Pax6 Regulates Proliferation and Apoptosis of Human Retinoblastoma Cells

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PURPOSE. To assess whether the Pax6 gene is involved in the development of retinoblastoma.

METHODS. Three human retinoblastoma cell cultures were transfected with human Pax6 specific double-stranded, small interfering siRNA molecules RH-1 and RH-2. In addition, untreated control groups and negative control groups (CT groups) transfected with siRNA without homology to the human genome were formed for all three cell culture lines.

RESULTS. After Pax6 gene was silenced by siRNA, the percentage of tumor cell survival decreased significantly ($P < 0.05$). Correspondingly, the percentage of apoptotic cells to total cells was significantly ($P < 0.05$) higher in the three retinoblastoma cell lines transfected with siRNA than in the CT control groups and the untreated control groups. In a parallel manner, the cell cycle was significantly ($P < 0.01$) altered in the transfected study groups, with reduced percentages of retinoblastoma cells in the S-phase. The cell-cycle-associated protein P21 was upregulated, and the protein P27 was slightly upregulated in the transfected retinoblastoma cell lines, in comparison to the control groups.

CONCLUSIONS. Silencing the Pax6 gene with short interfering RNA resulted in an inhibited growth and an increased apoptosis of cultured human retinoblastoma cells. It was paralleled by upregulation of the P21 and P27 proteins. (Invest Ophthalmol Vis Sci. 2011;52:4560–4570) DOI:10.1167/iovs.10-5487

Retinoblastoma is the most frequent primary intraocular cancer and has been gaining in importance rapidly, particularly in Asia.1–4 It affects approximately 1:14,000 to 1:22,000 live births.5 Studies have revealed that the retinoblastoma tumor is initiated by a bi-allelic inactivation of the retinoblastoma Rb1 gene in retinal cells in both the hereditary and sporadic types. However, a mutation of the Rb1 gene itself could not fully explain the genomic changes in retinoblastoma cells. Tumor cytogenetic and comparative genomic hybridization studies have suggested that an increase in potential oncogenes and a loss of tumor suppressor candidates along with epigenetic changes of aberrant methylation contribute to the development and progression of retinoblastomas.3 Additional genetic changes besides the mutation in the Rb1 gene may, therefore, be responsible in the transformation process of benign retinal cells into retinoblastoma tumor cells. Pax proteins are crucial for a normal embryogenesis in regulating cell proliferation and self-renewal, resistance to apoptosis, migration of embryonic precursor cells, and the coordination of specific differentiation programs.6,7 Although the precise role the Pax proteins play in the development of cancer has not been conclusively identified, an emerging hypothesis has been that the physiological functions of Pax proteins include maintaining tissue specific stem cells by inhibiting terminal differentiation and apoptosis. That may, however, also facilitate the development and progression of cancer cells. Evidence has been accumulating to support that hypothesis. Malexpression of Pax proteins has been detected in several different tumor types, such as the Pax3 and Pax7 proteins in sarcoma cells, the Pax2 protein in Kaposi’s sarcoma cells, and the Pax5 protein in B-cell lymphomas, to mention only few examples.8,9 It also holds true for the Pax6 gene, which is actively involved in the development of the central nervous system, including the eye. Its oncogenetic potential has been found in tumors of the exocrine pancreas and the intestine. In the embryologic development of the retina, the Pax6 gene is expressed to maintain the differentiation capabilities of retinal cells at an early stage. It is sharply downregulated before the terminal differentiation program, to ensure the correct formation of the retinal cells. Assuming that the Pax6 gene along with the retinoblastoma Rb1 gene contributes synergistically to the development and progression of retinoblastoma tumors, we conducted the present study to examine the influence of the Pax6 gene on the growth and apoptosis rate of cultured human retinoblastoma by silencing the gene with short interfering (si)RNA.

