The effects of siRNA-mediated inhibition of *E2A-PBX1* on *EB-1* and *Wnt16b* expression in the 697 pre-B leukemia cell line

Giovanna Casagrande
Geertruy te Kronnie
Giuseppe Basso

Background and Objectives. A common non-random translocation in childhood pre-B acute lymphoblastic leukemia (ALL) is t(1;19)(q23;p13), usually resulting in the expression of the chimeric gene *E2A-PBX1*. The role of this fusion gene during leukemogenesis is not yet fully understood; one approach to investigate its function is to selectively deplete the E2A-PBX1 protein and examine the consequences.

Design and Methods. We tested the efficacy of anti-E2A-PBX1 siRNA in the 697 pre-B leukemia cell line. Transfection was monitored by fluorescence microscopy and FACS, while *E2A-PBX1* mRNA expression was measured using real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis. The reduction of the level of the corresponding fusion protein was assessed by western blot analysis and the expression of putative downstream target genes was detected by SYBR Green PCR.

Results. We demonstrated efficient downregulation induced by anti-E2A-PBX1 siRNA in 697 t(1;19)-positive leukemia cells. In particular, *E2A-PBX1* silencing affected the *EB-1* gene, which encodes for a protein that could contribute to the transformed phenotype of pre-B ALL. The detected *EB-1* expression was reduced to 25% of the normal expression level in non-transfected 697 cells. Furthermore, the significant decrease in *Wnt16b* mRNA levels (but not of the *Wnt16a* isoform of the *Wnt16* gene), observed following depletion of the fusion gene, confirms the hypothesis that *Wnt16b* is a target of *E2A-PBX1*. The siRNA inhibition was followed by an increase in apoptosis and similar results were obtained in two other ALL cell lines, one with and one without the t(1;19) translocation.

Interpretation and Conclusions. Targeted-E2A-PBX1 inhibition leads to reduced expression of the *EB-1* and *Wnt16b* genes; aberrant expression of these genes may be a key step in leukemogenesis in t(1;19)-positive pre-B leukemia.

Key words: *E2A-PBX1*, fusion gene, siRNA, *EB-1*, *Wnt16*.

Haematologica 2006; 91:765-771

From the Laboratory of Hematology and Oncology, Department of Pediatrics, University of Padova, Italy

Correspondence:
Geertruy te Kronnie, Laboratory of Hematology and Oncology Department of Pediatrics University of Padova, 35128 Padua, Italy.
E-mail: truustekronnie@unipd.it

The t(1;19) chromosomal translocation is detected cytogenetically in approximately 5-6% of childhood early B-lineage acute lymphoblastic leukemias (ALL). The translocation has, in the past, been associated with an increased risk of relapse,[1-3] although recent protocols have resulted in increased overall survival.[4,5] The resulting E2A-PBX1 protein has been investigated extensively[6,7] and shown to be capable of transformation in fibroblast,[8] myeloid[9] and T lineage cells.[10] This fusion protein alters the properties pre-B-cell leukemic homeobox1 (PBX1), converting it to a potent transcriptional activator.[11,12] PBX1 binds specific DNA elements forming heterodimers with the Hox family of transcription factors and has no transactivating activity by itself.[13] E2A-PBX1, instead, has strong transactivation potential, inappropriately activating genes normally regulated by the PBX-Hox complexes.[14] The other gene involved in this translocation, E2A, codes for two alternatively spliced transcription factors characterized by the presence of a basic helix-loop-helix (bHLH) motif, E12 and E47, which regulate developmental pathways in various organs and cell types.[15] Given the importance of E2A in B lymphopoiesis, the oncogenic potential of the t(1;19) translocation is suggested to be due, in part, to the reduction of wild-type *E2A* that may disrupt the maturation of lymphoid lineages and expand undifferentiated progenitor populations. In addition to activating the expression of lymphocyte-specific genes, there is growing evidence that *E2A* can function as a tumor suppressor. Since *E2A* gene products are required for B-cell formation[16,17] and *E2A-PBX1* is under the control of the E2A promoter, the fusion protein is expected to activate target genes at a certain stage of B-cell differentiation leading to pre-B ALL. To clarify the role of E2A-PBX1 in leukemia cells and to explore the possibility of E2A-PBX1-targeted therapy, RNA interference (RNAi) was applied to knockdown the fusion gene

expression. RNAi is a post-transcriptional gene silencing induced by small interfering RNA (siRNAs), double-stranded oligonucleotides that mediate sequence-specific mRNA degradation.[18,19] The silencing of the fusion gene in 697 leukemia cells also affected the expression of *EB-1* and *Wnt16* genes. *EB-1* is significantly upregulated in t(1;19)-positive cells and not expressed in cells that lack this translocation, indicating that *EB-1* transcriptional activation may require either a co-operating oncogene or the expression of genes within the specific t(1;19)-positive target cells.[20] *Wnt16* belongs to the Wnt family of secreted glycoproteins, a group of signaling molecules that have been shown to control a range of developmental processes including cell fate specification, cell proliferation, cell polarity and migration.[21] The E2A-PBX1 fusion protein consistently activates transcription of the *Wnt16* gene in pre-B ALL, raising the possibility that Wnt signaling may contribute to the initiation and/or progression of this malignancy.

