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

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 isofrom 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.
expression. RNAi is a post-transcriptional gene silencing induced by small interfering RNA (siRNAs), double-stranded oligonucleotides that mediate sequence-specific mRNA degradation. The silencing of the fusion gene in 697 leukemia cells also affected the expression of EB-1 and Wnt6 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. Wnt6 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. The E2A-PBX1 fusion protein consistently activates transcription of the Wnt6 gene in pre-B ALL, raising the possibility that Wnt signaling may contribute to the initiation and/or progression of this malignancy.

**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% CO₂. 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. 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. M31252.1). 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 GAA CGU GUC AGC 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 (Amazax, Köln, Germany) according to the manufacturer’s instructions. Briefly, 10⁶ 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 nucleasefication, 400 μL of pre-warmed 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) +

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**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, has a constant junction between the E2A exon 13 (gb: GenBank accession no. M31252.1) and the PBX1 exon 2 (gb M31522.1). 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).
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, Tuscon, 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, Cambridge, 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 GGC C-3', designed according to the *Europe Against Cancer* protocols. 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 Primer Express 1.0 software (Applied Biosystems). 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, 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.

**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

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

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>1. EB1-2316F</td>
<td>5'-AAC ACC ATC CCT ACC ACG GGA -3'</td>
</tr>
<tr>
<td>2. EB1-2420R</td>
<td>5'-CTT GTC TCA CCA TTC TCT CTC -3'</td>
</tr>
<tr>
<td>3. Wnt16a-59F</td>
<td>5'-ACC ACT TGC CTC AGG GAC ACC -3'</td>
</tr>
<tr>
<td>4. Wnt16a-168R</td>
<td>5'-AAA TGC GCG CAC CCC AG -3'</td>
</tr>
<tr>
<td>5. Wnt16b-319F</td>
<td>5'-TGC TGG CTC CTC AGG CAG C -3'</td>
</tr>
<tr>
<td>6. Wnt16b-430R</td>
<td>5'-TCC TCG G66 CTC TTC -3'</td>
</tr>
</tbody>
</table>

Primers were designed with Primer Express 1.0 software based on published cDNA sequences for EB1, Wnt16a and Wnt16b genes. "F" and "R" stand for Forward and Reverse primers, respectively.
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.3 A number of genes differentially expressed in t(1;19) cell lines were identified,24±28 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,29 while Wnt16 encodes a novel member of the WNT/WG family of growth factors.28 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,30 Wnt signaling has been demonstrated to have a role in early hematopoiesis and pro-B cell proliferation.31 The Wnt16 gene has two isoforms, generated from separate promoters rather than splicing;32 the Wnt16 gene originally described by McWhirter et al.,34 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.3 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 Wnt16b mRNA, following E2A-PBX1 silencing, showed a reduction of the b-isofrom, and the corresponding Wnt16b mRNA decreased to 57±8% (Figure 3B).

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**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 down-stream 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.
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 therapeutic strategies to treat cancer, 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 oncoproteins, 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

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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. 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 invariably expressed in the subset of t(1;19)-positive pre-B leukemias, 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), we found that only Wnt16b was upregulated in the 697 ALL cells. The results of very recent work by Mazieres et al. 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. GC 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 “Città della Speranza”.

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