6. RESULTS

6.1. Coexpression of WT1 and its regulators in carcinomas

6.1.1. WT1 and its putative regulators expression pattern in carcinoma cell lines

43% of the studied carcinoma cell lines expressed WT1 gene – namely: 10 out of 23 cell lines. It was expressed more often in breast cancer cell lines than in colon cancer cell lines – 63% versus 33%, p<0,18. The median expression was 0,06 with 5 logs of the range of expression. In 90% of cell lines that expressed WT1 the levels of WT1 expression were above the ratio 0,0002 – similar levels of WT1 expression were previously shown in leukemic, but not in healthy bone marrow (Siehl et al., 2003). 73% of studied cell lines expressed PAX2 – namely: 17 out of 23 cell lines. The PAX2 was expressed slightly more often in breast cancer than in colon cancer cell lines (88% versus 67%) but the difference was statistically insignificant (p<0,28). The expression levels of PAX2 between the samples varied to 5 log. 73% of studied cell lines expressed PAX8 – namely 17 out of 23 cell lines. The prevalence of expression was almost identical in breast and colon cancer cell lines (75% versus 73%). The expression in our series of samples varied to 4 log (Figures 6.1-6.3). The GATA1 expression was also investigated but it was not expressed in any of the cell lines.

6.1.2. WT1 and its regulators expression patterns in patients samples

The expression of WT1 was present in 85% of studied tumor tissue samples – namely in 33 out of 39 cases. The frequency of expression was higher in breast cancers than in colon cancers (96% versus 61%, p<0,005). WT1 expression levels ranged over 6 logs with a median ratio of 0,0053. In 80% of cases the expression level of WT1 was comparable to that observed in leukemic blasts. In 41% of studied tumor tissue samples the expression of PAX2 was detected – namely in 16 out of 39 cases. The expression was more frequent in breast cancers than in colon cancers (57% versus 7%, p<0,003). The PAX2 expression levels ranged over 5 log. PAX8 expression was found in 43% of studied samples – namely in 17 out of 39 cases.

The expression was more frequent in breast cancer than in colon cancers (57% versus 15%, p<0,013). PAX8 expression levels ranged over 5 log. GATA1 expression was found in 61% of cases – namely in 24 out of 39 cases. It was predominately present in breast cancers when compared to colon cancers (77% versus 30%). The GATA1 expression levels ranged over 4 log with a median ratio of 0,0003 (Figures 6.1-6.3).

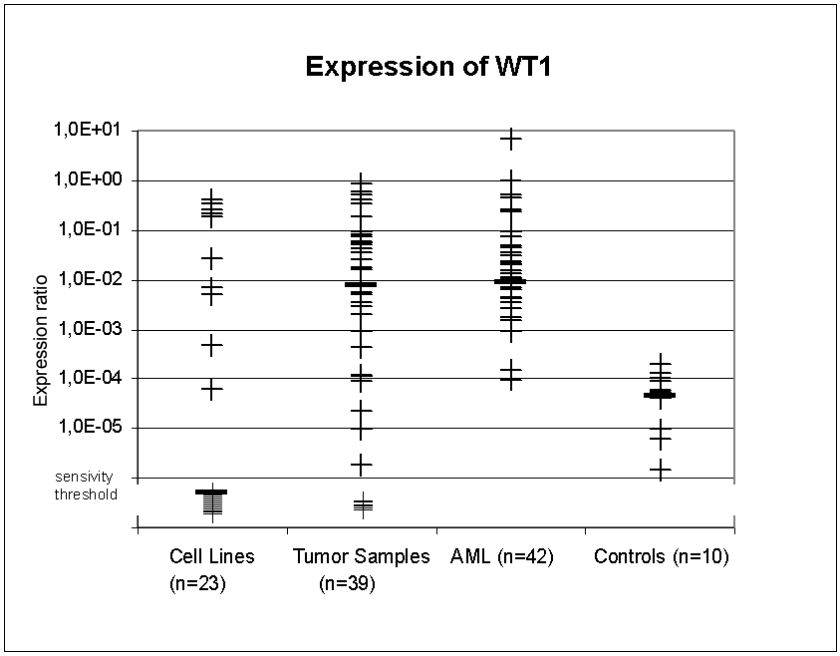

Figure 6.1: Expression levels of the WT1 gene in human carcinoma cell lines and tumor samples, compared to those of AML and healthy controls. Bold bar states median expression level.

6.1.3 Correlation between expression level of *WT1* and of its putative regulators.

Samples were divided into groups with positive and negative WT1, PAX2, PAX8 and GATA1 expression in order to examine a possible correlation between expression of studied genes. However no qualitative relations were observed.

As a next step of analysis expression levels of WT1, PAX2, PAX8 and GATA1 were compared. There are no known threshold value expression levels for studied genes therefore all of the results presented here have to be regarded as exploratory in nature.

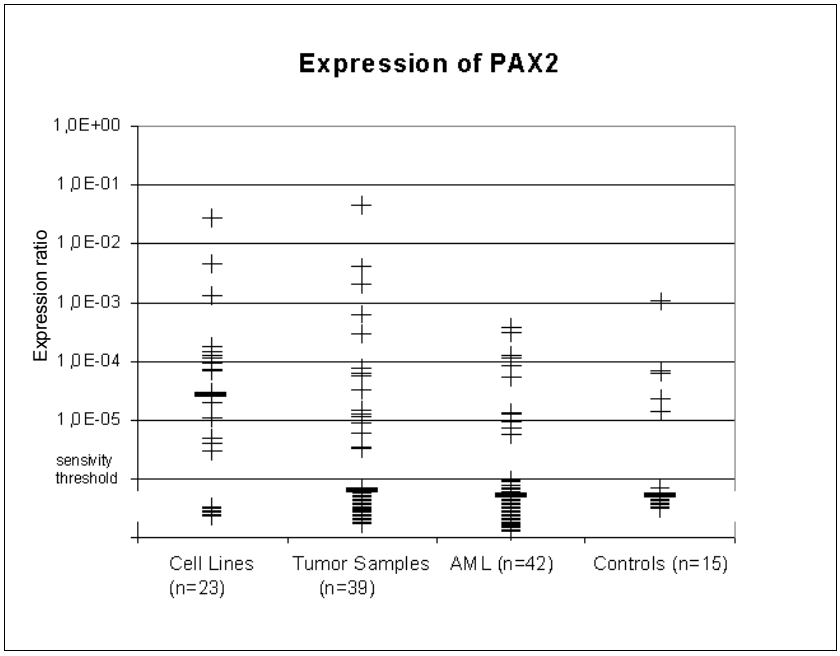

Figure 6.2: Expression levels of the PAX2 gene in human carcinoma cell lines and tumor samples, compared to those of AML and healthy controls. Bold bar states median expression level.

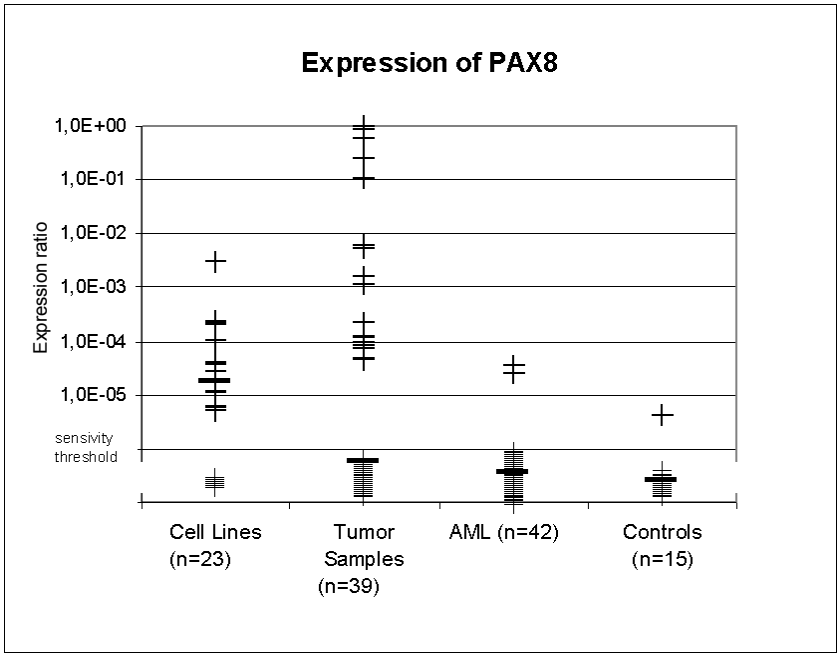

Figure 6.3: Expression levels of the PAX8 gene in human carcinoma cell lines and tumor samples, compared to those of AML and healthy controls. Bold bar states median expression level.

No obvious correlation between expression of WT1 and its regulators could be observed in cell lines (Figures 6.4-6.5). However, analysis of expression pattern in tissue samples revealed that overexpression of PAX2, PAX8 over the ratio of 0,0001 were connected with an upregulated expression of WT1 over the ratio of 0,01 ($p<0,01$ for PAX2 and PAX8) (Figures 6.6-6.7).

In 12 out of 14 cases expression of PAX genes over this threshold was connected with high expression of WT1. In 18 out of 20 cases with low or undetectable WT1 expression the PAX genes were expressed below this threshold. The most evident observation was made in breast cancers were high expression of *WT1* was present in every case of upregulation of the regulators ($p<0.0001$).

It must be noted that in 7 out of 39 samples high expression of WT1 was not accompanied by upregulation of the putative regulators.

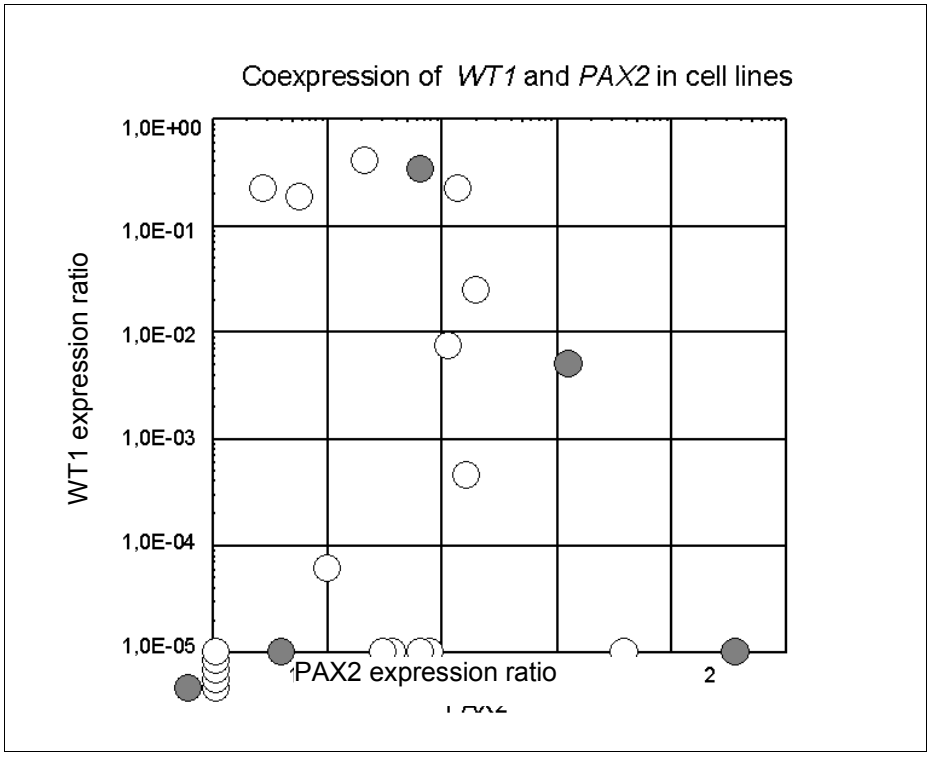

Figure 6.4: Coexpression of WT1 and PAX2 in carcinoma cell lines. Dark filed dots represent the cases with upregulation of PAX8 above postulated upregulation ratio.

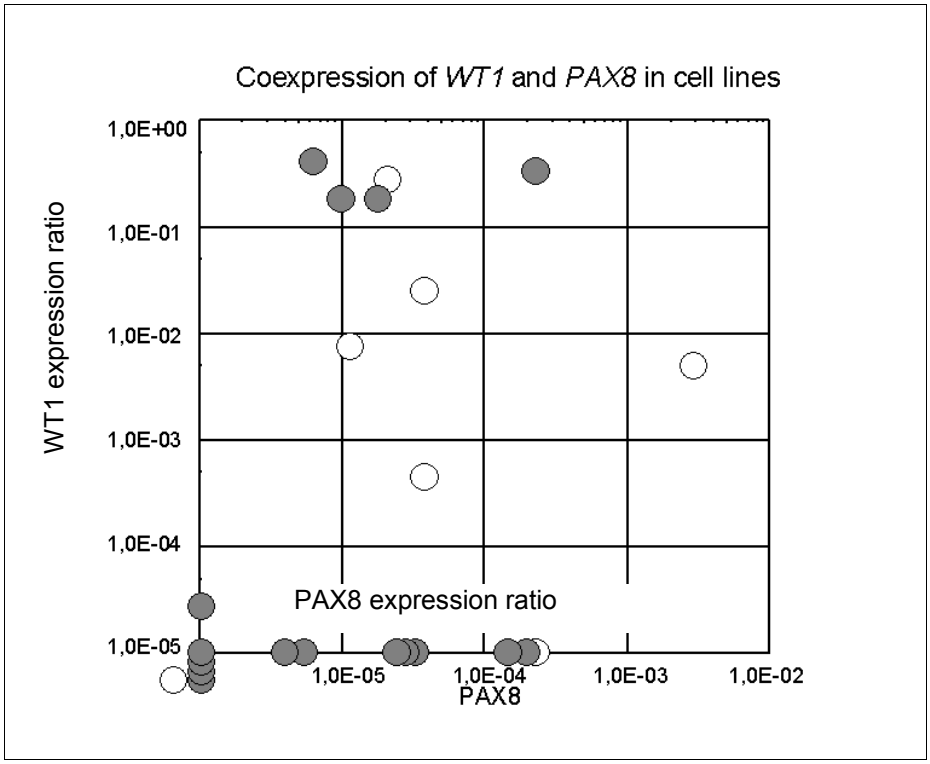

Figure 6.5: Coexpression of WT1 and PAX8 in carcinoma cell lines. Dark filed dots for the cases with upregulation of PAX2 above postulated upregulation ratio.

