NDRG2 in myeloid differentiation<sup>249</sup>. Recently, a report showed that NDRG2 regulated IL-10 production in myeloid cells through inhibitory mechanisms of p38 MAPK, with the underlying signal cascade yet unknown<sup>250</sup>.

Oncogenic consequences of upregulation of NDRG2 by enforced WT1 expression are not obvious from reported effects in some experimental models; NDRG2 is functioning as a candidate tumour suppressor gene in glioblastoma, where very low NDRG2 expression was detected as compared to normal brain, and with prohibited cell division when forced NDRG2 was introduced into the cells<sup>251</sup>. Interestingly however, NDRG2 has been identified as one of the genes

specific for the hematopoietic stem cell signature, associated with the quiescent state of the HSCs<sup>252</sup>. Both NDRG2 and WT1 have been suggested to be involved in the Wnt-signalling pathway. NDRG2 modulation of intracellular  $\beta$ -catenin with effects on T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcriptional activation of Wnt target genes have been reported in human carcinomas<sup>253</sup>. WT1 has been proposed to regulate Wnt components during kidney development<sup>152,153</sup> and deregulation of this pathway is probably involved in the pathogenesis of Wilms' tumour (reviewed in Nusse, 2007)<sup>254</sup>.

It is tempting to speculate that high WT1 levels in early hematopoietic stem cells affects the self-renewing capacity involving upregulation of NDRG2 in the quiescent HSCs. WT1 via NDRG2 may hypothetically affect Wnt-signalling, leading to leukemogenesis through activation of target genes promoting proliferation in the Wnt signalling pathway, as cyclin D1 and/or c-Myc? The absence of effects from forced expression of NDRG2 in colony formation assays of CD34<sup>+</sup> progenitor cells (**paper III**) might indicate that stem cells are not investigated, or that NDRG2 alone is not sufficient for effects. These issues need, however, to be further investigated.

### *Is WT1(del Z) an oncogenic gain-of-function mutation?*

As referred to in a previous passage, about 10% of the cytogenetically normal adult AMLs show presence of heterozygous WT1-mutations, which is an adverse independent prognostic predictor, regarding response to chemotherapy treatment and an indicator of increased risk of relapse and death<sup>199-201</sup>. The predominant mutations are frameshift mutations, a consequence of small insertions or deletions in exon 7, which introduce a premature stop-codon leading to a truncated WT1 protein devoid of zinc-fingers. The other main types of mutations in the WT1 gene are amino acid substitutions, due to point mutations, affecting exon nine. These types of mutations result in impaired/changed DNA-binding capacity for the WT1-mutant, resembling the altered zinc-finger configuration found in Denys-Drash syndrome<sup>116</sup>.

The relevance for protein expression of the insertion/deletion mutations in exon 7 of the WT1 gene detected in AMLs have been questioned in a recent report<sup>202</sup>. AML cases with WT1 mutations giving rise to premature termination codons (PTCs) produced WT1 transcripts that were degraded by translation-mediated nonsense-mediated RNA decay (NMD)<sup>202</sup>. NMD is a cellular control mechanism that functions to prevent translation of truncated and defect proteins by degradation of mRNA with PTCs<sup>255</sup>. The authors claim that most WT1

transcripts with PTCs are degraded and not expressing the predicted truncated WT1 protein<sup>202</sup>. However, in debate of this are studies with detected mRNA transcripts in primary childhood AML and pediatric and adult T-ALL cases harbouring WT1 mutations in exon 7<sup>204,205,207</sup>, suggesting that not all out-of-frame WT1 mutations are prone to the nonsense-mediated RNA decay. Indeed, in pediatric AML the cases harbouring WT1 mutations in exon 7 expressed significantly higher mutant WT1 mRNA levels than WT1 wild-type cases<sup>205</sup>.