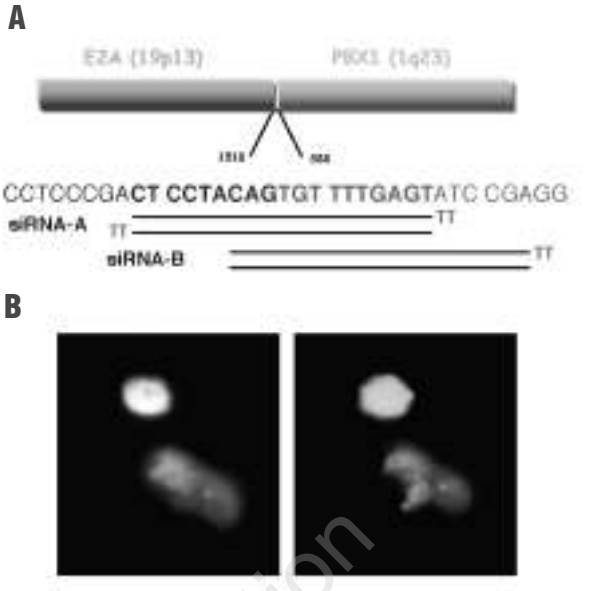

Figure 1. siRNA targeted against *E2A-PBX1* expression: sequences, target sites and siRNA internalization in 697 t(1;19)-positive cells. A. Schematic representation of the classical *E2A-PBX1* fusion gene transcript, siRNA sequences and their target sites. The *E2A-PBX1* transcript considered in this study, as in the majority of cases,[6] has a constant junction between the *E2A* exon 13 (*gb, GenBank accession no., M3122.1*) and the *PBX1* exon 2 (*gb M86546.1*). The sequence of the *E2A-PBX1* fusion site targeted by the dsRNA is indicated (blue letters, *E2A* gene; pink letters, *PBX1* gene; bold letters, anti-E2A-PBX1-A siRNA sense strain). The siRNA targeted against the fusion site (anti-E2A-PBX1-A and anti-E2A-PBX1-B, referred to in the figures for brevity as siRNA-A and siRNA-B) were 21-nt in length, with a 3'-overhanging TT. A non-silencing control siRNA oligonucleotide (NS-siRNA), designed for *Thermotoga maritima*, was used as a negative control. B. Intracellular distribution of transfected siRNA in 697 cells (100x original magnification). After delivery of 20nM 3'-FITC-labeled anti-E2A-PBX1-A siRNA, DAPI(4'6-diamidino-2-phenylindole-2HCl)-stained cells (left) were visualized by fluorescence microscopy (right).

Design and Methods

Cell culture and reagents

The pre-B leukemia cells were grown in RPMI 1640 medium (Invitrogen, Heidelberg, Germany) supplemented with fetal bovine serum (FBS), 1 unit/mL penicillin G, and 1 μg/mL streptomycin and maintained in a humidified 37°C incubator with 5% CO_2. The supplemented FBS was 10% (v/v) for the 697 cells and 20% (v/v) for the REH and RCH-ACV cell lines. All the cell lines were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

siRNAs

E2A-PBX1 gene expression was knocked down by siRNA gene-silencing. Duplexes of 21-nucleotide siRNA with 3'-overhanging TT were designed according to Elbashir *et al.*[22] and synthesized by Qiagen-Xeragon (Hilden, Germany). The sense strand of the first siRNA (anti-E2A-PBX1-A) was CUC CUA CAG UGU UUU GAG U and that of the second siRNA (anti-E2A-PBX1-B) was CAG UGU UUU GAG UAU CCG A, corresponding respectively to positions 615-633 and 621-639 of the *E2A-PBX1* mRNA relative to the start codon (GenBank accession no. M31522.1). Both siRNA carried a 3'-fluoresceinyl modification and their sequences and corresponding target sites are shown in Figure 1A. A non-silencing control siRNA oligonucleotide designed by QIAGEN-Xeragon for *Thermotoga maritima*, 5'-UUC UCC GAA CGU GUC ACG U d(TT)-3', has no target gene in mammalian cells and was used as a negative siRNA control for transfection.