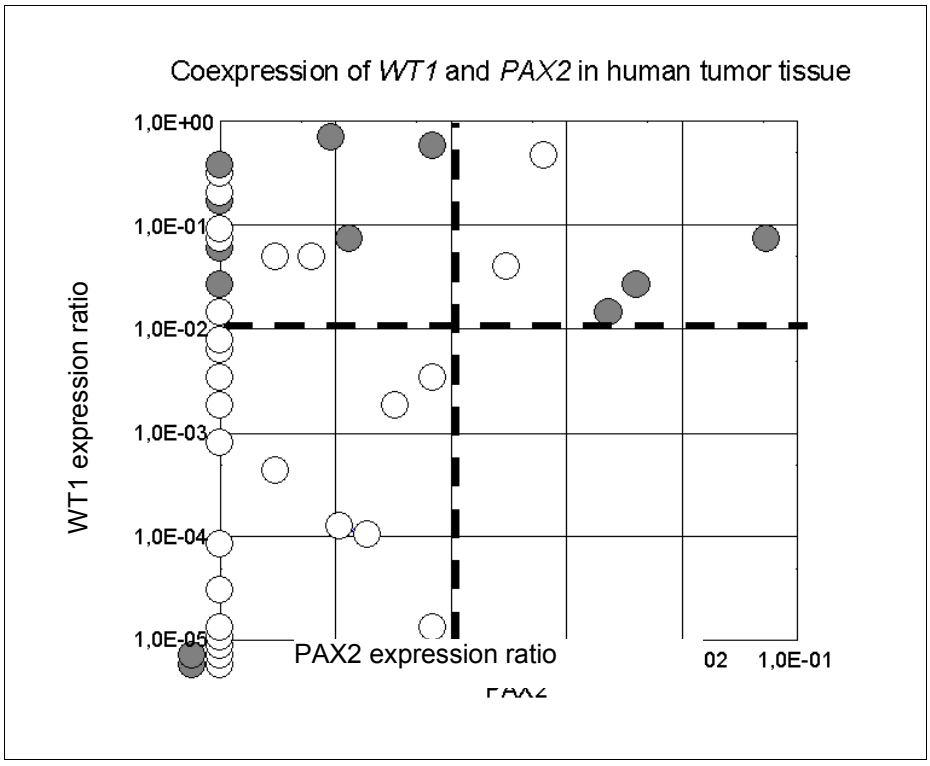

Figure 6.6: Coexpression of WT1 and PAX2 in tumor tissue samples. Dark filed dots represent the cases with upregulation of PAX8 above postulated upregulation ratio. Bold black lines state the putative upregulation thresholds.

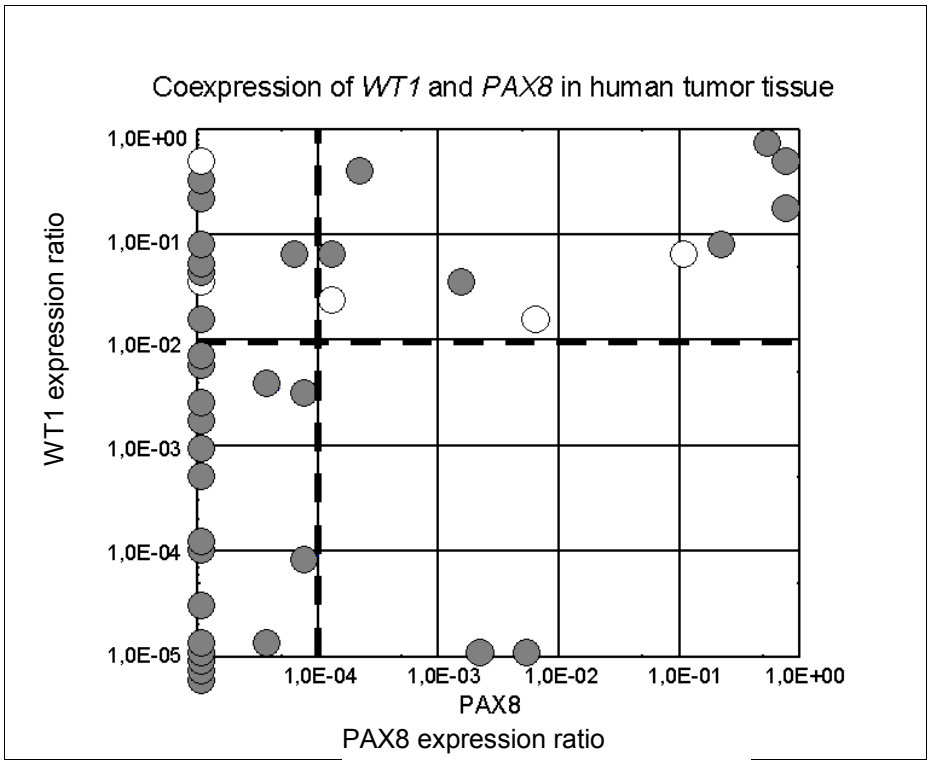

Figure 6.7: Coexpression of WT1 and PAX8 in tumor tissue samples. Dark filed dots represent the cases with upregulation of PAX2 above postulated upregulation ratio. Bold black lines state the putative upregulation thresholds.

6.2. RNA interference

6.2.1. Transfection with fluorescent labeled siRNA allows rapid optimization of transfection protocol

One of the major obstacles in RNA interference experiments is variability of transfection efficiency between the cell lines and individual experiments. The simplest and time saving way to overcome this problem is usage of fluorescently labeled siRNAs and analysis of transfection efficiency on FACS scanner. The results shown on the figure were taken from experiments on HEL cell line. HEL cells were transfected with siRNA stained with fluorescent dye and fluorescence was measured with FACS. The delivery of siRNA to the cells was only possible in the presence of transfection agent and the siRNA didn't enter or bind to the cells alone (Figure 6.8).
A rise of efficiency of transfection could be seen when the ratio of siRNA transfection agent complexes to cell number was increased (Figure 6.9). When the concentration

of siRNA was at 20nM a reduction of the cell number per well from 50 000 to 20 000 increased transfection rate from 54% to 78%. Further increase of transfection efficiency was achieved when the concentration of siRNA was raised. A raise in the siRNA concentration from 20nM to 50nM increased the efficiency to 98%. An additional rise of siRNA concentration to 100nM produced no extra increase in efficiency, but the median fluorescence per sample increased to 200% (from 153FU to 333FU). The most likely explanation is an increase in the number of complexes that are transfected per cell.

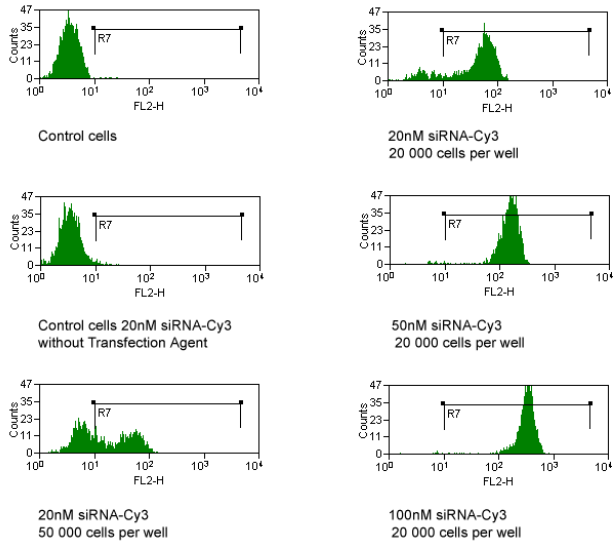

Figure 6.8: Transfection efficiency of HEL cells with siRNA-Cy3 and Oligofectamine Transfection Agent visualized on FACS.

6.2.2 Kinetic experiments

Time passing between the transfection and FACS analysis is a factor of crucial importance when the transfection efficiency is analyzed with a fluorescent marker.

As the fluorescent dye looses its activity over time it is important to state the time frame of the transfection process. It seems that two hours are sufficient for transfection complexes to enter the cells (Figure 6.9). An additional increase of the incubation time may cause the fluorescent dye to reduce its fluorescence and lead to false interpretation of experiment.

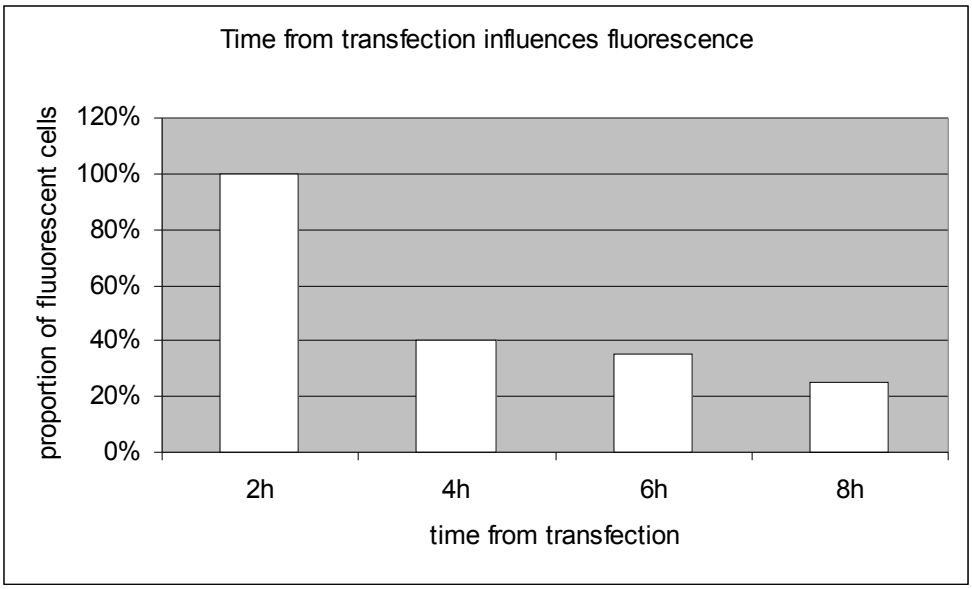

Figure 6.9: Reduction in fluorescence of the transfected cells over time. HL 60 cells transfected with siRNA-Cy3 complexes.

6.2.3 Factors influencing efficiency of transfection

There are at least two simple ways to improve the transfection rate of the cells. The first one is an incubation of cells on ice prior to transfection. 10 minutes of such incubation preceding transfection was shown to render cells more susceptible to transfection and improved the efficiency of transfection by 42% when compared to

a protocol without incubation (Figures 6.10 and 6.11). At the same time, the median fluorescence per sample increases over 179% as compared to untransfected cells. The second method is centrifugation of the cells with siRNA – transfection agent complexes. It was shown that this method greatly facilitates transfection in adherent cells, however, no consistent results were observed in not adherent cells used in siRNA experiments (data not shown).

Another important factor influencing the transfection in the RNA interference experiments is siRNA-transfection agent complex formation. The order and the time frame of mixing the reagents may have crucial importance for siRNA experiments.
A modification of the standard protocol for Oligofectamine and siPORT Lipid and addiction of stock siRNA to fully dissolved transfection agent created 34% increase

in transfection rate and 159% increase in fluorescence, as compared to controls (Figures 6.10 and 6.11).

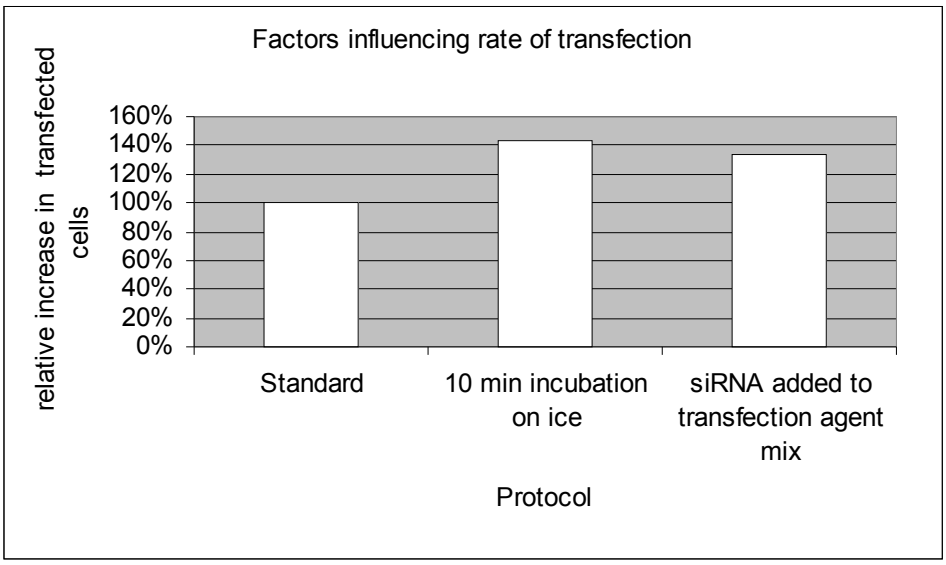

Figure 6.10: Influence of standard protocol modifications on transfection rate. HEL cells siRNA-Cy3 complexes, Oligofectamine.