Oncogenic effects mediated by truncated WT1 proteins are indicated because of the association with WT1 mutations to poor clinical outcome. If the mutant WT1 mRNA was degraded by NMD, the oncogenic effect must be dependent on a tumour suppressor function and haploinsufficiency of wild-type WT1. However, as described above, several lines of experimental evidence rather support an oncogenic function of WT1 in leukemia. NMD of mutant WT1 mRNA is not compatible with oncogenic effects of full-length WT1. Rather, aggravated or new oncogenic functions of mutant WT1 are more consistent with existing data. The most abundant WT1 mutation encoded protein, with no DNA-binding capacity, have an intact N-terminal part with functional domains and may possess changed properties. The full-length WT1 protein may be bound to the WT1-mutant via the self-association domain and this may lead to effects on remaining full-length WT1. The WT1-mutant may also compete with wild-type WT1 protein in sequestering of critical co-factors important for various transcriptional regulations, such as CBP/p300.

Does the truncated WT1-mutant with lack of zinc-fingers then indeed have oncogenic properties? In **paper IV**, we demonstrate that human progenitor cells with forced expression of a WT1-mutant devoid of the zinc-finger domain, WT1(delZ), show prolonged and increased proliferation in suspension cultures and increased clonogenic potential in methylcellulose cultures, as compared to wild-type WT1-expressing cells or to control cells. The WT1(delZ) expressing cells had enhanced proliferation rate both in absence or presence of added erythropoietin. Interestingly, the WT1(delZ) expressing cells without added erythropoietin differentiated towards an erythroid phenotype, as judged by erythroid clonogenic potential, elevated amounts of adult hemoglobin (HbA) and CD45 negativity (CD45 is expressed on all hematopoietic cells except erythrocytes and platelets). This suggests that WT1(delZ) activates some signalling pathways downstream of the erythropoietin receptor (EpoR). Apparently, WT1(delZ) did not fully substitute for erythropoietin signalling, since mRNA levels of the erythroid-specific transcription factors GATA-1,

erythroid Krüppel-like factor (EKL) and TAL-1/SCL were not affected by WT1(delZ), although they did respond to added erythropoietin.

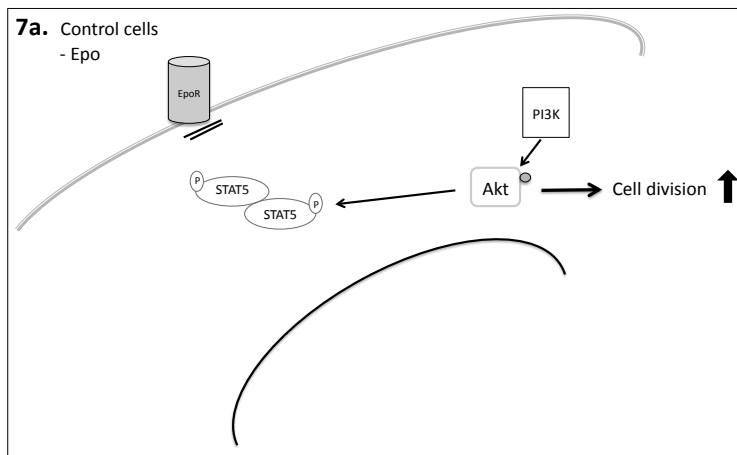
In the presence of added erythropoietin, HbA levels were raised in the WT1-expressing progenitor cells after two weeks in culture, possibly as a result of upregulation of the EpoR, a target gene activated by WT1<sup>92</sup>. In consistence with absence of transcriptional activation capacity, levels of the EpoR were not affected by the WT1(delZ) mutant. Notably, however, WT1(delZ) expressing cells induced with erythropoietin showed less erythroid differentiation (as judged by hemoglobin content), when compared to control cells. Therefore, data indicate that WT1(delZ) inhibits differentiating signals from the EpoR in the presence of erythropoietin, but in the absence of erythropoietin WT1(delZ) induces some signalling for erythroid differentiation. These effects of WT1(delZ) might appear contradictory to one another. We hypothesize that WT1(delZ) binds to specific proteins in the cytoplasm, since previous findings have shown a predominant cytoplasmic localization of the WT1-mutant<sup>115</sup>. One candidate for WT1 binding in the context of signals for erythroid differentiation is STAT5, which is a transcription factor involved in the cell signalling cascade downstream the EpoR and which is critical for proper erythroid differentiation (reviewed in Hennighausen and Robinson, 2008)<sup>256</sup>. Indeed, we found elevated STAT5A and STAT5B levels and phospho-STAT5A/B in WT1(delZ) transduced cells, even in the absence of erythropoietin (**paper IV**). The raised amount of STAT5 protein was not correlated to an increase of STAT5 mRNA, indicating a post-translational stabilization of STAT5 protein by WT1(delZ). The N-terminal part of full-length WT1 has previously been shown to directly interact with activated STAT3, thus promoting cell proliferation, probably through upregulation of cyclin D1 and bcl-X<sub>L</sub><sup>126</sup>. Besides its function as a nuclear transcription factor, it was recently reported that cytoplasmic activated STAT5 participates in a PI3K/Akt pathway leading to cell proliferation<sup>257</sup>. STAT5 is to a large extent localized to the cytoplasm in myeloid leukemias, consistent with a cytoplasmic function in enhancement of proliferation in those cells<sup>257</sup>. We speculate on that WT1(delZ) binds and stabilizes STAT5 in the cytoplasm, thus increasing signals for cell division, which might explain the increased proliferation of WT1(delZ) expressing cells. Further we speculate that the raised STAT5 levels contribute to some increase of nuclear STAT5, explaining the slight increase of erythroid differentiation in the absence of exogenous erythropoietin. How can it then be explained that WT1(delZ) rather inhibits erythroid maturation in the presence of erythropoietin? Hypothetically, a sequestering of STAT5 in the cytoplasm could interfere with the prominent