Transfection of 697 cells with siRNA

The duplex RNAi ribonucleotides were hybridized at a final concentration of 20 μM in hybridization buffer (25 mM Tris [tris(hydroxymethyl)aminomethane]-Cl pH 7.5, 100 mM NaCl) by heating them to 95°C and then incubating for 1 hour at 37°C. Transfection was carried out with the Nucleofection™ system (Amaxa, Köln, Germany) according to the manufacturer's instructions. Briefly, 10^6 cells were resuspended in 100 μL of Nucleofector solution R (Cell line Nucleofector™ kit R) and, immediately before the transfection step, double-stranded siRNA were added, yielding a final concentration of 20 nM. After nucleofection, 400 μL of prewarmed culture medium were added to the cuvette and the cells were transferred to prewarmed culture plates.

siRNA uptake studies

The amount of fluorescently stained cells was determined by flow cytometry using a FACS Vantage SE (Becton Dickinson, Heidelberg, Germany) at 4 and 16 hours after electroporation of fluorescein-labeled siRNA; the flow cytometer provides this quantification directly, as a percent of positively-fluorescent cells (therefore successfully transfected with siRNA) upon the total cell population. The cells were previously washed with fluorescence activated cell-sorting (FACS) buffer, consisting of phosphate-buffered saline (PBS) +

1% bovine serum albumin (BSA) + 0.1% sodium azide. A Leica DMRB fluorescence microscope (Wetzlar, Germany) was used to examine the intracellular distribution of siRNA and images were acquired with a SenSys camera (Photometrics, Tucson, AZ, USA) and SC Casti Imaging software (SC Processing, Venice, Italy).

Apoptosis assay

Apoptosis was detected using an Annexin-V-Fluos staining kit (Roche Diagnostics GmbH, Penzberg, Germany). Briefly, 10^6 cells were washed once with PBS and then resuspended in 100 μL of HEPES buffer containing annexin-V-fluorescein labeling reagent and propidium iodide solution, accordingly to the manufacturer's instructions. The suspension was gently vortexed and incubated at room temperature for 15 min. After addition of 500 μL of HEPES buffer the cells were analyzed using a Cytomics 500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) and RxP software (Becton Dickinson, San José, CA, USA).

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted using the PARIS kit (Ambion, Cambridgeshire, UK) according to the manufacturer's instructions and reverse transcription was performed with GeneAmp PCR System 2400™ (Applied Biosystems, Foster City, CA, USA). The E2A-PBX1 primers and probe (BD Biosciences, Erembodegen, Belgium) used for *E2A-PBX1* amplification were: primers (forward, PBX1) 5'-GGG CTC CTC GGA TAC TCA AAA-3' and (reverse, E2A) 5'-CCA GCC TCA TGC ACA ACC A-3', and TaqMan FAM/TAMRA hybridization probe 5'-CCC TCC CTG ACC TGT CTC GGC C-3', designed according to the *Europe Against Cancer* protocols.[23] The amount of E2A-PBX1 product was normalized to the expression of Abelson gene (*ABL*). SYBR Green PCR was conducted for *E2A-PBX1* downstream genes using the HPSF-purified primers (MWG-Biotech, Ebersberg, Germany) listed in Table 1, which had been designed with the Primer Express 1.0 software (Applied Biosystems) and selected upon testing their efficacy. The mRNA levels detected by SYBR Green analysis were normalized to beta-glucoronidase (*GUS*) mRNA levels. *GUS* is a valid control gene for quantitative PCR in leukemia studies and has been used as the first choice control gene for SYBR Green PCR experiments; *ABL* was preferred as the control gene for the *E2A-PBX1* mRNA measurements with TaqMan real-time PCR, since, according to the *Europe Against Cancer* protocols,[23] it is considered the most reliable control gene for quantification of fusion gene transcripts. Quantitative RT-PCR and SYBR Green PCR were performed with an ABI Prism 7700 Sequence Detector (Applied Biosystems) and the quantification was made using the *relative standard curve Method*.

Analysis of protein expression

Western blot was carried out using the monoclonal antibodies anti-E2A-PBX (BD Pharmingen, Franklin Lakes, NJ, USA) and anti-β-actin (Sigma Aldrich, Saint Louis, MO, USA), and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The analysis was performed on total lysates with equal amounts of protein (2 μg), quantified by colorimetric detection based on the bicinchoninic acid (BCA) test. Samples were heated at 95°C for 5 min and loaded in a 12.5% Phast-gel (Amersham Bioscience, Piscataway, NJ, USA). After electrophoresis, the separated protein fractions were transferred to a methanol-activated Hybond-P membrane (Amersham Bioscience). A double-antibody procedure and chemiluminescence (ECL Advanced Western Blotting Detection Kit, Amersham Bioscience) were used to detect the proteins.

Table 1. List of primers used for *EB1* and *Wnt16* gene amplifications.