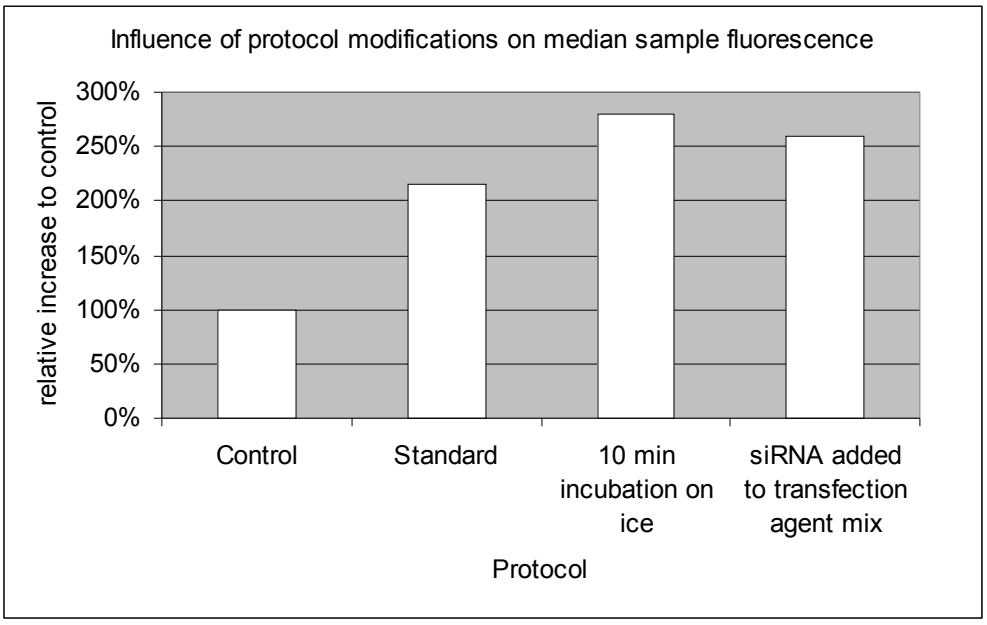

Figure 6.11: Influence of protocol modifications on fluorescence of the transfected samples. HEL cells siRNA-Cy3 complexes, Oligofectamine.

6.2.4 siRNAs synthesized with Silencer siRNA Construction Kit are able to efficiently silence mRNA expression

siRNAs targeting two housekeeping genes (PBGD and GAPDH) were synthesized with the siRNA Silencer Kit. siRNA against PBGD were designed and synthesized. siRNA against GAPDH was synthesized on matrices provided by Ambion, Cambridgeshire. K562 cells were transfected according to a modified protocol with 100nM siRNAs. The mRNA expression was measured 36 hours after the transfection. A reduction of the PBGD expression was observed by all of the designed PBGD siRNAs (Figure 6.12). The strongest action and reduction of PBGD expression to 30% of control was exhibited by siRNA PBGD sequence 2 localized 293pb downstream from the start codon. Other siRNAs reduced mRNA expression to 44% and 71% of controls. siRNA against GAPDH reduced the expression of GAPDH mRNA to around 62% of control (Figure 6.13).

Figure 6.12: siRNAs against PBGD efficiently reduce mRNA expression. K562 cells.

Figure 6.13: siRNA against GAPDH reduces mRNA expression. K562 cells.

6.2.5 Toxicity of different commercially available transfection agents

During the transfection procedure of not adherent cells the transfection agent is not washed away after the transfection. This creates a necessity to use transfection agents with low toxicity. Two groups of transfection agents were analyzed – first based on lipids, such as Oligofectamine and siPORT Lipid, and second based on polyamines such as siPORT Amine. Cells were transfected according to standard protocols and supplemented with fresh medium after 0, 4, 7 and 10 hours. The cell viability was assessed after 24 hours. Both lipid based transfection agents exhibited little toxicity on not adherent cells and incubation of cells with transfection complexes up to 10 hours caused no significant cell death. This effect was just slightly influenced by serum content in medium. On the other hand polyamine based transfection agent was very toxic from the moment of addiction (Figures 6.14 and 6.15) and the serum content in medium did not influence the toxicity. The cell survival analyses were performed as a standard procedure during all of the transfections, and no signs of toxicity of lipid-based agents were ever noted (data not shown).

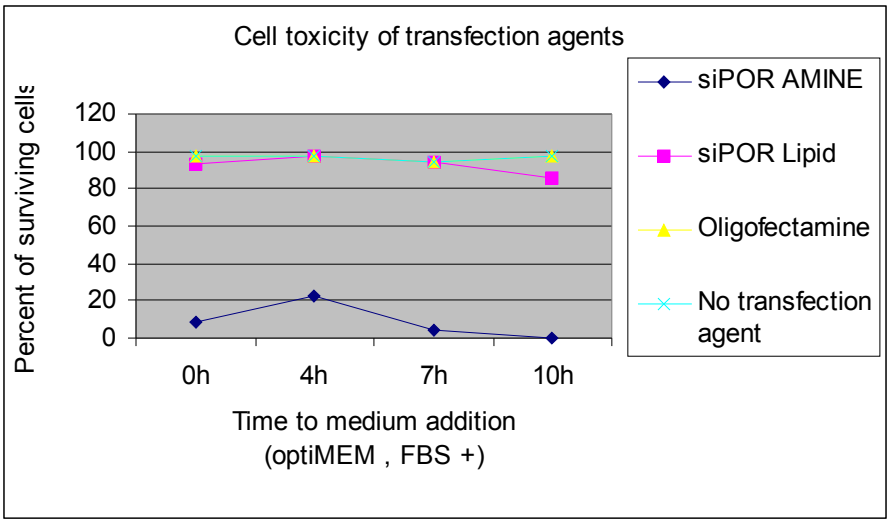

Figure 6.14: Toxicity of different transfection agents to K563 cells. Cells were supplemented with medium containing FBS after a time noted. Cell survival analyzed 24 hours from transfection.

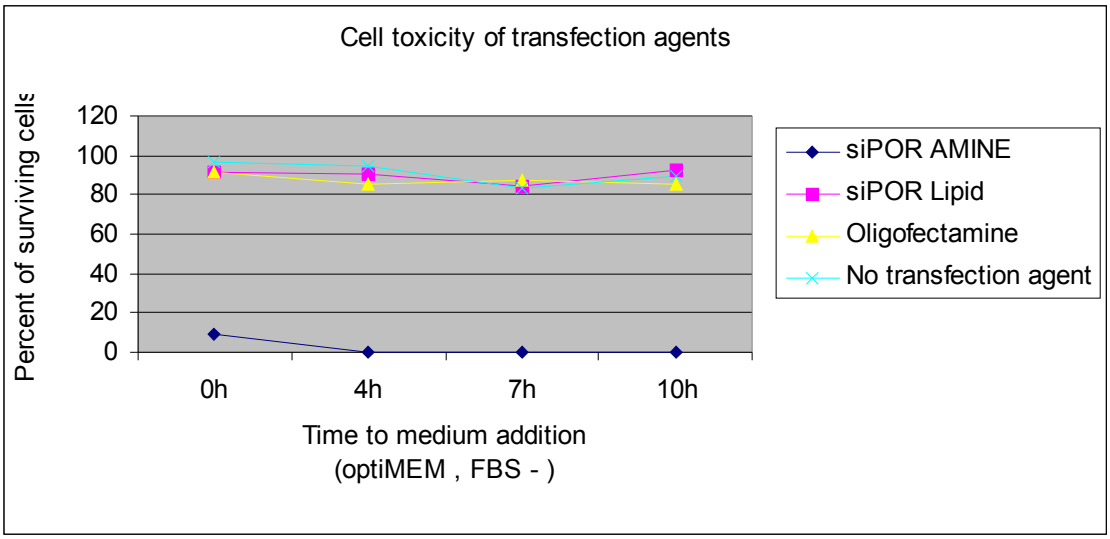

Figure 6.15: Toxicity of different transfection agents to K563 cells. Cells were supplemented with medium without FBS. Cell survival analyzed 24 hour from transfection.

6.2.6.1 WT1 RNA interference in K562

K562 and HEL cell lines were chosen as the examples of cell lines expressing WT1 and GATA1 transcription factors at moderate levels and not expressing PAX2 or PAX8 at the same time (Siehl et al., 2003). The transfection of K562 cells with siRNAs against WT1 caused relative growth inhibition (Figure 6.16). All tested siRNAs against WT1 exhibited some antiproliferative activity. The most effective one

– siRNA WT1 seq2 – suppressed cell number up to 50% as compared to negative control ($p<0,05$). The Negative Control siRNA (siRNA of a sequence without homology to any of the known genes) and transfection agent alone had no influence on growth of the studied cells – moreover, at the levels used they did not suppress the expression of other housekeeping genes – thus when gene expression was analyzed all the controls were combined together and were shown as one in analyses.

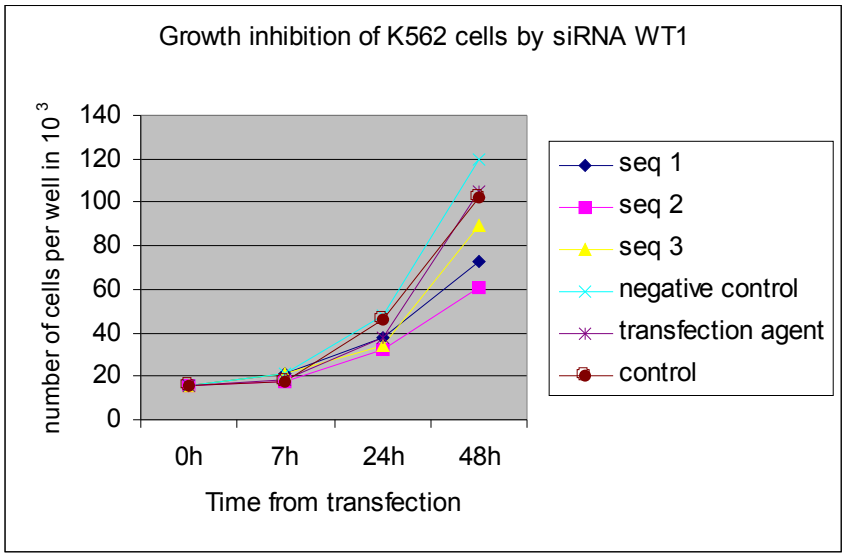

Figure 6.16: siRNAs against WT1 inhibit growth of K562 cells.

Since the effects on gene expression after siRNA transfection are seen as soon as 8 hours and are strongest 24-36 hours post transfection a time point of 24 hours was chosen for qRT-PCR. 24 hours post transfection was also a time point when first effects on proliferation were seen, thus it was reasonable that effects on gene expression should already be detectable at that time as well. However, the relative expression of WT1, when compared to housekeeping genes, was reduced to only around 80% of controls. The expression of GATA1 also didn't change much and had a similar ratio as in the control or was slightly downregulated same as WT1 gene (Figure 6.17).

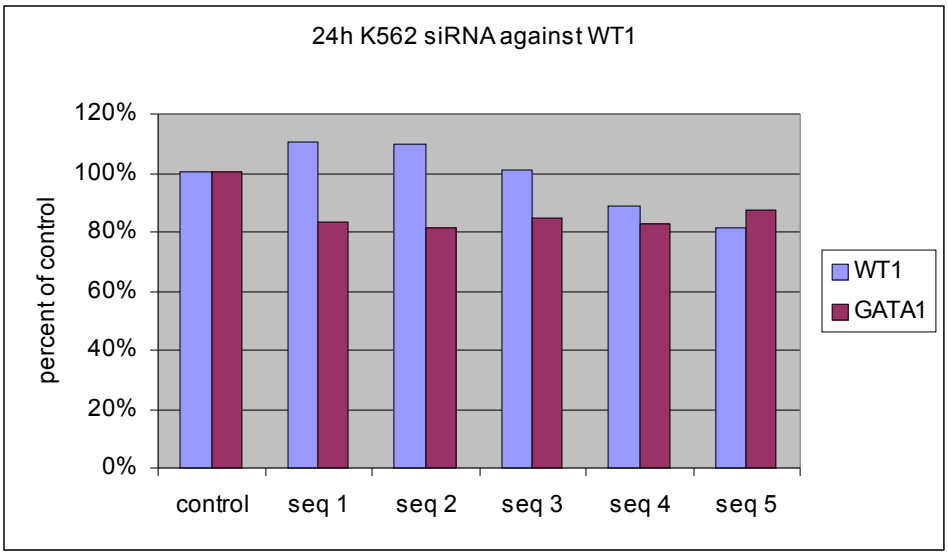

Figure 6.17: Effect of RNAi on relative ratios of gene expression. 24 hours post transfection siRNAs against WT1 used in K562 cell line.

When absolute gene expression was measured (absolute mRNA quantity per sample) downregulation of all of genes was evident. The expression of WT1 was reduced to 48% and other studied genes exhibited an analogous downregulation. The downregulation pattern under RNA interference of WT1 gene matched the growth inhibition pattern (Figure 6.18).

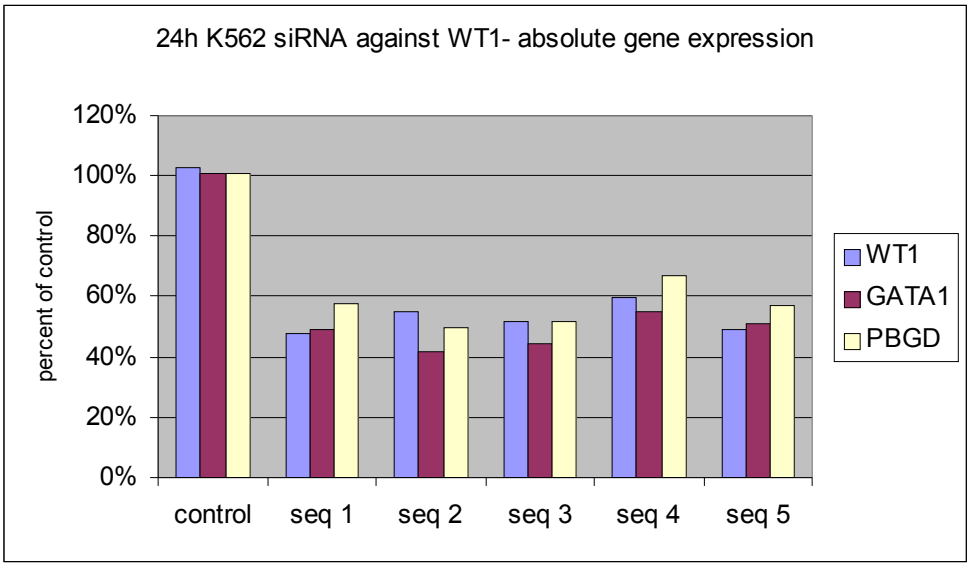

Figure 6.18: Absolute gene expression per sample measured 24 hours after the transfection. siRNAs against WT1 in K562 cell line.