nuclear translocation of STAT5, induced by EpoR-signalling, thus interfering with differentiation inducing signals (but not with signals promoting cell division). A speculative model on signals from cytoplasmic and nuclear STAT5 and how they are affected by EpoR-signalling and by WT1(delZ) is presented in fig 7.

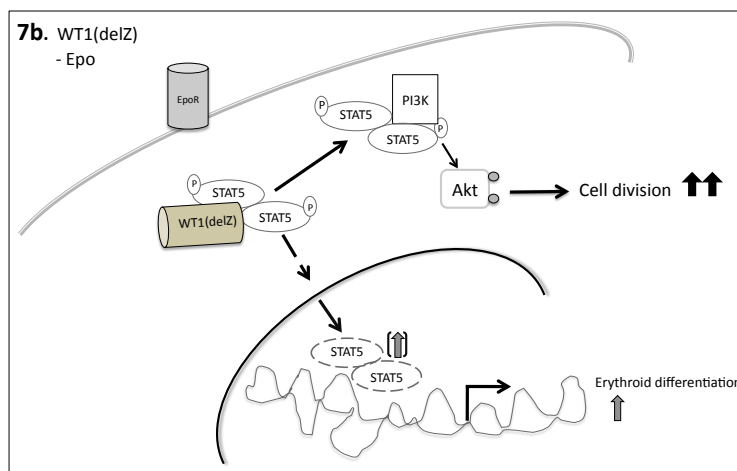
Other observations also support a functional role of mutated WT1 in hematopoietic cells: expression of a mutant WT1, truncated within zinc-finger three in embryonic stem cells resulted in delayed hematopoiesis into myeloid lineages in an *in vitro* study, suggesting impairment of hematopoietic development mediated by the WT1-mutant<sup>258</sup>. In accordance with this, our observation in **paper IV**, that WT1(delZ) expressing progenitor cells showed more sustained CD33 positivity, may reflect a delayed maturation. Is it possible that such a delay in progenitor cell differentiation may confer prolonged proliferation and an enhanced risk of genetic instabilities, leading to increased risk for leukemia development?

In conclusion, the novel properties of WT1(delZ) mediating increased and prolonged cell division capacity detected in our progenitor cells, the lineage choice towards erythroid differentiation and the sustained maturation shown by us and others<sup>258</sup> is in accordance with a gain-of-function for the WT1-mutant. It might be that full-length WT1 normally has a role to play in this context (the highest expression of WT1 was found in megakaryocyte/erythrocyte progenitor cells (MEPs)<sup>61</sup>) and that the oncogenic potential of WT1(delZ) depends on an increase of the cytoplasmic function of WT1, while a nuclear function in regulation of transcription of target genes is lost.