METHODS

Cell Culture and Transfection

The human retinoblastoma cell lines (Y79, WERI-RB-1, and SO-RB50) were provided by the Department of Pathology, Zhongshan Ophthalmic Center, Sun Yat-sen University and the Chinese University of Hong Kong. The human retinoblastoma cells were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium (HyClone Co., Logan, UT) supplemented with 10% fetal bovine serum, 100 U/L penicillin, and 100 U/L streptomycin at 37°C in a humidified atmosphere of 95% air/5% CO2. The culture medium was replaced every 3 days.

For the experiments with RNA interference (RNAi), a human Pax6-specific, double-stranded, small interfering (si)RNA was synthesized (Shanghai GenePharma Co., Ltd, Shanghai, China). Two of the siRNA molecules, RH-1 and RH-2, were selected: RH-1: (forward) 5-GGCAAUCG-

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and RH2: (forward) 5-CAAGCGUUCAUAAATTT-3 and (reverse) 5-UUAUGAUCGCUGGTT-3.

Since siRNA can nonspecifically suppress the expression of other genes leading to unintended, nonspecific side effects, we used two types of Pax6-targeted siRNA molecules in our study to avoid the misinterpretation of such nonspecific side effects. We also wanted to confirm the results of the investigations using the first molecule with the results of examinations using the second siRNA molecule. In addition, siRNA molecules that exhibited no homology to the human genome sequence were selected as negative controls (forward) 5-UUUCCUCGAAGGGUCACGUUTT-3 and (reverse) 5-AGGUACAGCUUCCGGAGAAATT-3 (CT control group). A further untreated group of retinoblastoma cell cultures served as the untreated control.

A transfection system (Lipofectamine 2000; Invitrogen Co., Carlsbad, CA) was used to introduce the siRNA into the retinoblastoma cells by means of the following protocol: The retinoblastoma cells were resuspended in RPMI medium 1640 with a density of 10^5 cells/mL and were placed into 24-well culture plates of 0.4 mL for each well. The siRNA and the transfection reagent were diluted in serum-free 1640 medium (1:50 and 2:50, respectively), mixed, and incubated for 25 minutes at room temperature. The mixtures of siRNA and the transfection reagent were added drop-wise to the retinoblastoma cells. On the third day after the siRNA transfection, the following experimental procedures were performed.

**Real-Time Polymerase Chain Reaction**

The total RNA was extracted from the cells using (TRizol reagent; Invitrogen): The mRNA was then reverse-transcribed (TaqMan Reverse Transcription Reagents, obtained from Tiangen, Beijing China; Applied Biosystems, Inc. [ABI], Foster City, CA). The detection of Pax6 mRNA levels was performed by real-time RT-PCR (PRISM 7900 Sequence Detection System; ABI). A 25-µl reaction mixture contained 12 µl mix (SYBR Green PCR Master Mix; ABI), 1 µl cDNA template, and 5-CT-TGGGAAAATCAGGACAGATT-3 forward and 5-GCTAGCCAGGTTCGGAAGAA-3 reverse primers for Pax6 (Invitrogen), which were designed on computer (Primer Express software; ABI). The PCR running conditions were: 2 minutes at 50°C and 10 minutes at 95°C of initial denaturation followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, 15 seconds at 95°C for annealing, 15 seconds at 60°C, and 15 seconds at 95°C. The threshold cycle (CT), which was the cycle number at which the amount of amplified gene of interest reached a fixed threshold, was subsequently determined. Relative quantification of the Pax6 mRNA expression was calculated with the comparative threshold cycle method. The relative quantification value of target, normalized to an endogenous control-18sRNA gene, was expressed as 2^ΔΔCT (ΔΔCT = CT of target genes (Pax6) − CT of endogenous control gene (18sRNA)).