This could be explained when downregulation of studied genes was an earlier event that happened before the analysis of gene expression. Therefore new time

points of 12 and 20 hours were set to investigate the kinetics of the RNA interference process (Figures 6.19 and 6.20). The most active siRNA sequence 2 was chosen for this experiment. The ratios of the gene expression of WT1 and GATA1 also in this case remained almost unchanged, when compared to housekeeping genes.

A second housekeeping gene (GAPDH) was in included in this assay to exclude potential bias that may arise when only one housekeeping gene is used. When absolute gene expression per sample was analyzed at these new time points the downregulation of all of the studied genes under 50% was observed when compared to control.

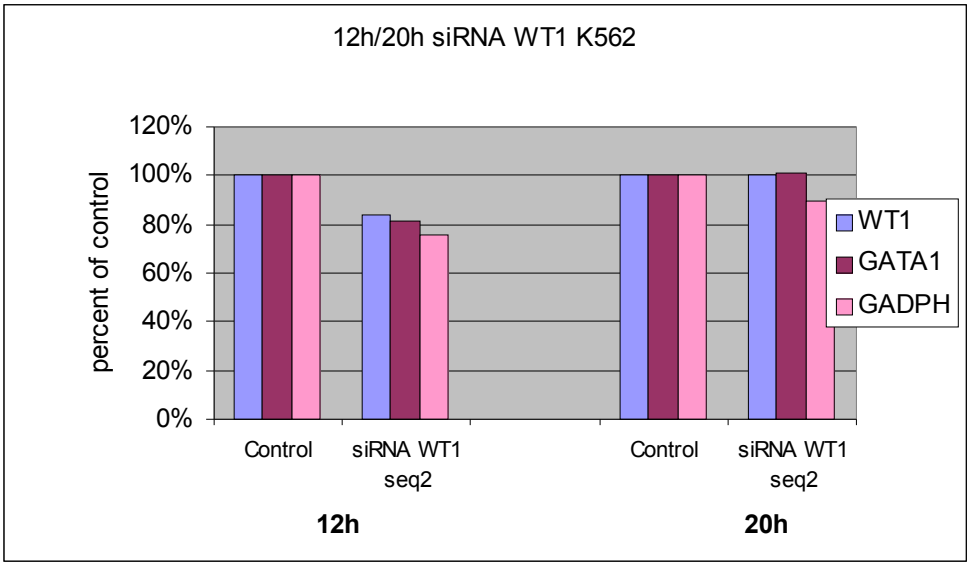

Figure 6.19: Relative ratios of gene expression 12 and 20 hours after the transfection. siRNA against WT1 in K562 cells.

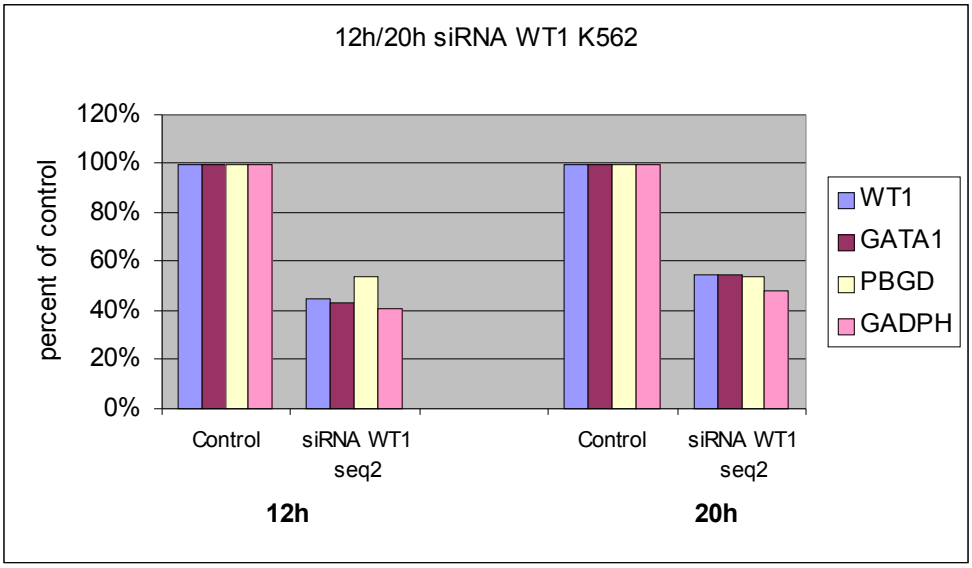

Figure 6.20: Absolute gene expression levels per sample measured 12 and 20 hours post transfection. siRNA against WT1 in K562 cells.

6.2.6.2 WT1 RNA interference in HEL

RNA interference of WT1 expression in HEL cells caused downregulation of WT1 mRNA ratio to 46% of controls and slight downregulation of GATA1 ratio to 85% already after 12 hours (Figure 6.21). However, after 20 hours the ratio of WT1 seemed to return to the levels of controls and reached 75% of their WT1 expression. When absolute gene expression per sample (mRNA quantity) was analyzed WT1 expression was downregulated just to 16% of controls after 12 hours and to 30% of controls after 20 hours (Figure 6.22). Ratios of other studied genes remained stable around 30% of control 12 and 20 hours after transfection with siRNAs. Apoptosis was measured in the transfected cells 24 hours post transfection but there was no increase in apoptosis ratio as compared to control (data not shown).

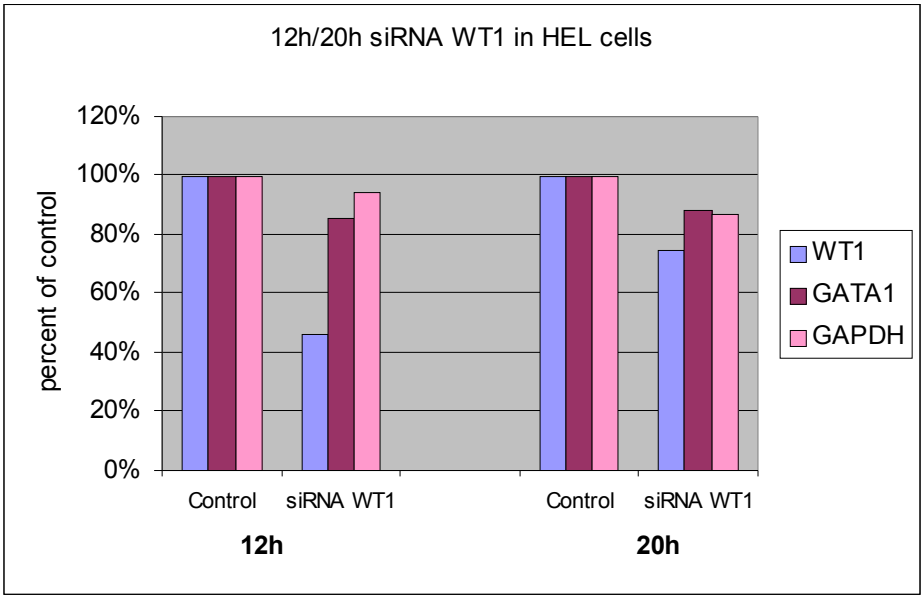

Figure 6.21: Relative ratios of gene expression 12 and 20 hours after the transfection. HEL cells transfected with siRNA against WT1 seq 5.

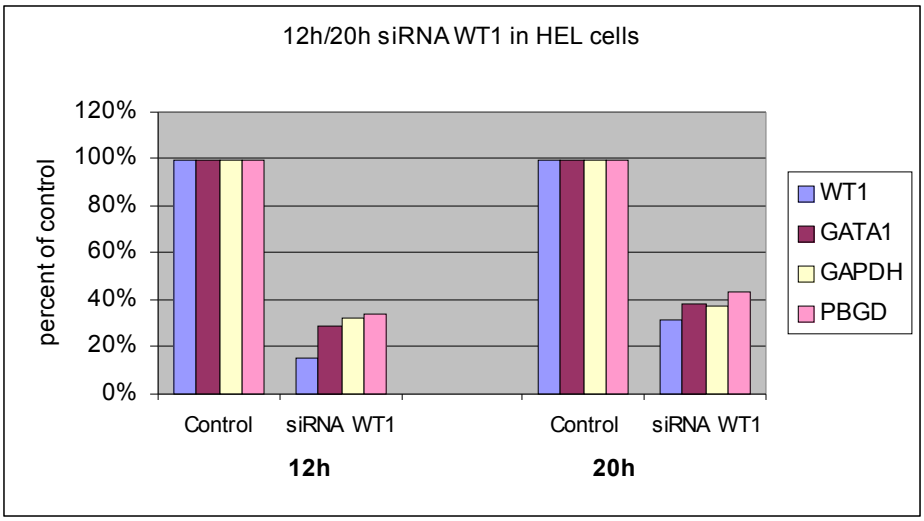

Figure 6.22: Absolute gene expression levels per sample measured 12 and 20 hours post transfection. HEL cells transfected with siRNA against WT1 seq 5.

6.2.6.3 GATA1 interference in K562

siRNAs against GATA1 caused growth inhibition in transfected K563 cells (Figure 6.23). GATA1 RNA interference reduced the cell number per well down to 50% of controls. The anti proliferation effect was seen only after 48 hours post transfection. This stays in contrast to siRNAs against WT1 where the growth inhibition was already seen after 24 hours (Figure 6.24).

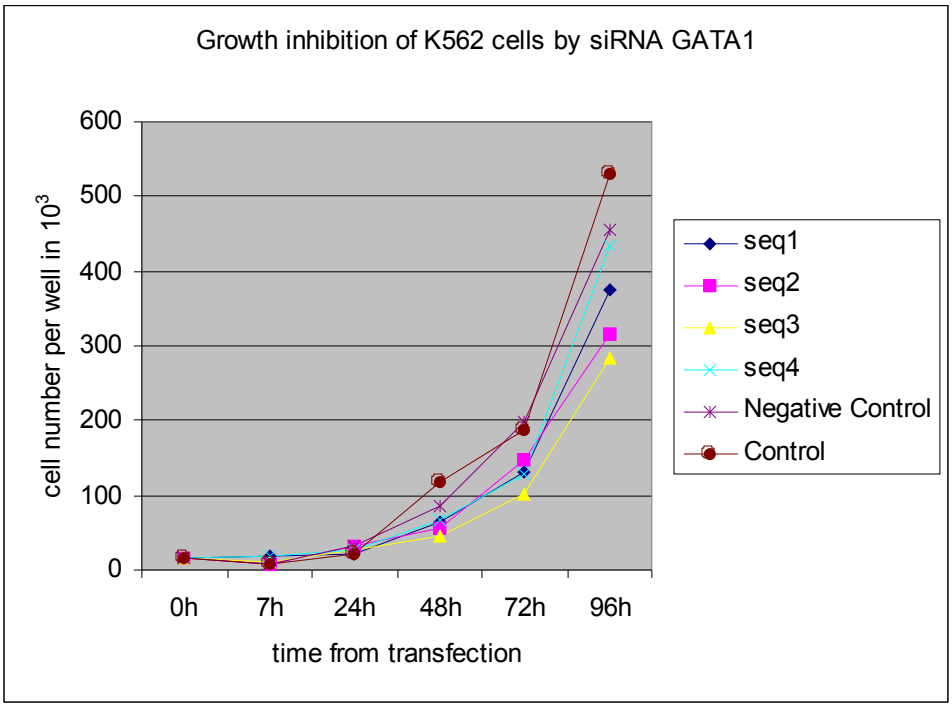

Figure 6.23: siRNAs against GATA1 inhibit growth of K562 cells.

When the gene expression profile was analyzed 24 hours post transfection an elevation of WT1 ratio per cell was found with an unchanged ratio of GATA1 expression (Figure 6.25). This observation is explained when absolute expression per sample is analyzed (Figure 6.26). The siRNAs against GATA1 cause downregulation of GATA1 and PBGD genes under 20% of control expression. This is accompanied by decrease of WT1 expression to 43%. A similar picture emerges when expression is analyzed 48 hours after the transfection (Figure 6.27 and 6.28). The GATA1 downregulation is accompanied by equal PBGD downregulation to around 40% and slight downregulation of WT1 expression to 80% of controls. Nevertheless, ratios of WT1 expression to PBGD expression relatively rise under GATA1 interference, which is caused by strong downregulation of the PBGD housekeeping gene.

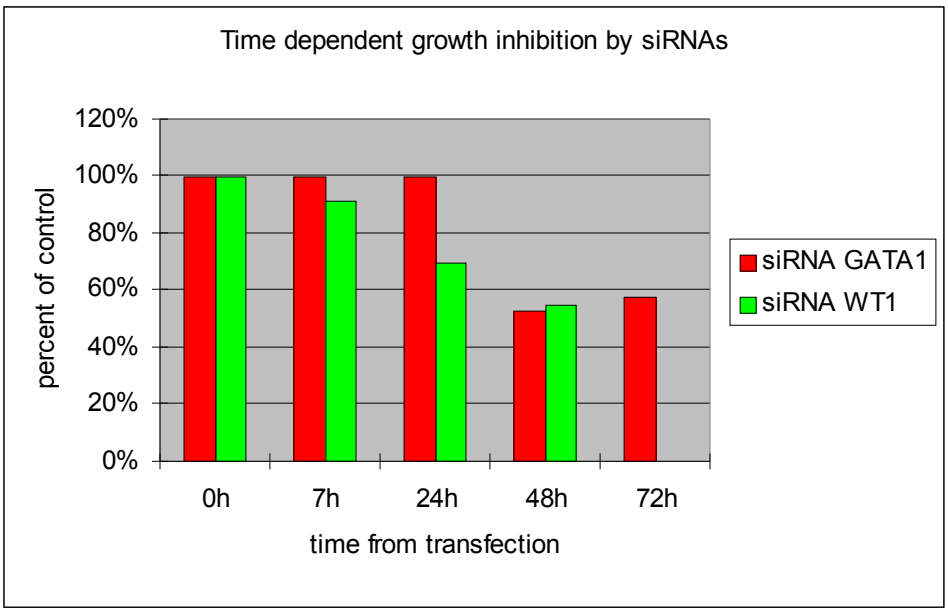

Figure 6.24: The dynamics of growth inhibition of K562 cells by the most active siRNAs against WT1 and GATA1. Values of RNAi for WT1 only from the first 48h.