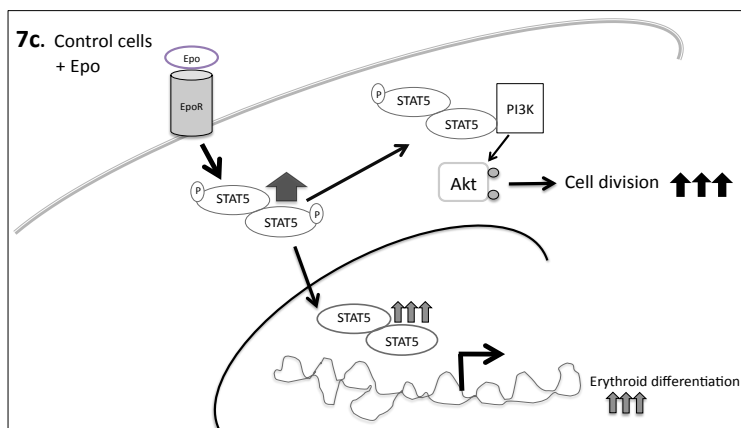
**Figure 7. Highly speculative models on signals from cytoplasmic and nuclear STAT5 and how they are affected by WT1(delZ).** STAT5 is to a large extent localized to the cytoplasm and cytoplasmic activated STAT5 participates in a PI3K/Akt cell signalling pathway. The presence of WT1(delZ) in the human progenitor cells confers altered phenotype as compared to control cells; erythropoietin (Epo) induced canonical signal may be affected regarding cell division and nuclear STAT5 transcriptional activity leading to perturbed erythroid differentiation programme induction.



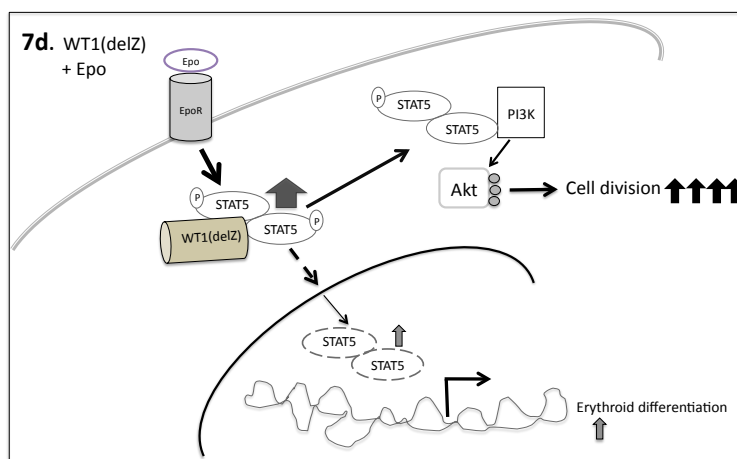
**Figure 7a.** In control cells with no exogenous Epo induction, cytoplasmic activated STAT5 participates in a PI3K/Akt pathway leading to cell division.



**Figure 7b.** We speculate on that WT1(delZ) binds and stabilizes STAT5 in the cytoplasm, thus increasing signals for cell division, which is a possible explanation for the increased proliferation of WT1(delZ) expressing progenitor cells. Raised STAT5 levels may contribute to some increase of nuclear STAT5, explaining the slight increase of erythroid differentiation in the absence of introduced exogenous Epo.



**Figure 7c.** A canonical Epo-induced signalling from the Epo-receptor confers both increased amounts of cytoplasmic activated STAT5, that binds to PI3K leading to enhanced cell division through the PI3K/Akt pathway, and a prominent nuclear translocation of STAT5 giving raise to strong erythroid differentiation induction in the control cells.



**Figure 7d.** We speculate on that canonical Epo-induced signalling from the Epo-receptor mediates increased cytoplasmic activated STAT5, stabilized by WT1(delZ) binding, thus increasing signals for cell division. A hypothetical explanation for the inhibited erythroid maturation in the WT1(delZ) expressing progenitor cells is that the sequestering of STAT5 in the cytoplasm interferes with the nuclear translocation of STAT5 induced by Epo-R signalling, thus interfering with erythroid differentiation signals (but not with signals promoting cell division).