**Western Blot Analysis**

The retinoblastoma cells were seeded on 24-well plates, transfected with the siRNA and analyzed after being cultured for 3 days. The cells (1.5 × 10^5) were washed twice with cold PBS (phosphate-buffered saline) and harvested in 1 mL lysis buffer containing 10 mM Tris, 150 mM NaCl, 1% NP-40, 0.1% Triton X-100, 5 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 4 mM sodium orthovanadate, 1 mM PMSF (phenylmethylsulfonyl fluoride), 10 µg/mL leupeptin, and 20 µg/mL aprotinin for 30 minutes on ice. The resulting cell lysates were preclarified by centrifugation at 12,000g for 10 minutes. The protein content was determined according to the instructions in a BCA (bicinchoninic acid) protein assay kit. Cellular proteins were fractionated by electrophoresis in 8% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel. Cellular proteins were then transferred to polyvinylidene difluoride (PVDF) membranes that were incubated with primary antibodies including rabbit anti-Pax6 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-P21 antibodies (1:1000; Cell Signaling, Beverly, MA) rabbit anti-P27 antibodies (1:1000; Cell Signaling), rabbit anti-cyclin D1 antibodies (1:1000; Cell Signaling), and rabbit anti-cdc2 antibodies (1:200; Santa Cruz Biotechnology), with anti-rabbit antibody conjugated with HRP (1:3000; Santa Cruz Biotechnology) used as the secondary antibody. Equal protein loading was confirmed by reprobing the membranes with β-actin antibodies (1:1000). Densitometric values of the protein bands were quantified (Quantity One Software; Bio-Rad Laboratories Inc., Hercules, CA).

**Cell Proliferation Assay**

The retinoblastoma cells were seeded at a volume of 90 µL of cell suspension (5000 cells/well) into 96-well plates, transfected with the siRNA, and analyzed after being cultured for 3 days. According to the manufacturer’s instructions, we added 10 µL of a standard colorimetric cell counting assay (CCK8) to each well and incubated the wells in a humidified incubator for 3 hours. The optical density was measured by a microplate reader at 450 nm.

**Cell Cycle Analysis**

The retinoblastoma cells were seeded on 24-well plates, transfected with siRNA and analyzed after being cultured for 3 days. Cells (1.5 × 10^6) were washed in PBS (phosphate-buffered saline), resuspended in 1 mL of cold alcohol and incubated for 12 hours at 4°C. The cells were then washed in PBS, resuspended in 1 mL of PBS containing 0.1% Triton X-100, and 100 µg/mL RNase at 37°C in a humidified incubator for 30 minutes. We then added 50 g/mL propidium iodide in the dark for 15 minutes. The cells were then analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Diego, CA). The data analysis was performed (CellQuest, ModFit LT software; BD Biosciences). The results is expressed as percentages of elements detected in the different phases of the cell cycle: G0/G1 (no DNA synthesis), S (active DNA synthesis), and G2 and M (premitosis to mitosis).

**Assessment of Apoptosis**

The rate of apoptosis was evaluated using the TUNEL assay analysis (Roche Inc., Basel, Switzerland). Cells were transfected in PBS and fixed in 1% paraformaldehyde in PBS (pH 7.6) for 1 hour at room temperature. The cells were incubated with a solution containing 0.1% Triton X-100 and 0.1% sodium citrate for 2 minutes on ice and then were then treated with terminal deoxynucleotidyl transference enzyme and incubated in a humidified atmosphere at 37°C for 1 hour. After washes in PBS, the cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA). The results were expressed as a percentage of green fluorescence-emitting (apoptotic) cells. According to the TUNEL staining, the cells were stained by DAPI (4′,6-diamidino-2-phenylindole) and were then analyzed by immunofluorescence.

**Statistical Methods**

Statistical analysis was performed (SPSS for Windows, version 17.0, SPSS, Chicago, IL) and the results are expressed as the mean ± SD of three independent experiments. Statistical analysis of differences was performed by one-way analysis of variance (ANOVA). P < 0.05 indicated statistical significance.