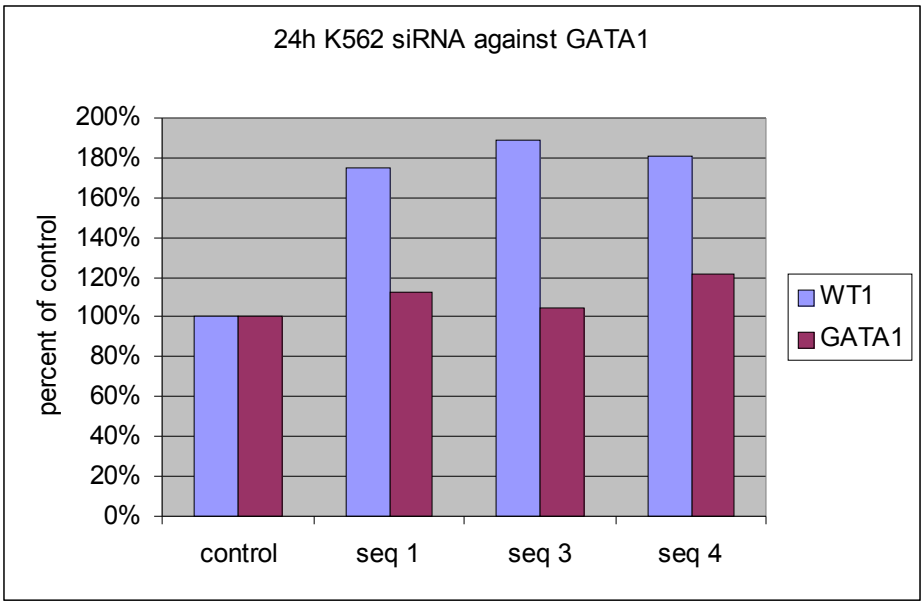

Figure 6.25: Effect of RNAi on ratio of gene expression. 24 hours post transfection siRNAs against GATA1used in K562 cell line.

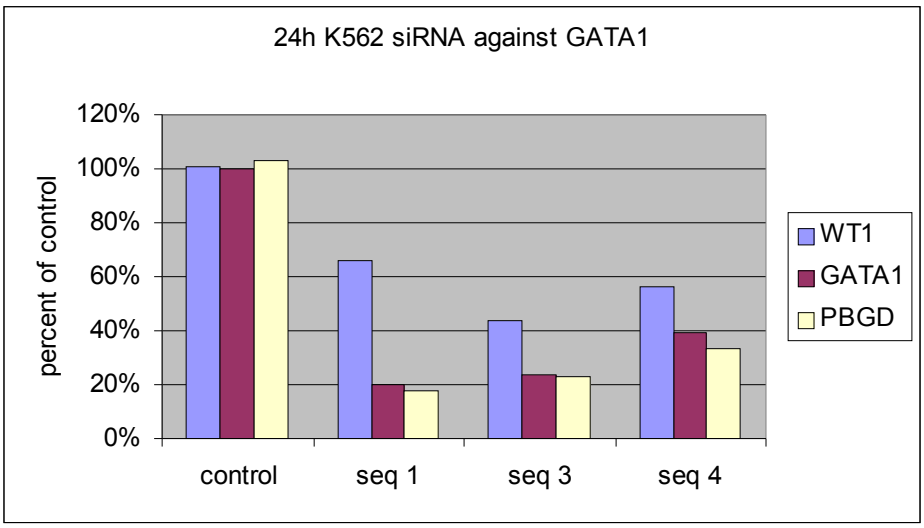

Figure 6.26: Absolute gene expression per sample measured 24 hours after the transfection. siRNAs against GATA1 in K562 cell line.

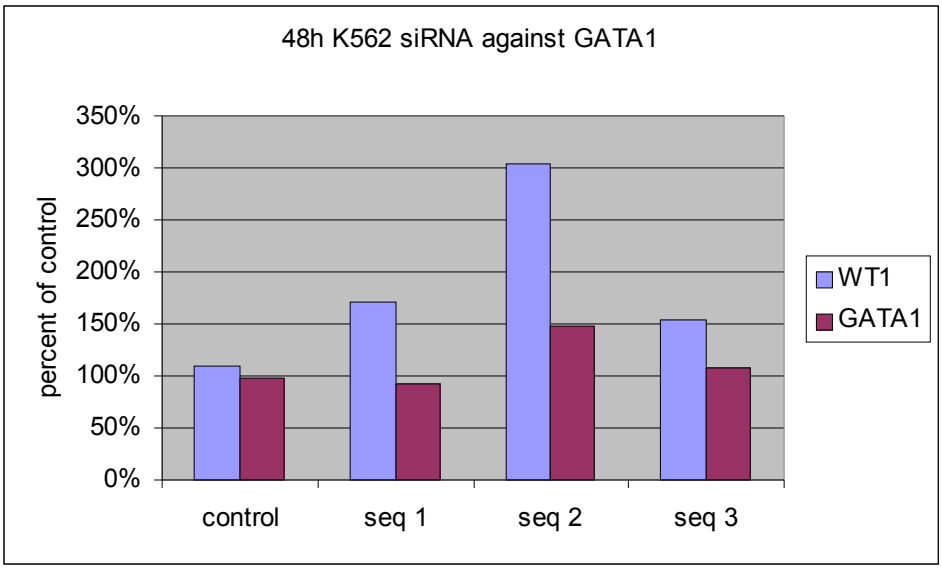

Figure 6.27: Effect of RNAi on ratio of gene expression. 48 hours post transfection siRNAs against GATA1used in K562 cell line.

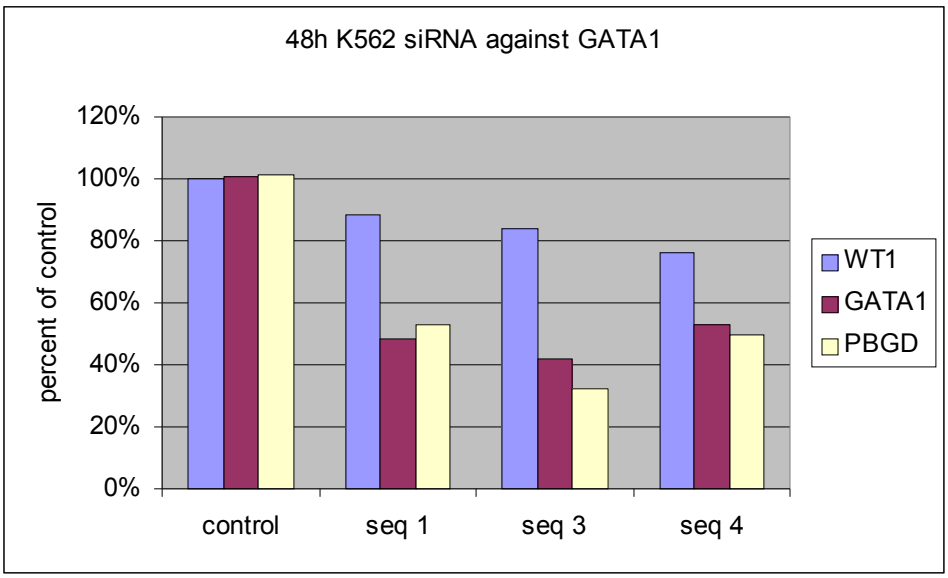

Figure 6.28: Absolute gene expression per sample measured 48 hours after the transfection. siRNAs against GATA1 in K562 cell line.

6.2.6.4 GATA1 RNA interference in HEL

The most efficient siRNA in K562 cells was used for RNA interference in HEL cells. The response of HEL cells to siRNAs against GATA1 was very rapid (Figure 6.29). Already after the 12 hours ratio of GATA1 gene decreased to around 50% of controls with slight changes in ratios of WT1 and GAPDH. When absolute gene expression was analyzed GATA1 downregulation under 20% was even more

evident. This was accompanied by downregulation of WT1 expression to 30%. The housekeeping genes were also down regulated: PBGD to 36% and GAPDH to 34% (Figure 6.30). After 20 hours ratios of GATA1 and GAPDH had similar values, and ratio of WT1 rose to over 140%. The expression of GATA1, GAPDH and PBGD analyzed after 20 hours had the similar levels to those measured after 12 hours. The absolute expression of WT1 seemed to recover and rose to 54% of control after 20 hours (Figure 6.30).

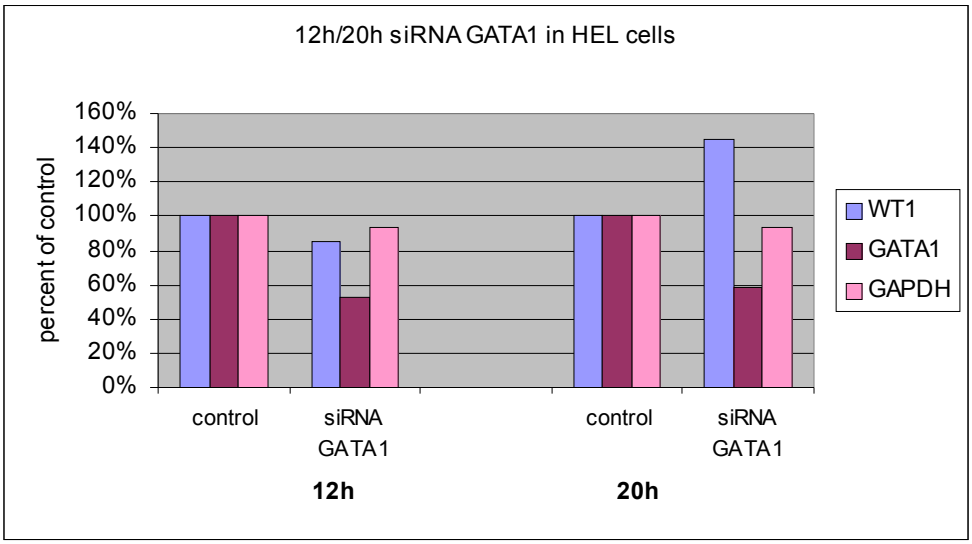

Figure 6.29: Effect of RNAi on ratios of gene expression. 12 and 20 hours after transfection. siRNA against GATA1 used in HEL cell line.

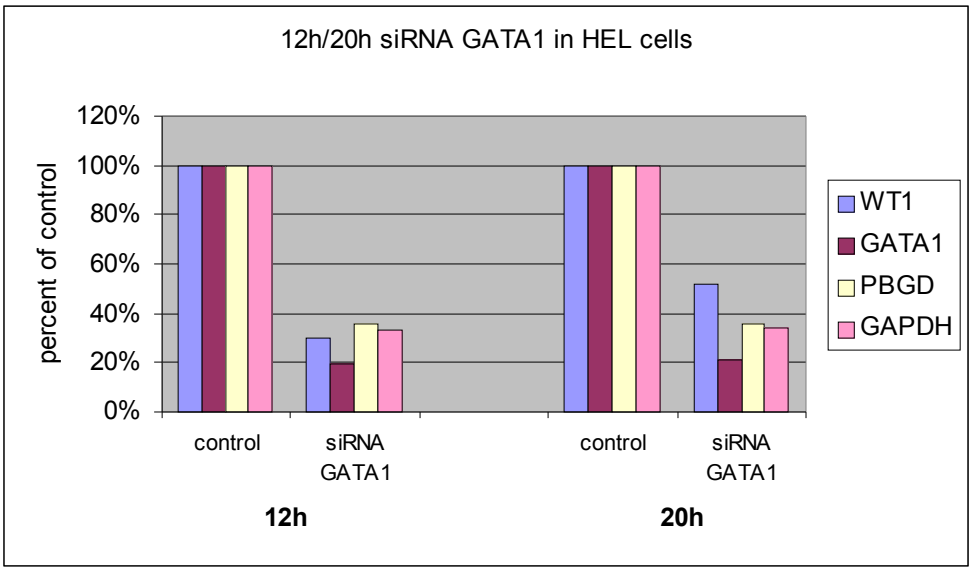

Figure 6.30: Absolute gene expression per sample measured 12 and 20 hours after the transfection. siRNA against GATA1 in HEL cell line.

6.2.6.5 PAX8 RNA interference in HBL100

The HBL 100 cell line was chosen for the RNA interference of PAX8 gene. This cell line lacks expression of other putative regulators of WT1 and expresses PAX8 at the levels comparable to leukemia (Siehl et al., 2003). During the methodological experiments with siRNA the most effective siRNA against PAX8 was chosen. The expression of studied genes was measured 24 and 48 hours post transfection. After 24 hours the ratios of studied genes didn't change much when compared to control (Figure 6.31). After 48 hours a drop of PAX8 ratio to 20% of control was observed accompanied by reduction of WT1 ratio to 56% of controls. When absolute expression levels were analyzed similar picture appeared (Figure 6.32). Absolute amounts of PAX8 expression were reduced to 90% of controls after 24 hours and to 27% of controls after 48 hours. Absolute WT1 expression hasn't changed after 24 hours from transfection, but was reduced to 65% of control after 48 hours. PBGD expression was reduced in transfected cells to 90% of control after 24 hours. However, after 48 hours the quantity of PBGD mRNA that was harvested from the RNAi sample was higher than from control.