Primer name	*Sequence*
1. EB-1-2316F	5'-AAC ACC ATC CTT CAC ATC GGA -3'
2. EB-1-2420R	5'-CTT GTG GTC TCA CCA TTC ATC TCT -3'
3. Wnt16a-59F	5'-ACC ACT TGC CTC AGG GAG ACC -3'
4. Wnt16a-168R	5'-AAA TTG GCG CAG CCC AG -3'
5. Wnt16b-319F	5'-TGC TGT TCC CCT ACG GAG C -3'
6. Wnt16b-430R	5'-TCC TTC TGG CGG CTG TTC -3'

Primers were designed with Primer Express 1.0 software based on published cDNA sequences for EB-1, Wnt16a and Wnt16b genes. "F" and "R" stand for Forward and Reverse primers, respectively.

Results

Transfection efficiency and intracellular distribution of siRNA

For the siRNA uptake studies we used 3'-fluorescein-labeled siRNAs delivered to 697 cells by Nucleofection™. Almost all cells had FITC-fluorescence 4 and 16 hours after transfection, as judged by FACS analysis (approximately 90% of viable cells were efficiently loaded with fluorescein-labeled siRNA, *data not shown*). The cellular internalization of siRNA was determined by fluorescence microscopy, which revealed the fluorescent label in a perinuclear localization, whereas the nuclear regions were only weakly stained (Figure 1B). The maximum silencing effect on mRNA occurred at 24 hours (*data not shown*), so quantitative RT-PCR, protein extraction, and western blotting were performed for samples harvested 24 hours after siRNA delivery. The maximum effect on apoptosis occurred 24 hours later and apoptosis was, therefore, measured at this time.

Selection of anti-E2A-PBX1 siRNA

Two different siRNAs targeted against the fusion site of *E2A-PBX1* and a non-silencing control (NS-siRNA) were transfected into the 697 cells. Of the chemically synthesized 21-nt siRNA tested (Figure 1A), anti-E2A-PBX1-A was the most efficient, as shown in Figure 2A. The siRNA concentration used was 20 nM: this was selected from a range of different concentrations (from

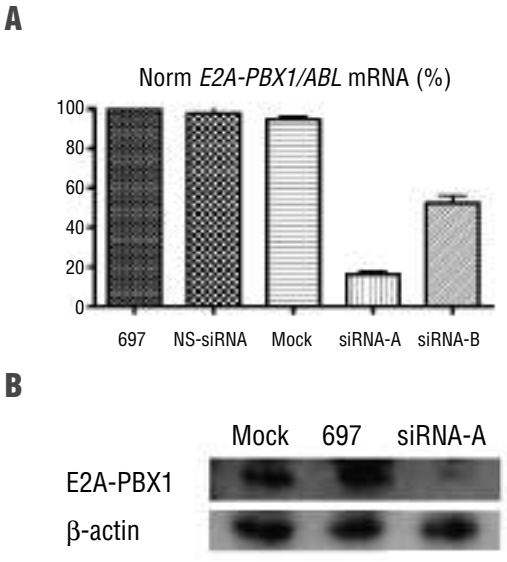

Figure 2. siRNA-mediated inhibition of *E2A-PBX1* expression in t(1;19)-positive cells. A. Normalized *E2A-PBX1/ABL* mRNA levels were measured by TaqMan quantitative RT-PCR 24 hours after transfection and are shown compared to levels in non-transfected 697 control cells (100%). Two independent transfection experiments were performed and each of the mRNA levels was measured in duplicate. B. Western blot of whole cell lysates 24 hours after transfection. Cells were transfected with anti-E2A-PBX1-A siRNA (named *siRNA-A*, in the third lane) and without siRNA (*Mock*) and were compared with the 697 cell line. β-actin blotting was carried out as a control.

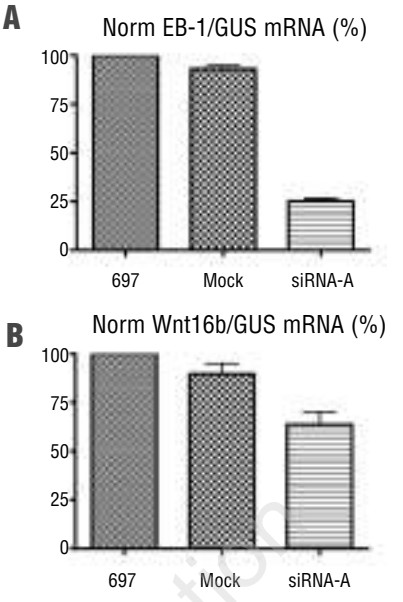

Figure 3. SYBR Green PCR conducted for the *E2A-PBX1* downstream genes *EB-1* and *Wnt16b*. The mRNA levels were measured 24 hours after transfection and are shown compared to the normal level in the 697 cell line. *GUS* was used as the housekeeping gene for normalization and the values are means ± SD of three independent experiments. *Mock* represents the mock control and *siRNA-A* the cells transfected with anti-E2A-PBX1-A siRNA. A. *EB-1* mRNA levels were detected by SYBR Green PCR and the primers used were EB-1-2316F and EB-1-2420R, selected as the most efficient from four tested *EB-1* primers. *E2A-PBX1* silencing also produced a significant decrease in *EB-1* mRNA. B. *Wnt16b* mRNA was reduced as a consequence of the fusion gene inhibition. There was no amplification signal for *Wnt16a* in 697 cells. All the primers used for the amplifications are listed in Table 1.