**Results**

**Pax6 Gene Silencing in the Retinoblastoma Cells**

To investigate the Pax6 gene function, we tried to silence this gene in the human retinoblastoma cell lines with siRNA targeting Pax6 mRNA. The elimination of the Pax6 mRNA in Y79, WERI-RB-1, and SO-RB50 cell cultures was achieved as determined by RT-PCR (Figs. 1A–C). The effectiveness of these siRNA molecules was confirmed by examining the Pax6 protein levels by Western blot analysis (Fig. 1D). The protein expression level of Pax6 decreased significantly (P < 0.05) in the RH-1 and -2 groups compared with that in the CT groups.
and untreated groups (Fig. 1D). For comparison, the β-actin protein did not vary significantly between the groups.

**Rb Cell Survival Inhibition by Pax6 Gene Silencing**

A significant inhibition of the retinoblastoma cell survival rate was found by silencing of the Pax6 gene in the human retinoblastoma cell lines Y79, WERI-RB-1, and SO-RB50. The percentage of cells surviving decreased (P < 0.05) from 81% ± 8%, 85% ± 4%, and 76% ± 6% in the CT control groups of the three retinoblastoma cell lines, respectively, to 36% ± 7%, 36% ± 7%, and 35% ± 8% in the RH-1 groups, and to 34% ± 8%, 40% ± 8%, and 44% ± 7% in the RH-2 groups, respectively; (Fig. 2).

**FIGURE 1.** (A–C) Real-time PCR of three human retinoblastoma cell lines (Y79, WERI-RB-1, and SO-RB50) in which the Pax6 gene function was silenced by siRNA (RH-1 and -2) targeting Pax6 mRNA. The mRNA expression level of Pax6 in the RH-1 and -2 groups decreased significantly (P < 0.05), compared with that in the CT groups and the untreated groups. RH-1 and -2 groups: cells treated with siRNA molecules RH-1 and -2, respectively; CT group: cells treated with an siRNA that had a randomized nucleotide sequence with no significant homology to any part of the human genome; untreated groups: cells with no treatment. (D) Examination of the protein expression level of the Pax6 gene by Western blot after treatment with the indicated siRNA molecules RH-1 and -2. The protein expression level of Pax6 decreased in the RH-1 and -2 groups compared with that in the CT and untreated groups. The data present the most prominent Pax6 isoform. RH-1 and -2 groups: cells treated with siRNA molecules RH-1 and -2, respectively; CT group: cells treated with a siRNA that had a randomized nucleotide sequence with no significant homology to any part of the human genome; untreated groups: cells without any treatment.
Changes in the Cell Cycle Induced by Pax6 Gene Silencing

To assess the change of the cell cycle induced by silencing the Pax6 gene in the three retinoblastoma cell lines Y79, WERI-RB-1 and SO-RB50, the cell nuclei were stained with propidium iodide and the cell cycle was analyzed by flow cytometry. In all three cell lines, the percentages of cells in the S-phase were significantly ($P < 0.01$) lower in the RH-1 groups (0.0% ± 0.0%, 0.6% ± 1.0%, and 1.3% ± 1.2%, respectively) and in the RH-2 groups (0.0% ± 0.0%, 2.5% ± 0.7%, and 0.02% ± 0.04%, respectively) than in the untreated group (21% ± 3%, 26% ± 4% and 30% ± 1%, respectively) and than in the CT control group (22% ± 1%, 26% ± 3% and 28% ± 1%, respectively; Figs. 3A–C, 4). As a corollary, the percentages of cells in the G$_0$–G$_1$-phase were significantly ($P < 0.01$) higher in the RH-1 groups (81% ± 1%, 89% ± 3%, and 86% ± 1%, respectively) and in the RH-2 groups (79% ± 2%, 86% ± 0.4%, and 89% ± 1%, respectively) than in the untreated group (69% ± 1%, 72% ± 3%, and 58% ± 1%, respectively) and than in the CT control groups (68% ± 1%, 75% ± 3%, and 58% ± 2%, respectively; Figs. 3A, 3B, 4A, 4B). For the retinoblastoma cell lines Y79 and WERI-RB-1, the percentages of cells in the G$_2$–M-phase were significantly ($P < 0.01$) higher in the RH-1 groups (19% ± 1% and 11% ± 2%, respectively) and in the RH-2 groups (21% ± 2% and 12% ± 1%, respectively) than in the untreated groups (10% ± 2% and 3% ± 1% respectively) and than in the CT control groups (11% ± 1% and 1% ± 0%, respectively; Figs. 3A, 3B, 4A, 4B). For the retinoblastoma cell line SO-RB50, the percentages of cells in the G$_2$–M-phase did not vary significantly between the RH-1 group, the RH-2 group, the untreated group, and the CT group.