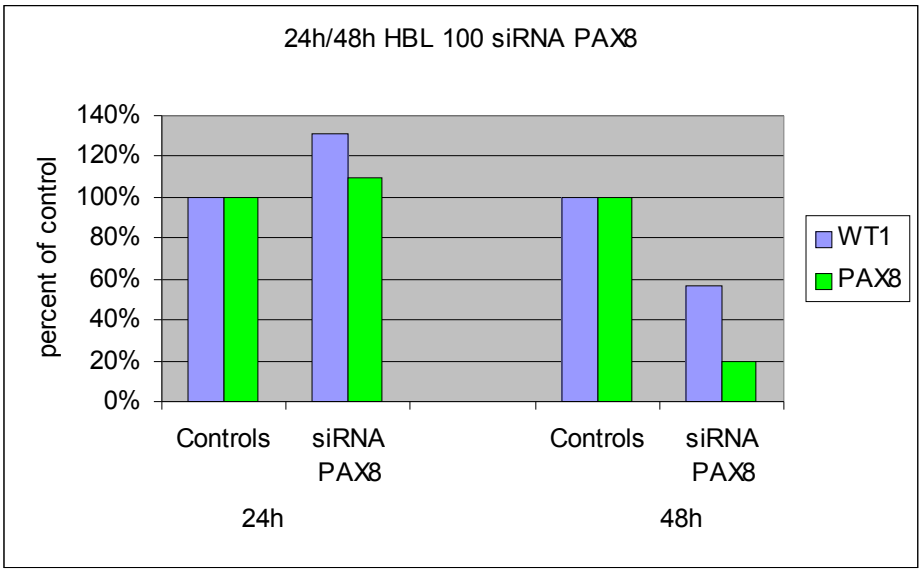

Figure 6.31: Effect of RNA interference against PAX8 gene on ratios of gene expression. Gene expression measured 24 and 48 hours after transfection. siRNA against PAX8 used in HBL 100 cell line.

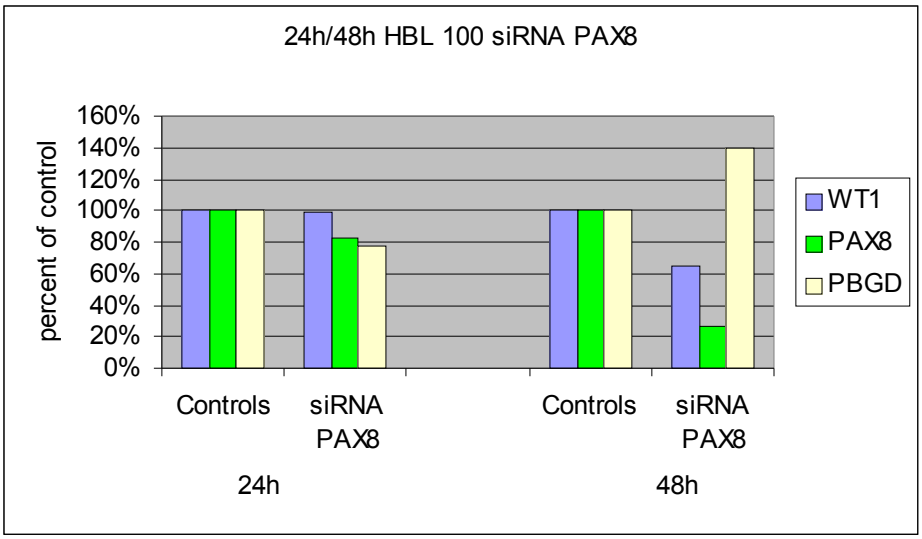

Figure 6.32: Absolute gene expression per sample measured 24 and 48 hours after the transfection. siRNA against PAX8 in HBL 100 cell line.

6.2.6.6 PAX2 RNA interference in HL60

The RNA interference against PAX2 was studied in HL60 cell line. This cell line expresses only PAX2 and lacks expression of other WT1 regulators (Siehl et al., 2003). The PAX2 is expressed at the levels corresponding to leukemia. The most effective siRNA against PAX2 suppressed ratio of PAX2 expression down to 44% of controls after 24 hours post transfection. The ratio of WT1 was reduced to 74% of controls at the same time. After 48 hours expression of both studied genes had similar ratios of 60% that of controls (Figure 6.33). When absolute expression levels were measured (Figure 6.34) the downregulation of absolute PAX2 expression to 50% after 24 hours and 44% after 48 hours was seen. This was accompanied by the downregulation of WT1 to 84% after 24 hours and 39% after 48 hours. The following reduction of PBGD expression was also seen from 114% of control after 24 hours to 65% of control after 48 hours.

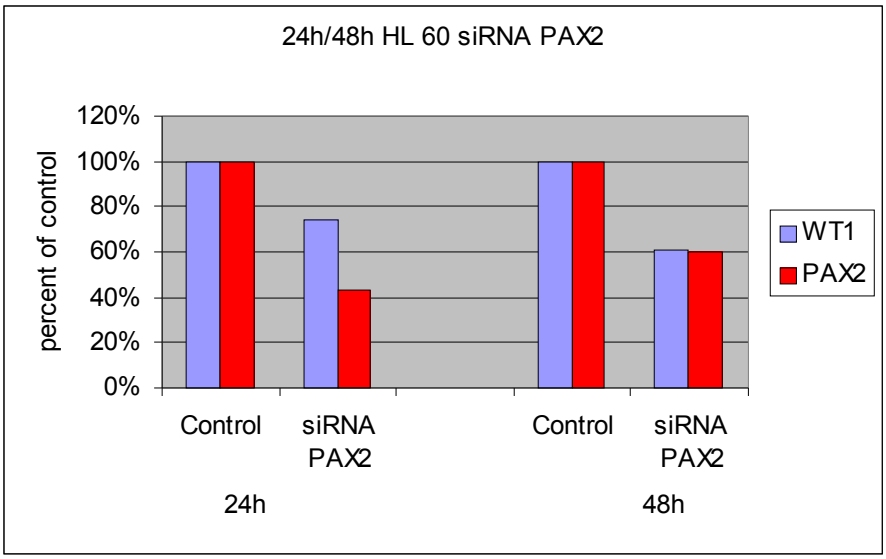

Figure 6.33: Effect PAX2 RNA interference on ratios of gene expression. 24 and 48 hours after transfection. siRNA against PAX2 used in HL 60 cell line.

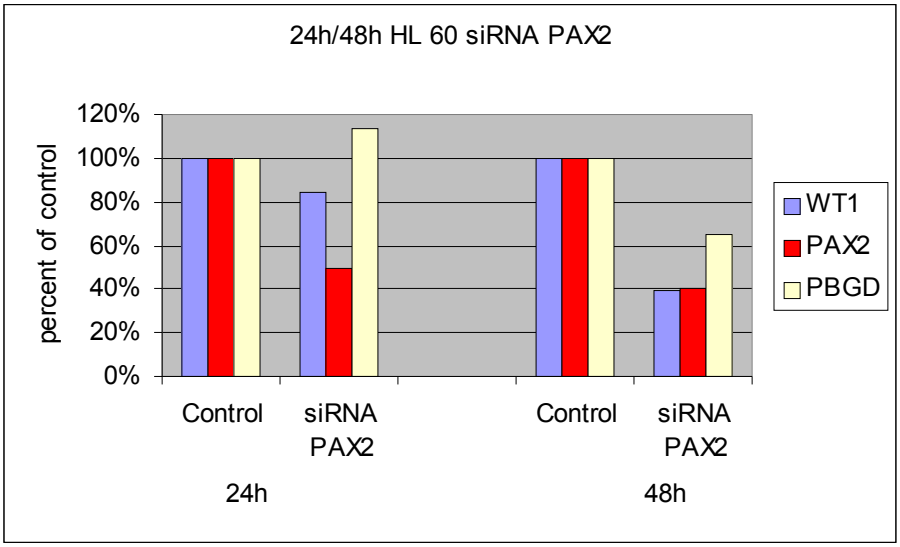

Figure 6.34: Absolute gene expression per sample measured 24 and 48 hours after the transfection. siRNA against PAX2 in HL 60 cell line.

6.3 Mutation analysis of promoter regions of studied genes in patients with AML

6.3.1 WT1 promoter

The two fragments of WT1 promoter were amplified and sequenced. In the analyzed group of AML patients one know variation was found at the position 842

(figure 6.35). The PubMed Nucleotide database states the frequency at 0,46 T and 0,54 C. In the analyzed group of patients frequencies were similar: 0,56 for T and 0,44 for C. The rest of the promoter region of WT1 was not scanned for variations in the healthy controls so far. There were two new variations in that group. First one found at the position 972: 0,96 G and 0,04 C; and second one at the position 1378: 0,56 T and 0,44 for C. The variations are situated apart from binding places for most of the known regulators of WT1 expression. It seems unlikely that such variations could have influence on binding of known WT1 expression regulators (Transcription factor binding sites taken from Dressler and Douglas, 1992, Faisst and Meyer, 1992, Fraizer et al., 1994, Pavletich and Pabo, 1991, Zannini et al., 1992). There were no other sequence changes to be found. PCR products from all samples had the same length and there were no additional bands that could represent rearrangements in promoter sequence.

6.3.2 WT1 enhancer

There are four known variations in the region of WT1 enhancer: 50292: 0,13 T; 50319: 0,02 A; 50320: 0,01 C; 50540: 0,05 A (PubMed Nucleotide). In the analyzed series of AML samples the most common variation at the position 50292 was also found – with a similar frequency to the control population in the database: 0,81 C and 0,19 T. Two new variations were found in the material from AML. First at the position 50164: 0,91 A and 0,09 C, and second at the position 50319: 0,92 G and 0,08 A. Also here the binding places for GATA1 are not affected by the variations. The PCR products always had predicted size and there were no additional bands to found during gel electrophoresis.

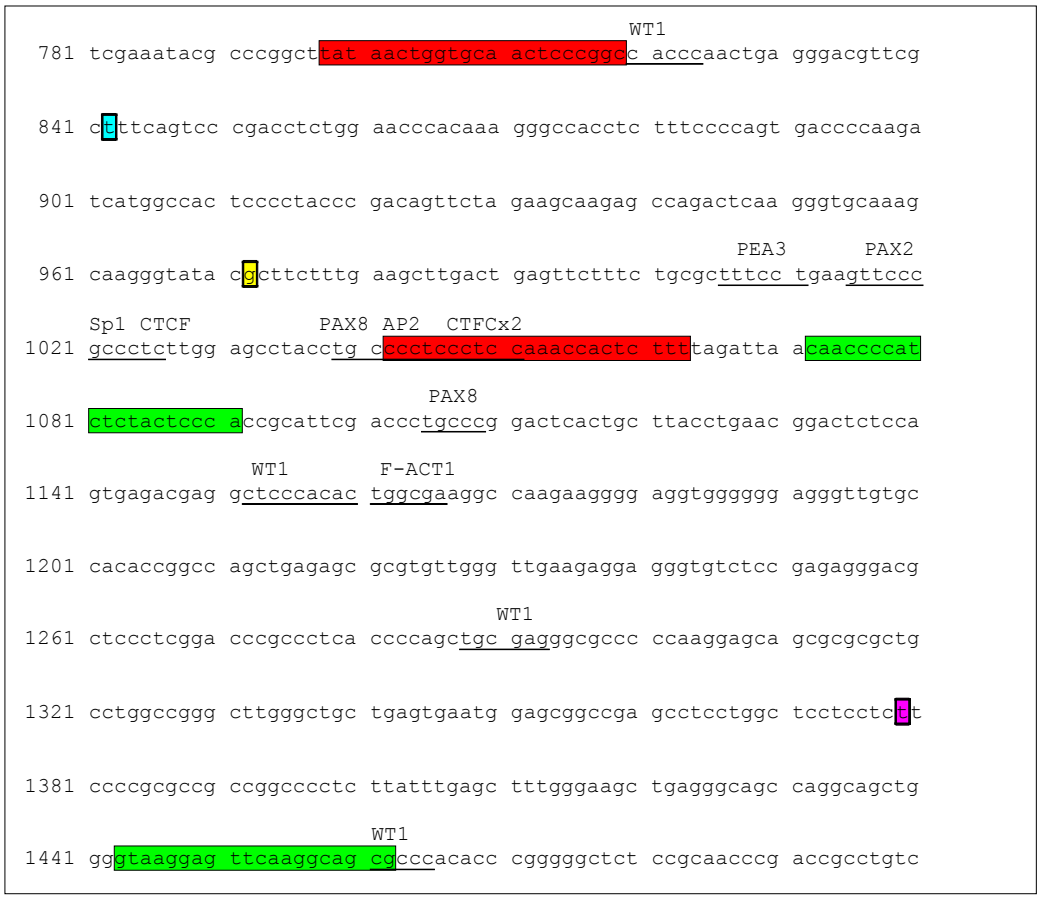

Figure 6.35: Fragment of WT1 promoter sequence. Primary transcript starts at 1430. The PCR primer binding sites shown in green for proximal promoter in red for distal promoter. Locus HSWT1PRO. Variations: position 842: CT – 50%, T- 30%, C – 20% (n=24), position 972: G – 96%, C – 4% (n=24), position 1378: CT – 50%, T- 30%, C – 20% (n=24).

Figure 6.36: Fragment of WT1 enhancer. The PCR primer binding sites shown in green. Locus AY245105. Variations: position 50164: A – 83%, AC – 17% (n=24), position 50292: C – 67%, TC – 29%, T – 4% (n=24), position 50319: G – 84%, GA – 16% (n=24).