1 nM to 100 nM) as the lowest amount able to induce the most significant silencing effect (*data not shown*). mRNA levels were quantified in relation to the amount of the *ABL* housekeeping gene by real-time RT-PCR. After 24 hours, anti-E2A-PBX1-A and anti-E2A-PBX1-B siRNA reduced *E2A-PBX1* mRNA levels to 15±5% and 50±6%, respectively, of the expression levels of t(1;19)-positive 697 cells (Figure 2A). *E2A-PBX1* mRNA was not affected by NS-siRNA and the expression levels in the cells that underwent the transfection conditions (mock control) were similar to those of the non-transfected cell line (Figure 2A). siRNA-dependent reduction of *E2A-PBX1* mRNA corresponded to a major decrease of the chimeric protein, as shown by western blot analysis (Figure 2B).

E2A-PBX1 suppression affects downstream genes

Recently, human pre-B cell lines containing the t(1;19) translocation were compared to transformed pre-B cell lines lacking this translocation.[24] A number of genes differentially expressed in t(1;19) cell lines were identified,[20,24] two of which were considered in this study: *EB-1* and *Wnt16*. The first encodes a phosphotyrosine binding domain protein and as such may play a role in the regulation of cell proliferation,[20] while *Wnt16* encodes a novel member of the WNT/WG family of growth factors.[25] *EB-1* transcripts are expressed exactly where *PBX1* is most highly expressed; normal *EB-1* expression could be regulated by PBX proteins, while its aberrant expression in pre-B ALL could be influenced by the E2A-PBX1 protein (through a mechanism in which E2A-PBX1 replaces endogenous PBX proteins bound to enhancer elements). As detected by quantitative SYBR Green PCR (Figure 3A) inhibition of *E2A-PBX1* leads to a significant 4-fold decrease in *EB-1* mRNA levels, compared to the levels in the mock control. Although *Wnt16* is not usually expressed in pre-B cells,[24] Wnt signaling has been demonstrated to have a role in early hematopoiesis and pro-B cell proliferation.[26] The *Wnt16* gene has two isoforms, generated from separate promoters rather than splicing;[27] the *Wnt16* gene originally described by McWhirter *et al.*[24], constitutively expressed by *E2A-PBX1* corresponds to the *Wnt16b* isoform. A previous assumption, considering the tandem E2A-PBX1 response elements in the putative Wnt16a promoter, suggested that *Wnt16a* rather than *Wnt16b* was specifically upregulated in ALL.[27] However, in this study *Wnt16b* was the only isoform detected in the 697 human pre-B cell line. There was no detectable signal for the *Wnt16a* isoform, indicating its absence from the 697 cell line. Quantitative SYBR Green PCR of *Wnt16* mRNA, following *E2A-PBX1* silencing, showed a reduction of the b-isoform, and the corresponding *Wnt16b* mRNA decreased to 57±8% (Figure 3B).

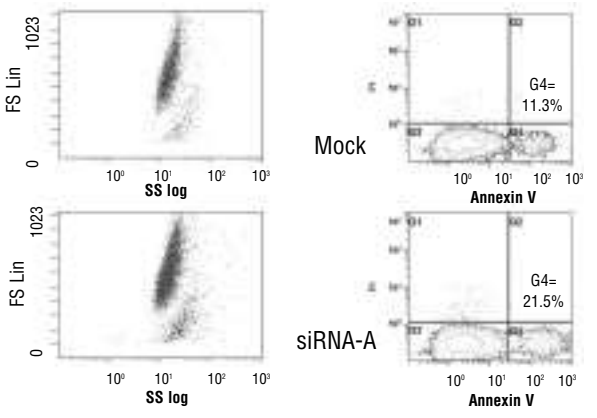

Figure 4. ***E2A-PBX1*** **siRNA-mediated apoptosis in 697 cells. Apoptosis was measured 48 hours after transfection of 697 cells with 20nM of anti-E2A-PBX1-A siRNA and without siRNA (Mock control). Annexin V was used as a sensitive probe for phosphatidylserine (PS), which is translocated to the outer cell plasma membrane during apoptosis. Simultaneous staining with propidium iodide (PI), which is not cell membrane permeable, distinguishes apoptotic cells (annexin-positive but PI-negative) from necrotic cells. The analysis was performed with a Cytomics 500 flow cytometer (Beckman Coulter), and the light scatter graphs represent one of three independent experiments with similar results. Each sample is plotted as *FS (Lin.) vs. SS (Log)* and as *PI vs. annexin V*.**