Apoptosis Induced by Pax6 Gene Silencing

To examine the apoptosis induced by silencing the Pax6 gene in the three retinoblastoma cell lines Y79, WERI-RB-1, and SO-RB50 Rb (Figs. 5, 6, and 7), we performed TUNEL staining (green), followed by a FACS analysis and fluorescence microscopic image analysis. The flow cytometric analysis revealed an enhanced TUNEL fluorescence signal in the RH-1 and -2 groups compared with the untreated and control groups. The percentage of apoptotic cells to total cells was significantly ($P < 0.05$) higher in the three retinoblastoma cell lines transfected with siRNA (RH-1: 25% ± 3%, 33% ± 2%, and 26% ± 2%, in the Y79, WERI-RB-1, and SO-RB50 cells, respectively; RH-2: 25% ± 4%, 36% ± 3%, and 30% ± 1%) than in the CT control groups (3.8% ± 2%, 5.4% ± 1%, and 4.7% ± 1%) and in the untreated control groups (0.2% ± 0.2%, 0.9% ± 0.4%, and 0.8% ± 0.4%). Correspondingly, Western blot analysis showed that the level of cleaved-caspase3 as the active form of caspase 3 was slightly upregulated in all three cell lines transfected with RH-1 and -2 in comparison to the untreated groups and the CT groups (Fig. 8).

Analysis of Cell Cycle-Related Molecules Induced by Pax6 Gene Silencing

To identify signaling pathways for the growth inhibition, the change in the cell cycle, and the apoptosis induced by silencing the Pax6 gene in the human retinoblastoma cell lines, we performed a Western blot analysis of the cell cycle–associated proteins P21, P27, cdc2, and cyclinD1. The level of the P21 protein was upregulated in the cell lines transfected with RH-1 and -2 in comparison to the untreated groups and the CT groups (Fig. 8). The level of the P27 protein was slightly upregulated. The level of the cdc2 protein was downregulated in the Y79 and WERI-RB-1 cell lines, and it was not affected in the SO-RB50 cell line. The level of the cyclinD1 protein did not decrease in the RH-1 and RH-2 groups. For comparison, the level of β-actin protein did not vary markedly between the groups (Fig. 8).

Discussion

Pax6 is a member of the Pax gene family, which encodes transcription factors. Many studies have suggested that the downregulation of Pax proteins is essential for physiological cell differentiation, and moreover that the Pax gene is expressed in a variety of different cancers. In the evolution of the eye, the highly conserved transcription factor Pax6 plays a key role in both flies and mammals. In our study, we examined whether silencing of the Pax6 gene by siRNA exerted an effect on the growth and apoptosis of human retinoblastoma cells in cell culture. We found that silencing the Pax6 gene with siRNA resulted in an inhibition of the growth of the retinoblastoma cells and, in a corollary manner, in an increased apoptosis of the tumor cells. These effects were paralleled by an upregulation of the P21 and P27 proteins and a downregulation of the cdc2 protein.