## CONCLUSIONS

In this thesis project leukemic cell lines and primary human CD34<sup>+</sup> progenitor cells, extracted from umbilical cord blood, were used as experimental model system to characterize the impact and molecular mechanisms of WT1 and the WT1-mutant, WT1(delZ) in hematopoietic cells. The main conclusions of the present study may be summarized as follows:

- Oncogenic BCR/ABL1 tyrosine kinase activity in chronic myeloid leukemia (CML) cells induces high WT1 mRNA and protein expression via the PI3K/Akt pathway.
- Forced expression of WT1 in CML cells provides resistance against cytotoxic effects induced by the ABL tyrosine kinase inhibitor imatinib.
- Oncogenic FLT3-ITD tyrosine kinase activity confers enhanced WT1 levels and forced expression of WT1 in FLT3-ITD expressing cells mediates resistance to decreased proliferation induced by the FLT3 inhibitor, AG1296.
- WT1 decreases the expression of the myeloid tumour suppressor interferon regulatory factor 8 (IRF8) by direct binding to the IRF8 promoter.
- An anticorrelation between WT1 mRNA and IRF8 mRNA is present in leukemic cell lines and in mRNA expression data sets from primary leukemias.
- N-myc downstream regulated gene 2 (NDRG2) is induced by WT1 and is a putative WT1 target gene.
- Expression of the leukemia-associated WT1-mutant WT1(delZ) in human progenitor cells promotes proliferation, increased clonogenic growth, and erythroid differentiation, indicating oncogenic effects.
- STAT5 protein levels are increased in the WT1(delZ) expressing progenitor cells at the post-transcriptional level, suggesting stabilization of STAT5 as a possible oncogenic mechanism for WT1(delZ).

## SVENSK SAMMANFATTNING (SUMMARY IN SWEDISH)

### *Hematopoes och leukemi*

Hematopoes är benämningen på den process varigenom alla blodceller genom hela livet kontinuerligt utvecklas i benmärgen, samt vid bildandet av lymfocyter även i lymfkörtlarna. Varje dygn bildas det ca  $10^{12}$  nya celler i benmärgen för att ersätta uttjänta blodceller som dör och bryts ned. Ett litet antal stamceller i benmärgen ansvarar för att hematopoesen kan fortgå. Nivån av stamceller hålls konstant genom att de vid celldelning bildar antingen nya stamceller eller förstadier till blodceller, som i en utmognadsprocess slutligen blir mogna röda blodkroppar (erytrocyter), vita blodkroppar (granulocyter, monocyter och lymfocyter) och blodplättar (trombocyter).

Hematopoesen regleras i ett komplext system med samspel mellan speciella proteiner som benämns tillväxtfaktorer och transkriptionsfaktorer, vilka är proteiner som binder till gener i cellernas DNA och aktiverar eller förhindrar uttryck från generna. De mogna blodcellerna transporteras ut i kroppen för att utföra sina specifika funktioner. De röda blodcellerna är syretransportörer. Vita blodceller är viktiga i immunförsvaret vid infektioner och blodplättarna är nödvändiga för att förhindra blödningar och deltar i blodets koagulering.

Leukemi är samlingsnamn för en rad elakartade cancerformer som drabbar de blodbildande cellerna. Omvandlingen från en normal cell till en leukemisk cell beror på uppkomna genetiska förändringar i de hematopoietiska stamcellerna eller i tidiga hematopoietiska celler, vilket leder till förhindrad utmognad av funktionella blodceller och en ansamling av vita omogna blodceller i benmärgen och i blodet. Obehandlade akuta leukemier leder snabbt till döden på grund av den kraftiga expansionen av leukemiceller i benmärgen och till följd av detta få normala blodceller i blodet. Patienterna lider ofta av blodbrist och stor infektionskänslighet. Akuta leukemier drabbar både barn och vuxna, medan kronisk leukemi förekommer främst bland vuxna. Kroniska leukemier omvandlas ofta med tiden till mer aggressiva, akuta leukemier. Troligen orsakas denna utveckling av ytterligare genetiska avvikelser med förändringar i funktionen för transkriptionsfaktorer samt aktivering av andra cellsignaleringsvägar.