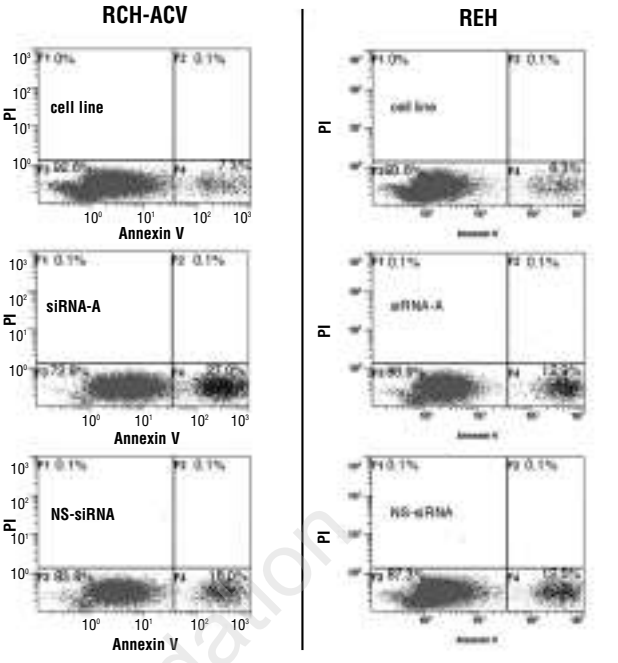

Figure 5. ***E2A-PBX1*** **siRNA-mediated apoptosis in the RCH-ACV and REH cell lines. Apoptosis was measured 48 hours after transfection of the cells with 20nM of anti-E2A-PBX1-A siRNA and without siRNA (Mock control). The light scatter graphs represent one of three independent experiments with comparable results. Each sample is plotted as *FS (Lin.) vs. SS (Log)* and as *PI vs. annexin V*. (*Left*). Apoptosis in the RCH-ACV cell line (top), in RCH-ACV cells transfected with anti-E2A-PBX1 siRNA-A (middle) and in RCH-ACV cells transfected with NS-siRNA (*bottom*). (*Right*) REH cell line (top), REH cells transfected with anti-E2A-PBX1 siRNA-A (*middle*) and REH cells transfected with NS-siRNA (*bottom*).**

Silencing effect on apoptosis

Apoptosis was assessed 48 hours after siRNA transfection and increased from 11.3% in the *E2A-PBX1* silenced cells to 21.5% in the mock control (Figure 4). Two more pre-B-ALL cell lines were used to validate these results. One of the cell lines, RCH-ACV, harbors the t(1;19) translocation while the other, REH, is t(1;19)-negative. After assessing the presence of the fusion, RCH-ACV was silenced following the procedure previously optimized for the 697 cells. siRNA inhibition of *E2A-PBX1* induced significant apoptosis also in the RCH-ACV cells, when compared to the non-silencing control (Figure 5). The viability of REH cells, transfected under the same conditions, with either anti-E2A-PBX1-A, which had no fusion gene target, or with NS-siRNA did not differ (Figure 5).

Discussion

RNA interference offers a powerful tool for silencing the expression of specific genes allowing loss-of-function analysis and the study of molecular mechanisms. It represents a very promising technology for developing tailored therapeutics in the treatment of cancers, especially because it has the advantage of being a natural process that the cell normally utilizes for its own regulation. Applications of RNAi in oncology have been focused mainly on mutated and overexpressed genes, viral oncogenes, and fusion oncogenes to elucidate the function of these genes and their interaction with other molecules.

One of the advantages of siRNA technology is its ease of application, aiming gene expression knock-down, when compared to traditional methods and to other RNAi technologies. So, when the protein encoded by the silenced gene has a sufficiently short half-time, as in the case of E2A-PBX1, it is the preferable method to use. Beside this, the intracellular siRNA expression obtained by transcription of shRNA from viral vectors could induce side effects such as cell adaptation to shRNA expression or deregulation of the shRNA cassette itself. Even if viral technology could be considered for studying oncogenic mechanisms, its future as eventual therapeutic option is uncertain, due to safety concerns. Another consideration to take in account, is that shRNA intracellular expression is usually driven by an RNA polymerase III promoter, such as the H1 or the U6 promoter. Many siRNA targeting fusion sites of chimeric mRNA are not suitable for expression by the polymerase III system because the four or more thymidine stretches that could be carried in the siRNA sequence also correspond to the terminal signals for RNA-polymerase III.