Our findings agree with those in previous studies in the literature and further extend their results. Consistent with our
results, recent studies have shown in a similar way, that in the mouse pancreas a sustained overexpression of the \( \text{Pax6} \) gene led to an increase in the ductal epithelium, a reduction of islet cells, and the development of a cystic adenoma.\(^{11,12} \) In contrast to these studies, Mayes et al.\(^ {13} \) reported that \( \text{Pax6} \) suppressed the invasiveness of glioblastoma cells. Shyr et al.\(^ {14} \) that \( \text{Pax6} \) may act as a prostate cancer repressor by interacting with the androgen receptor and by repressing the transcriptional activity and target gene expression of the androgen receptor to regulate cell growth and regeneration. These studies indicated that the \( \text{Pax6} \) gene plays a different role in different tissue cancers. An investigation by Ouyang et al.\(^ {15} \) considered that \( \text{Pax6} \) as a gene-encoding transcription factor and, being at the top of the genetic hierarchy for the development and morphogenesis of the eye, continues to be expressed in the ocular surface epithelia of the postnatal eye.\(^ {15} \) They therefore investigated the potential role for \( \text{Pax6} \) in controlling the dynamics of the ocular surface epithelia. They inserted full-length mouse \( \text{Pax6} \) cDNA, or truncated \( \text{mPax6Delta286} \) lacking the transcriptional activation domain, into a tetracycline-inducible vector (Tet-on). A rabbit corneal epithelial cell line SIRC was used to establish stable transfectants. The investigators found that the overexpression of full-length \( \text{Pax6} \) retarded the rate of cell proliferation, whereas the truncated form had no effect. Full-length \( \text{Pax6} \) affected the rate at which individual cells traversed the cell cycle and induced a caspase-3-independent apoptosis in a small percentage of cells. Transient transduction of cells with recombinant \( \text{mPax6} \) adenovirus also inhibited cell proliferation. The authors concluded that inhibition of cell proliferation in \( \text{Pax6}\)-overexpressing corneal epithelial cell lines and primary cell culture was consistent a role of \( \text{Pax6} \) in controlling the corneal epithelial cell dynamics in vivo. \( \text{Pax6} \) expression levels were crucial during proliferation of retinal progenitors as shown by Hsieh et al.\(^ {16} \) These investigators took into account that the paired homeobox protein \( \text{Pax6} \) is essential for proliferation and pluripotency of retinal progenitors. They examined the dynamic changes in \( \text{Pax6} \) expression among chicken retinal progenitors as they progressed through the neurogenic cell cycle and determined the effects of altered \( \text{Pax6} \) levels on retinogenesis. They found that during the preneurogenic to neurogenic transition, \( \text{Pax6} \) protein levels in proliferating progenitor cells were downregulated. Neurogenic retinal progenitors retained a relatively low level of \( \text{Pax6} \) protein, whereas postmitotic neurons either elevated or extinguished \( \text{Pax6} \) expression in a cell-type-specific manner. Cell imaging and cell cycle analyses showed that neurogenic progenitors in the S-phase of the cell cycle contained low levels of \( \text{Pax6} \) protein, whereas postmitotic neurons either elevated or downregulated \( \text{Pax6} \) protein levels in proliferating progenitor cells. Hsieh et al. also showed that M-phase cells contained varied levels of \( \text{Pax6} \), and some correlated with the onset of early neuronal marker expression, forecasting cell cycle exit and cell fate commitment. Furthermore, either elevating or knocking down \( \text{Pax6} \) attenuated cell proliferation and resulted in increased cell

![Figure 3](https://example.com/fig3.png)

**Figure 3.** Representative histograms of a flow cytometric analysis of human retinoblastoma cell lines Y79 (A), WERI-RB-1 (B), and SO-RB50 Rb (C) in the untreated groups, the CT groups (treated with a nonhomologous siRNA), and the RH-1 and -2 groups (treated with the siRNA RH-1 and -2). The DNA was labeled with propidium iodide and monitored by FACS.
control groups, whereas for the retinoblastoma cell lines Y79 and WERI-RB-1, the percentages of cells in the S-phase were significantly (P < 0.01) lower in the RH-1 and -2 groups than in the untreated groups and in the CT control groups. As a corollary, the percentages of cells in the G0–G1-phase were significantly (P < 0.01) higher in the RH-1 and -2 groups and in the RH-2 groups than in the untreated groups and in the CT control groups. For the retinoblastoma cell lines Y79 and WERI-RB-1, the percentages of cells in the G2–M-phase were significantly (P < 0.01) higher in the RH-1 and -2 groups than in the untreated groups and in the CT control groups, whereas for the retinoblastoma cell line SO-RB50, the percentages of cells in the G2–M-phase did not vary significantly between the RH-1, the RH-2, the untreated, and the CT groups.