Trots att det idag finns relativt mycket kunskap om genetiska förändringar som är förknippade med utveckling av leukemi, så är de faktorer som styr omvandlingen från en normal cell till en leukemisk cell till stor del fortfarande inte klargjorda.

## ***Wilms' tumör gen 1 och leukemi***

Wilms' tumör gen 1 (WT1), är en transkriptionsfaktor som uttrycks i en liten andel av tidiga stamceller, minskar under utmognaden av blodcellerna och som inte återfinns i normala mogna blodceller. Detta skulle kunna innebära att en minskning av WT1 proteinet under utmognad är det normala och är nödvändigt för att tillåta utmognadsprocessen.

Många leukemier hos både barn och vuxna har höga nivåer av WT1 vid diagnostillfället, vilket ofta innebär en sämre prognos för patienten. Genetiska förändringar, mutationer, i WT1 genen återfinns i en del av leukemierna, vilket innebär en ökad risk för resistens mot behandling. WT1-mutationerna leder ofta till ett WT1 protein som saknar den proteindelen som kan binda till DNA, vilket innebär att mutanten inte kan reglera de gener som WT1 normalt binder till. Förändrade egenskaper och förvärvade nya egenskaper för WT1-mutanten kanske bidrar till utmognadsblockaden och ger WT1 en onkogen roll i utvecklingen av leukemi.

## ***Min forskning***

För att undersöka effekter av WT1 och mekanismer för hur WT1 påverkar celldelning och utmognad av blodceller, så överuttrycktes WT1 och WT1-mutanten i tidiga omogna celler. I mina försök medförde WT1-mutanten, som inte kan binda till DNA, en förlängd ökad celltillväxt och utmognad i den riktning som leder till bildning av röda blodceller. Normalt WT1 medförde däremot minskad tillväxt av både vita och röda blodceller. Den exakta mekanismen som styr WT1-mutantens påverkan på celltillväxten är ännu inte klargjord, men jag har resultat som indikerar att transkriptionsfaktorn STAT5 kan vara involverad i denna process.

För att försöka identifiera gener som WT1 reglerar i leukemier med högt WT1 uttryck, gjordes en analys av förändrade genuttryck i humana tidiga blodceller med överuttryckt WT1 jämfört med kontrollceller. Jag hittade en gen som aktiveras av WT1, N-myc downstream regulated gene 2 (NDRG2). Betydelsen av detta fynd är inte helt uppenbar, men NDRG2 har senare visat sig vara en av de gener som styr hematopoetiska stamceller. Detta skulle kunna innebära att WT1 via NDRG2-reglering indirekt kan påverka stamcellernas funktion.

Jag identifierade även en transkriptionsfaktor, Interferon regulatory factor 8 (IRF8), vars gen WT1 proteinet binder till, vilket medför sänkt uttryck från IRF8 genen. Betydelsen av detta fynd bekräftades vid undersökning av ett stort

patientmaterial från primära leukemier, där jag fann en stark anti-korrelation mellan WT1 och IRF8 nivåer.

Mina studier bekräftade fynd presenterade i tidigare rapporter att onkoproteinet BCR/ABL1 ger förhöjda WT1 nivåer och jag påvisade dessutom vilken specifik cellsignalleringsväg som är aktiv i denna process. Celler med överuttryckt BCR/ABL1 visade förhöjda WT1 nivåer och sänkta IRF8 nivåer, både på genuttrycksnivå och proteinnivå i mina försök och detta speglar förhållandet i de leukemiceller där BCR/ABL1 finns närvarande.

Jag tror att WT1 proteinets roll i leukemogenesen är mångfacetterad, men genom att karakterisera olika molekylära mekanismer som WT1 proteinet styr, både i sin full-längds form och som WT1-mutant, så kan man så småningom använda denna kunskap i utvecklingen av riktad terapi som leukemibehandling.

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## ***Appendix: Papers I-IV***