In this study siRNA were used for efficient suppression of the E2A-PBX1 leukemia fusion protein. First, we validated the delivery of siRNA in the 697 pre-B ALL cell line: the cellular uptake was detected by fluorescent confocal microscopy using fluorescein-labeled siRNA and FACS analysis revealed that approximately 90% of viable transfected cells carried siRNA. The *E2A-PBX1*

mRNA and protein levels were analyzed 24 hours after the transfection in order to ensure sufficient time for specific mRNA cleavage and new synthesis of the fusion protein. The ability of the designed siRNA to reduce *E2A-PBX1* mRNA in 697 cells was estimated by quantitative real-time RT-PCR, which showed that the expression of the target gene was reduced to 15±5% compared to that in the non-transfected 697 cells (or the mock control that was characterized by a similar *E2A-PBX1* expression). Western blotting showed a notable reduction in the protein level.

Analysis of downstream signaling molecules showed that siRNA-induced downregulation of *E2A-PBX1* resulted in a considerable decrease of *EB-1* and *Wnt16b* mRNA. Representation display analysis (RDA) PCR performed in t(1;19)-positive and -negative human pre-B ALL cell lines identified *EB-1* as one of the mRNA specifically expressed in the cells containing the t(1;19) translocation.[20] This method can identify *E2A-PBX1* target genes whose transcription requires co-activation of genes encoding heterodimer partners of *E2A-PBX1*, but also genes transcriptionally independent from the fusion gene and whose products have a complementing function in altering cellular proliferation or differentiation. *EB-1* is known to be invariantly expressed in the subset of t(1;19)-positive pre-B leukemias,[20] and the decrease in its mRNA resulting from *E2A-PBX1* siRNA inhibition confirms that it is a gene transcriptionally activated by E2A-PBX1.

Contrary to the hypothesis that *Wnt16a* rather than *Wnt16b* is specifically upregulated in ALL (based on the tandem *E2A-PBX1* response elements in the putative Wnt16a promoter),[27] we found that only *Wnt16b* was upregulated in the 697 ALL cells. The results of very recent work by Mazieres *et al.*[28] concur with and confirm this finding. In our study, *E2A-PBX1* silencing reduced the mRNA level of *Wnt16b* to 57±8%. Therefore, *Wnt16b* specifically seems to be transcriptionally activated by *E2A-PBX1*, and its aberrant expression in pre-B ALL cells could be a key-step towards the development of t(1;19)-positive leukemias.

The efficient suppression of *E2A-PBX1* by siRNA was accompanied by an increase in apoptosis (almost 2-fold when compared to the mock control). This effect was also seen when the fusion gene was silenced in another t(1;19)-positive leukemia cell line, RCH-ACV, which showed a high percentage of apoptotic cells at 48 hours, compared to the cells transfected with NS-siRNA. In contrast, in a t(1;19)-negative pre-B leukemia cell line, apoptosis did not differ according to which siRNA was delivered.

The prospect of using siRNA as powerful small molecule inhibitors of a particular gene offers a new therapeutic approach for any pathology that occurs due to specific overexpression of the particular gene; RNAi-induced silencing of chimeric fusions is an ideal way to target tumor cells specifically, while leaving normal cells unaffected, and represents a promising principle to be developed further for clinical applications.

GC designed the project, conceived and performed the experiments, analyzed the data, and wrote the manuscript. GtK provided critical discussion and participated in the co-ordination of the study. GB supervised the project, provided analytical tools, interpreted the analyses, and contributed to the revision of the manuscript. All authors read and approved the final manuscript. The authors report no potential conflict of interest. There are no potentially redundant publications. The authors declare that they have no potential conflict of interests. *This study was supported by the Italian foundation "Citta' della Speranza".*

Manuscript received December 4, 2005. Accepted April 6, 2006.