A so far unique aspect of our investigation is that we previously reported the Pax6 gene with siRNA in retinoblastoma cells. We found that after the gene was silenced, tumor cell proliferation was significantly inhibited. In the cell lines Y79 and WERI-RB-1, the cells accumulated in the G0–G1-phase and G2–M-phase, and the cells of the SO-RB50 cell line accumulated in the G0–G1-phase. Correspondingly, the percentage of cells in the S-phase decreased. The results suggested that the Pax6 gene can be silenced by siRNA leading to a reduction in the proliferation of the retinoblastoma cells. Previous reports have suggested that an arrest of the cell cycle is followed by an apoptotic death of cancer cells. In accordance with our study, in which the percentage of apoptotic retinoblastoma cells increased after Pax6 gene silencing. These results may suggest that silencing the Pax6 gene can play a role in inhibiting the development and progression of retinoblastomas.

To further explore the potential mechanism underlying these results in our study and to develop a mechanistic model explaining how silencing of the Pax6 gene by siRNA works to induce cell cycle arrest and apoptosis, the P21 protein, P27 protein, cyclinD1 protein, and cdc2 protein were examined. These proteins influence the cell cycle, which is regulated by the expression of cyclins and sets of activating and inhibitory proteins. The level of the P21 protein was upregulated and the level of the P27 protein was slightly upregulated in the retinoblastoma cell lines transfected with the siRNA molecules RH-1 and -2 (Fig. 8). This upregulation of the P21 protein and P27 protein may have been related to the G0–G1 checkpoint and the P21 protein mediates both G0–G1 and G2–M arrest. In agreement with the results of our study, Kase et al. demonstrated that P27(KIP1) blocked the cell cycle transition from G1- to S-phase, and a functional loss of the retinoblastoma gene led to a downregulation of P27(KIP1) and an uncontrolled retinal cell proliferation in human retinas and in retinoblastoma tumors. The level of the cdc2 protein was downregulated in the Y79 and WERI-RB-1 retinoblastoma cell lines in our study, and it was not affected in the SO-RB50 cell line. These partially heterogeneous findings in the three retinoblastoma cell lines may be explained by differences between the cell lines in their origin, growth characteristics, and morphologic structure. The G0–M arrest seen in the retinoblastoma cell lines Y79 and WERI-RB-1 in our study, but not detected in the SO-RB50 retinoblastoma cell line, appeared to be associ-
FIGURE 5. (A) Flow cytometric analysis of the human retinoblastoma cell line Y79 in the untreated group, CT group (treated by a nonhomologous siRNA), and RH-1 and -2 groups (treated by siRNA RH-1 and -2). The apoptotic cells were visualized by TUNEL staining (green). After TUNEL staining, the cells were analyzed by flow cytometry. Representative flow cytometry graphics are shown. M1 indicates the percentage of apoptotic cells. (B) Typical fluorescence microscopy image of the human retinoblastoma cell line Y79 in the RH-1 group (treated with siRNA RH-1). After flow cytometry, the cells were stained with DAPI (blue) (4',6-diamidino-2-phenylindole). Merged images are also shown. The percentage of apoptotic cells in the RH-1 group was significantly ($P < 0.05$) higher than in the untreated group and the CT group (treated with a nonhomologous siRNA). (C) Typical fluorescence microscopy image of the human retinoblastoma cell line Y79 in the CT group, treated with a nonhomologous siRNA. After flow cytometry, the cells were stained with DAPI (blue) (4',6-diamidino-2-phenylindole). Merged images are also shown.