6.3.3 GATA1 promoter

So far there is no data available in PubMed Nucleotide Database on variation in the sequence of the GATA1 gene promoter. In studied series of samples the only variation was found at the position 164: 0,96 C and 0,04 T. No other variations or mutations were found. The PCR products always had expected length and no extra bands were found during gel electrophoresis. Thus GATA1 promoter seems to be unlikely site for mutation in AML.

```
  1 ctgggctggt ggttgcggag ggttcggccg ccttggggat gtggcaagct cagtgtgatc
 61 ccaggggggtg tcctggctgg ccttggcctt tgaggctccc ttcctccctc cctccctccc
121 tcccttctcc ctcctccctc ctccccaggg agtggctggt gcctcagtgg gtggggcagt
181 ccaggggagg gggcgggtcc catgcttgtg gggcacctct tgggcactgg aggccctctt
241 ccaccctccc tgcccaccct ggggattctt ggacctcgct cgacctaggc tcccgtggac
301 tgtagtgtcc agacaagcaa aataggctga aaccaagata aatgaaaata gcagatatgg
361 tttcatgact tcactgggct tttcagagag taaagccctt aatttgtgcg gggccggtgt
421 gggctagact cctgtgtatg tgcgtcttcc tggctgcagg gagagaaggg atgtggctgt
481 acccattttg caggtgagaa gagtgaggct cagagaggtt ctgctggatt tgaactagag
541 cctgtgggat accttgatga cccaggctaa gcctgcaggc caggccagtg ctggcgggag
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Figure 6.37: Fragment of GATA1 CDS. Promoter region 1-400. The PCR primer binding sites shown in green. Locus HSGATA1P. Variations: position 164: C 92%, CT 8% (n=23).

6.3.4 PAX2 promoter

There were two variations found in the PAX2 promoter in the studied panel of AML samples. First one at the position 1031: 0,9 T and 0,1 G, and the second one at the position 1314: 0,8 T and 0,2 G. There were no other mutations to be found in studied series of the samples.

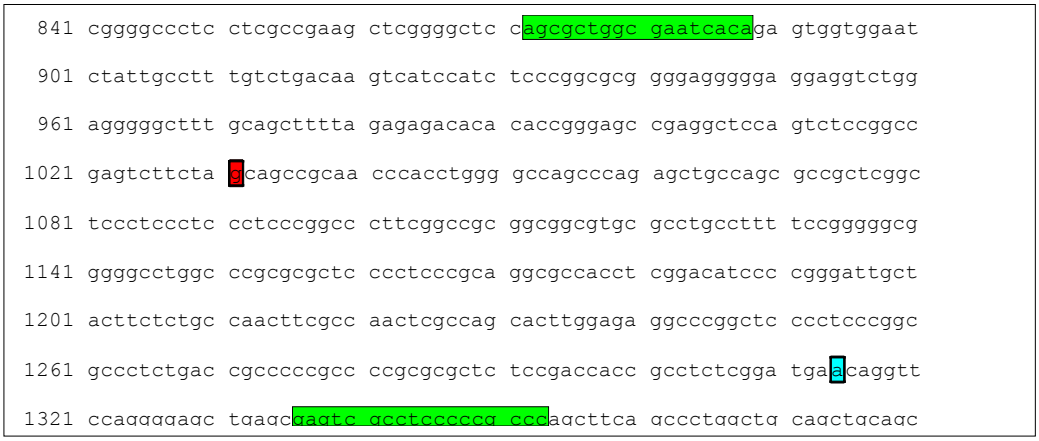

Figure 6.38: Fragment of PAX2 CDS. Primary transcript starts at 1002. The PCR primer binding sites shown in green. Locus HSPAXTWO01. Variations at positions:1031: 0,9 T and 0,1 G; 1314: 0,8 T and 0,2 G

6.3.5 PAX8 promoter

In the analyzed series of AML samples there were no variations found in the PAX8 gene promoter. There were also no additional bands found during gel electrophoresis analysis of PCR products.

```
2161 gaggagagac accgggccca gggcaccctc gcgggcggac ccaagcagtg agggcctgca
2221 gccggccggc caggtatgtc acccaggggt tagctggaag ctggctagca gtgaggacgg
2281 gggatggaag aaaggagagg gtcccaggat gcctggcagc cttttccctc ccaagttaaa
2341 cgggataaga ctgggacagc ggagggagtg ggcacggagg ttggagtctg gagcttcttc
2401 agcgcactcc caatccttga tcctcccggg aagcctgtta gctaggctag gctgaggttg
2461 gccctacttc gcctaaaaat cctcctactc ctggcagacg atgcaggtgt aaaggatgag
2521 gcctggggag ggggcctgag gatgcaggca tcgaatctca tcgcatctca tgcccttctc
2581 ctgggtttgt gcagggcagc ggcaggcgcg gcccggacct acgggaggaa gcccgagcc
2641 ctcggcgggc tgcgagcgac tccccggcga tg
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Figure 6.39: Fragment of PAX8 CDS. The PCR primer binding sites shown in green. Locus HUMPAX8A. No variations compared to PubMed and healthy controls.

6.4 Quantitative methylation analysis

A novel approach was developed to quantitatively analyze methylation of promoter regions of the studied genes. The method bases on combination of DNA digestion with methylation-sensitive enzyme and quantitative PCR. The basics of method are shown on figure 5.3. The examined sample was digested with restriction enzyme specific for unmethylated DNA (protocols in section 5.12). The ratio of DNA amount in digested and undigested sample measured with quantitative PCR reflected the percentage of methylated residues. As controls served methylated and unmethylated DNA samples. Methylated DNA was included as a control of PCR efficiency. Unmethylated DNA served as a control of effectiveness of digestion.

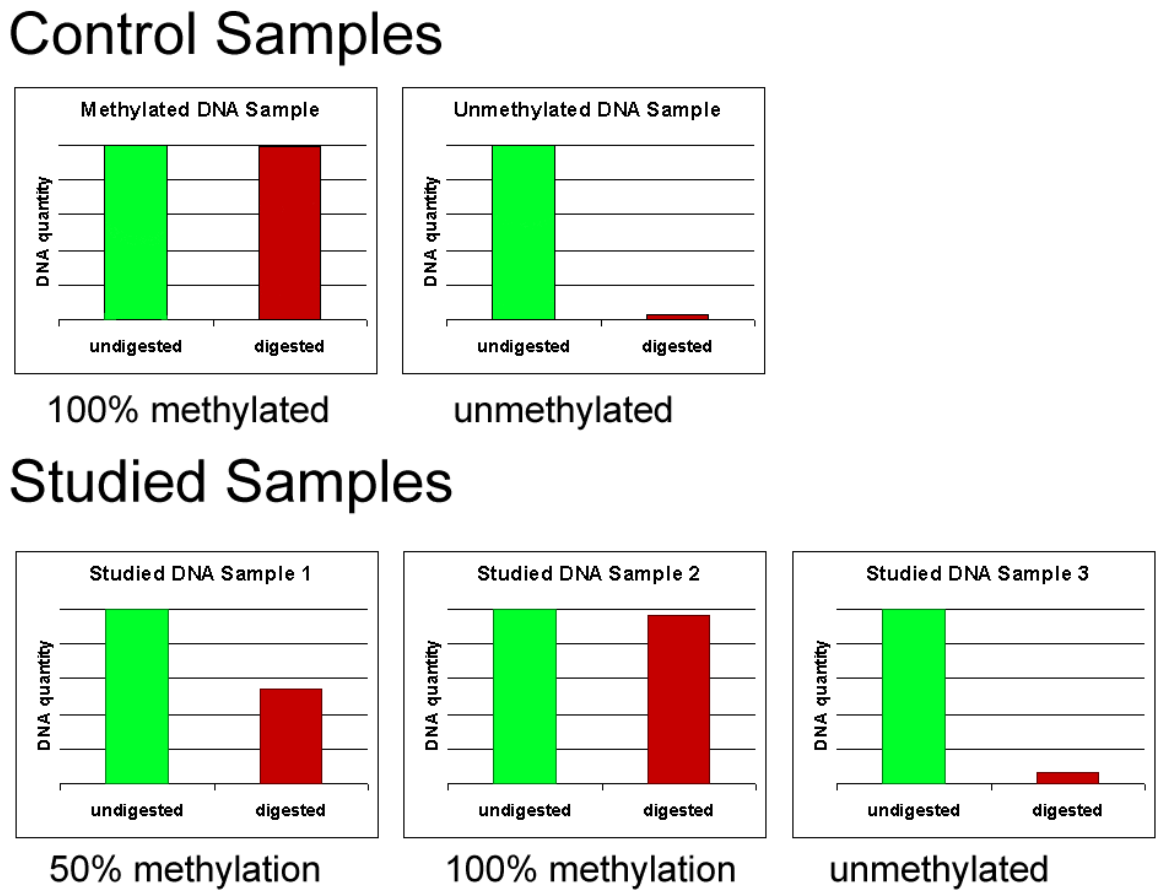

Figure 5.3: Schematic illustration of quantitative methylation analysis. Quantity of DNA in Methylated and Unmethylated sample are measured prior and post digestion with methylation sensitive restrictase (top). The studied samples are digested and quantified in the same way (bottom). The differences in DNA quantity prior and post digestion reflect methylation status of the samples.

The quantitative PCR was performed with Light Cycler and SYBR Green stain was used to obtain fluorescence of amplified sequences (protocols in sections 4.10 and 5.10). With this simple and rapid approach any pair of standard PCR primers can be adapted to give information about gene – promoter methylation.

The promoter and enhancer regions are not frequently mutated in the analyzed series of AML samples. If the genes are not mutated, the pathological expression of them could be explained by epigenetic changes, such as changes of promoter

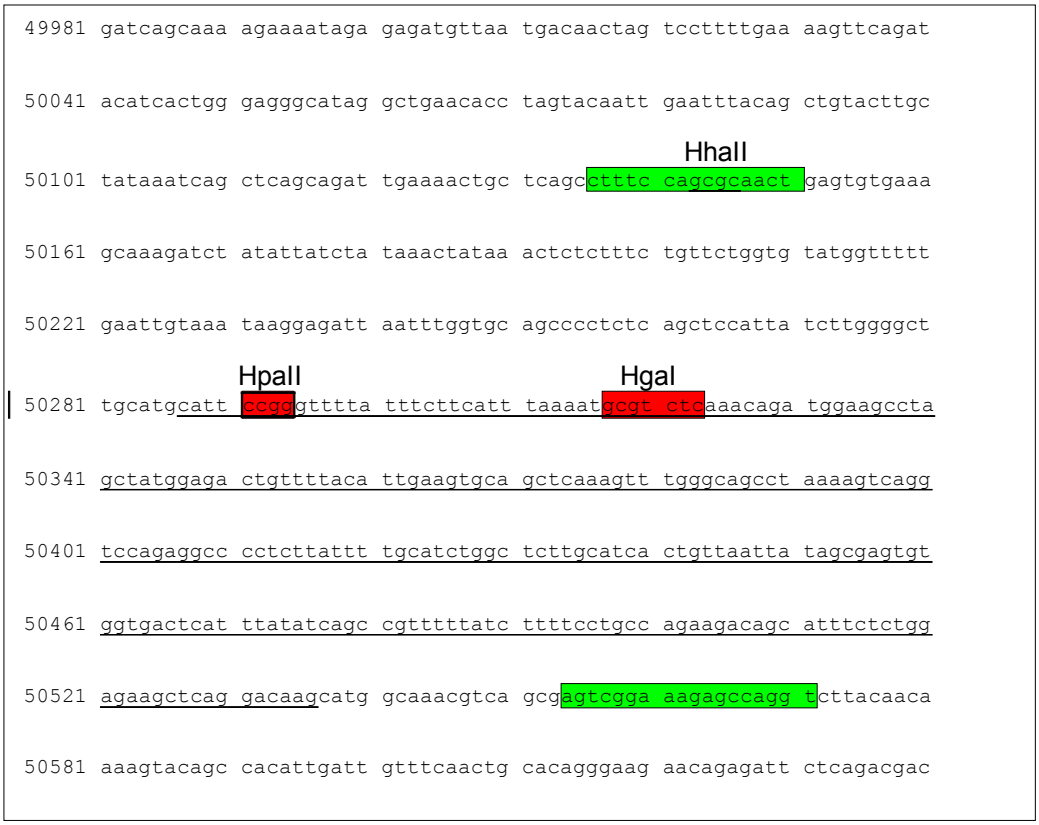

Figure 6.40: Fragment of WT1 enhancer. PCR primer binding sites shown in green. Locus AY245105. Minimal 250bp enhancer region underlined.

methylation, or upregulation of regulator genes. With a novel approach the methylation status of the two CpG residues in the WT1 enhancer was analyzed. Localization of the analyzed sites is shown on figure 6.40. The series of DNA samples from AML patients and healthy volunteers were studied. The three CpG sites can be analyzed with commercially available methylation sensitive restriction enzymes. These sites are 50144 restricted by Hha II, site 50293 restricted by Hpa II and site 50319 restricted by Hga II. The median methylation of the Hha II residue of

the AML samples didn't significantly differ from methylation of the healthy controls (figure 6.41). In AML median methylation was 70% compared to 65% in controls (p=0,27). The methylation at the Hpa II restriction site was 39% and was lower than in control – 50% (p=0,21). The big proportion of the AML samples seemed not to be methylated at this residue at all (figure 6.42). The median methylation of the Hga I position was 73% in AML. It differed significantly from median methylation healthy controls, which was 54% (p<0,03).

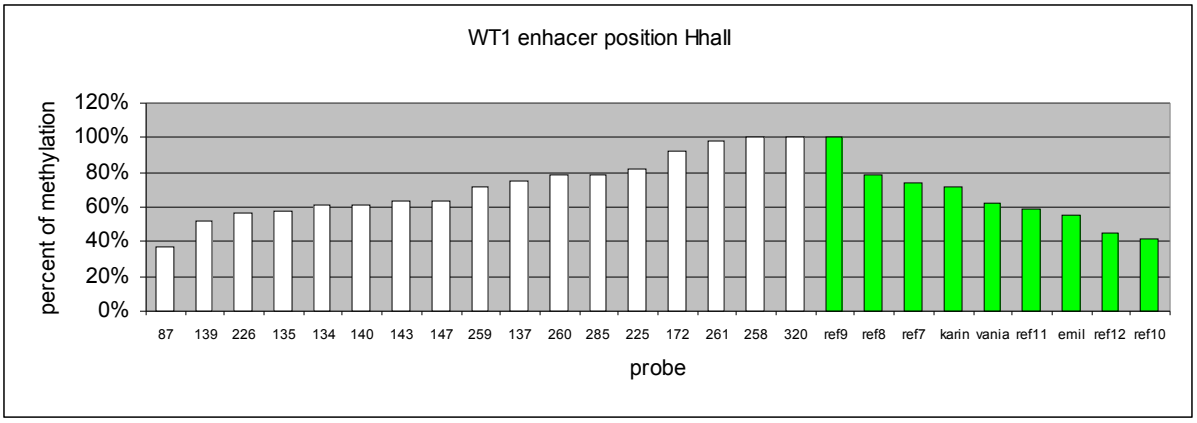

Figure 6.41: Methylation pattern of the HhaII residue of the WT1 enhancer. White bars AML cases, green bars healthy volunteers.