References

1. Carroll AJ, Crist WM, Parmley RT, Roper M, Cooper MD, Finley WH. Pre-B cell leukemia associated with chromosome translocation 1;19. Blood 1984;63:721-4.
2. Hunger SP, Fall MZ, Camitta BM, Carroll AJ, Link MP, Lauer SJ, et al. E2A-PBX1 chimeric transcript status at end of consolidation is not predictive of treatment outcome in childhood acute lymphoblastic leukemias with a t(1;19)(q23;p13): a Pediatric Oncology Group study. Blood 1998;91:1021-8.
3. Nourse J, Mellentin JD, Galili N, Wilkinson J, Stanbridge E, Smith SD, et al. Chromosomal translocation t(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. Cell 1990; 60:535-45.
4. Frost BM, Forestier E, Gustafsson G, Nygren P, Hellebostad M, Jonmundsson G, et al. Translocation t(1;19) is related to low cellular drug resistance in childhood acute lymphoblastic leukaemia. Leukemia 2005; 19:165-9.
5. Carroll WL, Raetz EA. Building better therapy for children with acute lymphoblastic leukemia. Cancer Cell 2005; 7:289-91.
6. Hunger SP, Galili N, Carroll AJ, Crist WM, Link MP, Cleary ML. The t(1;19)(q23;p13) results in consistent fusion of E2A and PBX1 coding sequences in acute lymphoblastic leukemias. Blood 1991;77:687-93.
7. Aspland SE, Bendall HH, Murre C. The role of E2A-PBX1 in leukemogenesis. Oncogene 2001;20:5708-17.
8. Fu X, Kamps MP. E2a-Pbx1 induces aberrant expression of tissue-specific and developmentally regulated genes when expressed in NIH 3T3 fibroblasts. Mol Cell Biol 1997;17:1503-12.
9. Kamps MP, Baltimore D. E2A-Pbx1, the t(1;19) translocation protein of human pre-B-cell acute lymphocytic leukemia, causes acute myeloid leukemia in mice. Mol Cell Biol 1993;13:351-7.
10. Dedera DA, Waller EK, LeBrun DP, Sen-Majumdar A, Stevens ME, Barsh GS, et al. Chimeric homeobox gene E2A-PBX1 induces proliferation, apoptosis, and malignant lymphomas in transgenic mice. Cell 1993;74:833-43.
11. LeBrun DL, Cleary ML. Fusion with E2A alters the transcriptional properties of the homeodomain protein PBX1 in t(1;19) leukemias. Oncogene 1994; 9: 1641-7.
12. Lu Q, Wright DD, Kamps MP. Fusion with E2A converts the Pbx1 homeodomain protein into a constitutive transcriptional activator in human leukemias carrying the t(1;19) translocation. Mol Cell Biol 1994;14:3938-48.
13. Van Dijk MA, Voorhoeve PM, Murre C. Pbx1 is converted into a transcriptional activator upon acquiring the N-terminal region of E2A in pre-B-cell acute lymphoblastoid leukemia. Proc Natl Acad Sci USA 1993;90:6061-5.
14. McWhirter JR, Goulding M, Weiner JA, Chun J, Murre C. A novel fibroblast growth factor gene expressed in the developing nervous system is a downstream target of the chimeric homeodomain oncoprotein E2A-Pbx1. Development 1997;124:3221-32.
15. Kadesch T. Helix-loop-helix proteins in the regulation of immunoglobulin gene transcription. Immunol Today 1992; 13:31-6.
16. Bain G, Maandag EC, Izon DJ, Amsen D, Kruisbeek AM, Weintraub BC, et al. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. Cell 1994;79:885-92.
17. Zhuang Y, Soriano P, Weintraub H. The helix-loop-helix gene E2A is required for B cell formation. Cell 1994;79:875-84.
18. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA

interference in cultured mammalian cells. Nature 2001;411:494-8.
19. Hannon GJ, Rossi JJ. Unlocking the potential of the human genome with RNA interference. Nature 2004; 431: 371-8.
20. Fu X, McGrath S, Pasillas M, Nakazawa S, Kamps MP. EB-1, a tyrosine kinase signal transduction gene, is transcriptionally activated in the t(1;19) subset of pre-B ALL, which express oncoprotein E2a-Pbx1. Oncogene 1999;18:4920-9.
21. Wodarz A, Nusse R. Mechanisms of Wnt signalling in development. Annu Rev Cell Dev Biol 1998;14:59-88.
22. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 2001;15:188-200.
23. Gabert J, Beillard E, van der Velden VH, Bi W, Grimwade D, Pallisgaard N, et al. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia: a Europe Against Cancer program. Leukemia 2003;17:2318-57.
24. McWhirter JR, Neuteboom ST, Wancewicz EV, Monia BP, Downing JR, Murre C. Oncogenic homeodomain transcription factor E2A-Pbx1 activates a novel WNT gene in pre-B acute lymphoblastoid leukemia. Proc Natl Acad Sci USA 1999; 96:11464-9.
25. Polakis P. Wnt signalling and cancer. Genes Dev 2000;14:1837-51.
26. Reya T, O'Riordan M, Okamura R, Devaney E, Willert K, Nusse R, et al. Wnt signalling regulates B lymphocyte proliferation through a LEF-1 dependent mechanism. Immunity 2000;13:15-24.
27. Fear MW, Kelsell DP, Spurr NK, Barnes MR. Wnt-16a, a novel Wnt-16 isoform, which shows differential expression in adult human tissues. Biochem Biophys Res Commun 2000;278:814-20.
28. Mazieres J, You L, He B, Xu Z, Lee AY, Mikami I, et al. Inhibition of Wnt16 in human acute lymphoblastoid leukemia cells containing the t(1;19) translocation induces apoptosis. Oncogene 2005;24:5396-400.