FIGURE 6. (A) Flow cytometric analysis of the human retinoblastoma cell line WERI-RB-1 in the untreated group, CT group (treated by a nonhomologous siRNA), and RH-1 and -2 groups (treated by siRNA RH-1 and -2). The apoptotic cells were visualized by TUNEL staining (green). After TUNEL staining, the cells were analyzed by flow cytometry. Representative flow cytometry graphics are shown. M1 indicates the percentage of the apoptotic cells. (B) Typical fluorescence microscopy image of the human retinoblastoma cell line WERI-RB-1 in the RH-1 group (treated with siRNA RH-1). After flow cytometry, the cells were stained with DAPI (blue) (4',6-diamidino-2-phenylindole). Merged images are also shown. The percentage of apoptotic cells in the RH-1 group was significantly ($P < 0.05$) higher than that in the untreated group and the CT group (treated with a nonhomologous siRNA). (C) Typical fluorescence microscopy image of the human retinoblastoma cell line WERI-RB-1 in the CT group, treated with a nonhomologous siRNA. After flow cytometry, the cells were stained with DAPI (blue) (4',6-diamidino-2-phenylindole). Merged images are also shown.
FIGURE 7. (A) Flow cytometric analysis of the human retinoblastoma cell line SO-RB50 in the untreated group, CT group (treated by a nonhomologous siRNA), and RH-1 and -2 groups (treated by siRNA RH-1 and -2). The apoptotic cells were visualized by TUNEL staining (green). After TUNEL staining, the cells were analyzed by flow cytometry. Representative flow cytometry graphics were shown. M1 indicates the percentage of the apoptotic cells. (B) Typical fluorescence microscopy image of the human retinoblastoma cell line SO-RB50 in the RH-1 group (treated with siRNA RH-1). After flow cytometry, the cells were stained with DAPI (blue) (4',6-diamidino-2-phenylindole). Merged images are also shown. The percentage of apoptotic cells in the RH-1 group was significantly ($P < 0.05$) higher than that in the untreated group and the CT group (treated with a nonhomologous siRNA). (C) Typical fluorescence microscopy image of the human retinoblastoma cell line SO-RB50 in the CT group, treated with a nonhomologous siRNA. After flow cytometry, the cells were stained with DAPI (blue) (4',6-diamidino-2-phenylindole). Merged images are also shown.
ated with the downregulation of the cdc2 protein in the same cell lines, Y79 and WERI-RB-1. The cdc2 protein is a master of the intracellular regulator entry into mitosis. In accordance with our study, a previous investigation showed that low doses of paclitaxel potently induced a G2–M arrest in human retinoblastoma Y79 cells, associated with the downregulation of the cdc2 protein. The level of the cyclinD1 protein was not affected by silencing the Pax6 gene in the RH-1 and -2 groups in our study. According to previous studies, cyclinD1 is essential for the progression through the cell cycle, and the Rb–cdc2 protein.24 The paradox between an increased level of p21.24,26 The paradox between an increased level of p21, P27, cyclinD1, and cleaved-caspase3 in the three human retinoblastoma cell lines Y79 (left column), WERI-RB-1 (middle column), and SO-RB50 (right column) in the untreated groups, CT groups (treated by a nonhomologous siRNA), RH-1 groups (treated by the siRNA molecule RH-1), and RH-2 groups (treated by the siRNA molecule RH-2).

FIGURE 8. Western blot analysis to describe the levels of the proteins P21, P27, cdc2, cyclinD1, and cleaved-caspase3 in the three human retinoblastoma cell lines Y79 (left column), WERI-RB-1 (middle column), and SO-RB50 (right column) in the untreated groups, CT groups (treated by a nonhomologous siRNA), RH-1 groups (treated by the siRNA molecule RH-1), and RH-2 groups (treated by the siRNA molecule RH-2).

In summary, silencing the Pax6 gene with siRNA resulted in a growth inhibition and an increase in apoptosis in cultured human retinoblastoma cells, in parallel with an upregulation of the P21 and P27 proteins and a downregulation of the cdc2 protein. The findings indicate that the Pax6 gene sustains cell growth and regulates the cell cycle in retinoblastoma cells in culture. The Pax6 gene may be a molecular target for antican- cancer therapies.

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