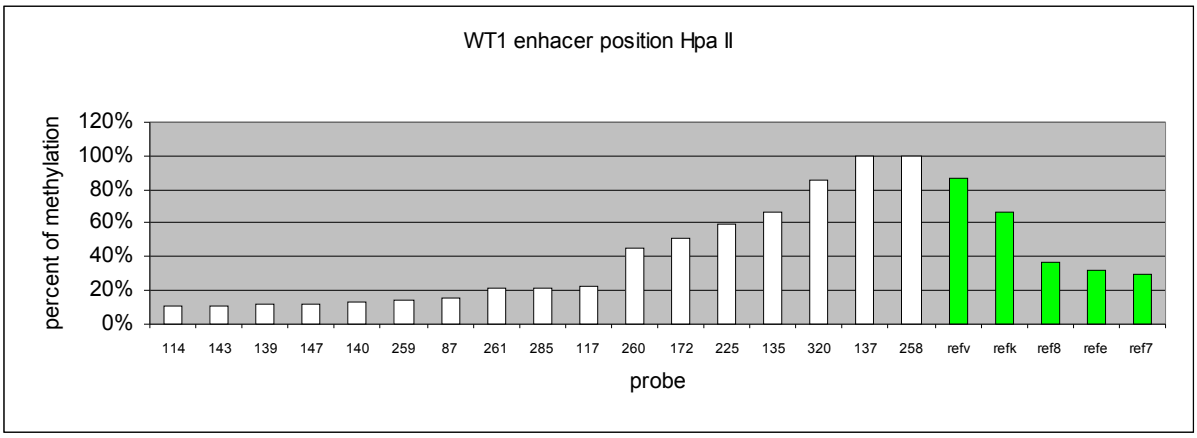

Figure 6.42: Methylation pattern of the HpaII residue of the WT1 enhancer. White bars AML cases, green bars healthy volunteers.

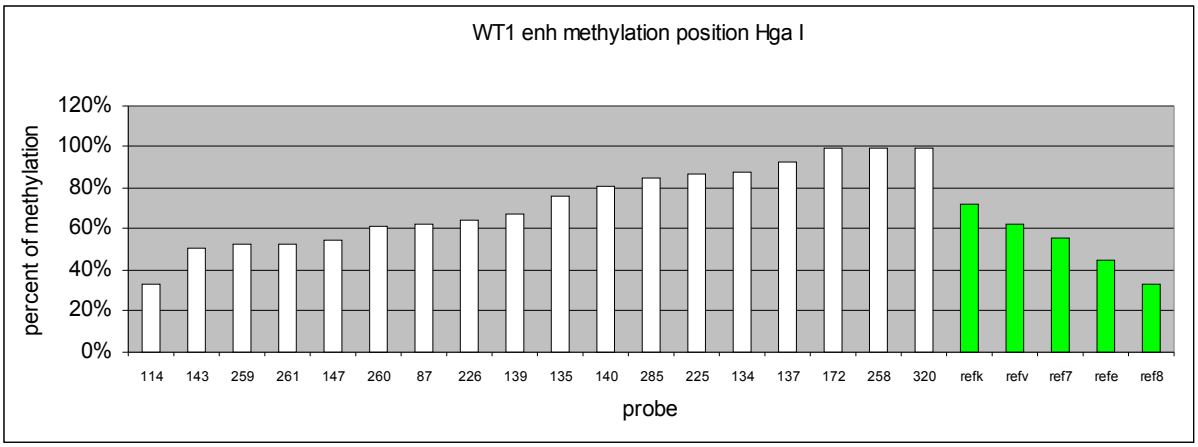

Figure 6.43: Methylation pattern of the Hga I residue of the WT1 enhancer. White bars AML cases, green bars healthy volunteers.

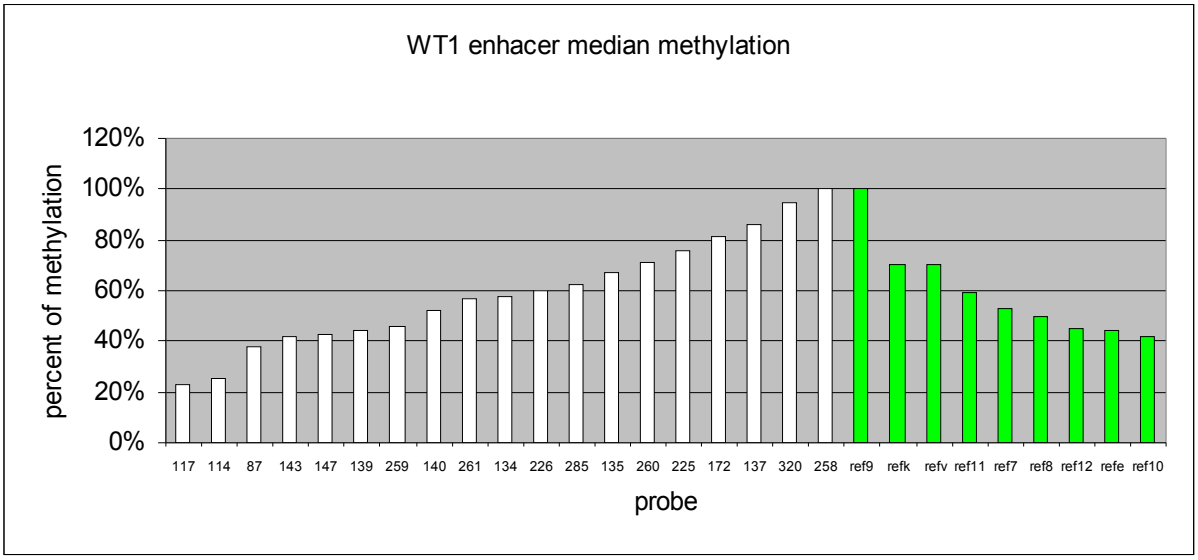

Figure 6.44: Median methylation of the WT1 enhancer. White bars AML cases, green bars healthy volunteers.

The median methylation of the enhancer was identical for the AML and control samples and was 59%. There was no correlation between blast number and the methylation profile of the samples analyzed on all residues.

6.5 Enhancer methylation status and gene expression levels

The expression levels of WT1 and GATA1 were analyzed in groups of AML cases with low and high methylation profile. The sample was stated as low methylated when methylation level at a given CpG residue was lower than the average methylation in the control group of healthy volunteers. The sample was referred to as high methylated if methylation was higher than the average of control. The methylation of both CpG residues localized in the minimal enhancer region (Frazier G.C. et al., 1994) – Hga I and Hpa II exhibited similar relation between expression of studied genes and promoter methylation. The expression levels of WT1 were higher in cases with high methylation than in cases with low promoter methylation (figures 6.45 and 6.46). The difference between expression ratios of WT1 at the Hga I residue in low and high methylated groups was statistically significant with p=0,049. The expression ratios of GATA1 gene were higher in cases with low methylation and lower in cases with high methylation (figures 6.45 and 6.46). However the difference was statistically insignificant.

In case of the Hha II residue, which lies outside the minimal enhancer of the WT1 gene, the relation between expression of studied genes and residue methylation was opposite (figure 6.47). The WT1 was expressed at higher levels in cases with low enhancer methylation and at lower levels in cases with high methylation status. The mean GATA1 expression was also higher in cases with low enhancer methylation. On the other hand, median GATA1 expression was lower in cases with low enhancer methylation. The differences were statistically insignificant.

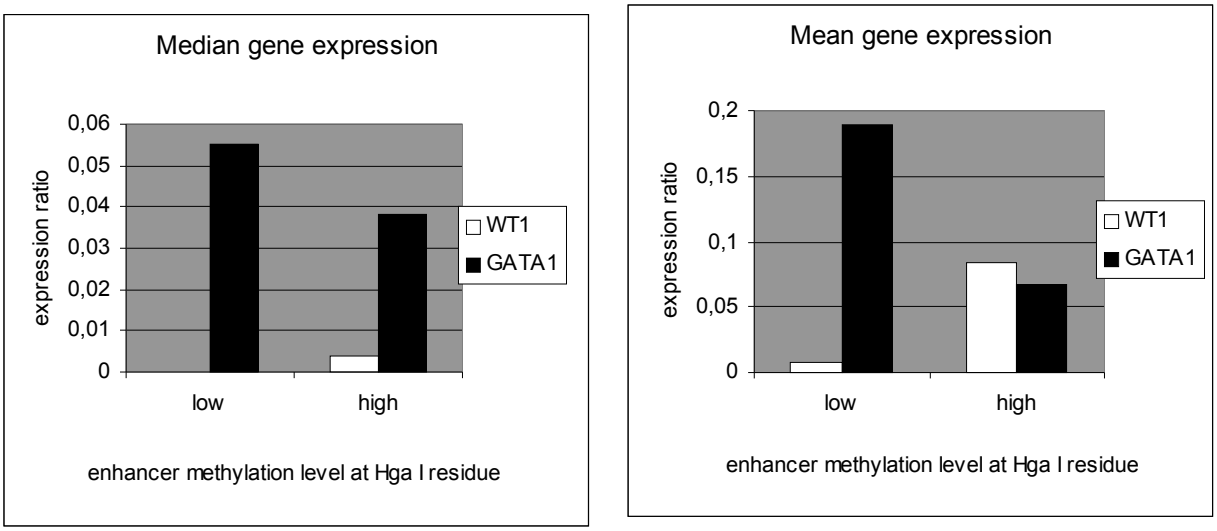

Figure 6.45: WT1 enhancer methylation status at the Hga I residue and gene expression ratios in AML patients. Black bars represent WT1 gene expression, white bars represent GATA1 gene expression. The cases were divided in two groups with low (thus lower than mean control methylation) and high methylation (thus higher than mean control methylation).

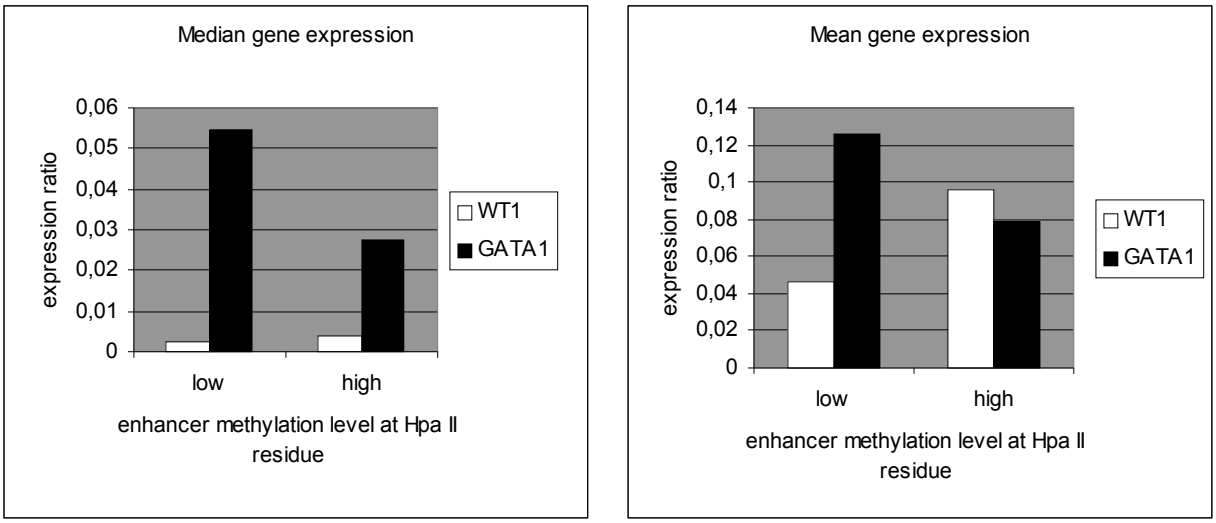

Figure 6.46: WT1 enhancer methylation status at the Hpa II residue and gene expression ratios in AML patients. Black bars WT1 gene expression, white bar GATA1 gene expression. The cases were divided in two groups with low (thus lower than mean control methylation) and high methylation (thus higher than mean control methylation).

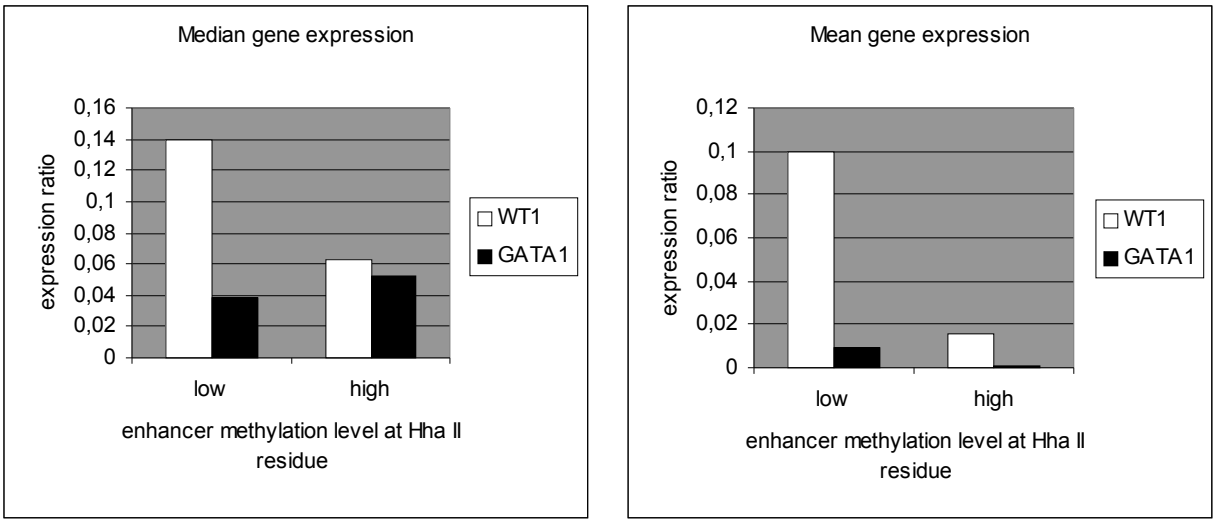

Figure 6.47: WT1 enhancer methylation status at the Hha II residue and gene expression ratios in AML patients. Black bars WT1 gene expression, white bar GATA1 gene expression. The cases were divided in two groups with low (thus lower than mean control methylation) and high methylation (thus higher than mean control methylation).