KLF5 Interacts with p53 in Regulating Survivin Expression in Acute Lymphoblastic Leukemia* 

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The Kruppel-like factor 5 (KLF5) is a transcription factor that regulates cellular signaling involved in cell proliferation and oncogenesis. Here, we report that KLF5 interacts with tumor suppressor p53 in regulating the expression of the inhibitor-of-apoptosis protein survivin, which may play a role in pathological process of cancer. The core promoter region of survivin contains multiple GT-boxes that have been characterized as KLF5 response elements. Deletion and mutation analyses as well as chromatin immunoprecipitation and electronic mobility shift assay indicated that KLF5 binds to the core survivin promoter and strongly induces its activity. Furthermore, we demonstrated that KLF5 protein is able to bind to p53 and abrogate the p53-regulated repression of survivin. Transfection of KLF5 into a KLF5-negative acute lymphoblastic leukemia cell line EU-8 enhanced survivin expression, and conversely, silencing of KLF5 by small interfering RNA in a KLF5-overexpressing acute lymphoblastic leukemia cell line EU-4 down-regulated survivin expression. The KLF5 small interfering RNA-mediated down-regulation of survivin sensitized EU-4 cells to apoptosis induced by chemotherapeutic drug doxorubicin. These findings identify a novel regulatory pathway for the expression of survivin under the control of KLF5 and p53. Deregulation of this pathway may result in overexpression of survivin in cancer, thus contributing to drug resistance.

Survivin is a unique member of the inhibitor-of-apoptosis protein family. It plays an important role not only in inhibiting apoptosis but also in regulating mitosis (1, 2). Moreover, it is highly expressed in almost all types of human cancer but undetected in most adult tissues (3). High levels of survivin expression have been associated with cancer progression, drug resistance, poor prognosis, and short patient survival (4, 5). On the other hand, inhibition of survivin expression or interference with survivin function has been demonstrated to reduce cancer cell growth, induce cancer cell death, and sensitize cancer cells to radiation or chemotherapy (6, 7). Therefore, survivin is regarded as a promising target for cancer treatment, and modulating the expression of survivin seems to be one important approach.

Much effort has been made to explore the mechanisms by which survivin expression is regulated. It is well established that survivin expression is repressed by wild-type p53 (8–10), while how survivin expression is activated remains less clear. Recent studies in colon cancer cells suggest that regulation of survivin expression is at least partially T-cell factor (TCF)/β-catenin-dependent (11–13). Furthermore, a previous study to analyze the basal transcriptional requirement of survivin gene expression has indicated that the survivin gene promoter contains GC-rich sequences, and the Sp1 transcription factor induces survivin expression in HeLa cells (14). In addition to GC-rich sequences, the core promoter of survivin contains multiple CACCC or GGGTG motifs (also called GT-boxes) for Sp1-like proteins and Kruppel-like factors (Sp/KLF).2 The Sp/KLF family contains at least 20 members with highly related zinc finger proteins that are important components of the eukaryotic cellular transcriptional machinery (15). Individual members of the Sp/KLF family have preferences for binding different DNA sequences in the target gene promoter. For example, Sp1, Sp3, and Sp4 bind with higher affinity to GC-boxes than to GT-boxes, whereas many of the KLF proteins bind preferentially to GT-boxes over GC-boxes (16, 17). Sp/KLF can function as activators or repressors depending on which promoter they bind and the co-regulators with which they interact.

Although the Sp/KLF family is considered a major transcriptional activator for survivin expression, the ability of individual members of this family, and in particular those binding GT-boxes, to regulate survivin transcription in a given cell type has not been evaluated. Previous studies have suggested that members of the Sp/KLF family can interact with oncogenes and tumor suppressors, they can be oncogenic themselves, and altered expression of family members has been detected in tumors (18). Recently, attention has been given to the involvement of Sp/KLF family member KLF5 in oncogenesis. KLF5 is also known as basic transcription element-binding protein 2 (BTEB2) and intestine-enriched Kruppel-like factor (IKLF). Although KLF5 expression was originally found in gut and epithelial tissues, recent studies showed that KLF5 is also expressed in other types of tissues and cells such as skin, vascular smooth muscle cells, and lymphoid cells (19–21). In lymphoid cells, KLF5 is expressed mostly in pro-T-cell and at much lower levels in normal thymus and bone marrow (21). KLF5 is expressed mainly in proliferating cells and its expression is inducible (22, 23). KLF5 as a transcription factor activates expression of genes such as SM22α and γ-globin through GGGTG or CACCC motif within the gene promoters (24, 25). Previous studies (26–29) have demonstrated that KLF5 is a positive regulator of cell proliferation and mediates cell survival and tumorigenesis.

In the present study, we examined the expression of KLF5 in leukemia bone marrow cells from children with ALL as well as in a panel of leukemia cell lines derived from pediatric ALL. We found that KLF5 is widely expressed in both ALL lines and fresh ALL samples and that

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3 The abbreviations used are: KLF, Kruppel-like factor; CHIP, chromatin immunoprecipitation; EMSA, electronic mobility shift assay; ALL, acute lymphoblastic leukemia; BCP, B-cell precursor; PBS, phosphate-buffered saline; FBS, fetal bovine serum; siRNA, small interfering RNA.
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KLF5 expression is consistently associated with high levels of survivin expression in this disease. By analyzing the survivin promoter activity, we further found that KLF5 is an stimulator for survivin expression and that tumor suppressor p53 can down-regulate survivin expression by binding to KLF5. These findings suggest that KLF5 may be a novel target for therapy of ALL and other tumors with inactivated p53.

EXPERIMENTAL PROCEDURES

Patient Cells and Cell Line—Bone marrow aspirates were studied from 11 patients with B-cell precursor (BCP) ALL, diagnosed by standard immunologic, morphologic, and cytochemical criteria. Mononuclear cells were separated by centrifugation on Ficoll-Hypaque (1.077 g/ml), washed twice in phosphate-buffered saline (PBS), and resuspended at 10⁶/ml in RPMI 1640 containing 10% fetal bovine serum (FBS). The cells were incubated on plastic Petri dishes for 1 h at 37 °C to remove monocytes, and nonadherent cells were recovered by gently washing the dishes. All specimens for studies of gene expression contained more than 90% blasts following purification.

Six BCP-ALL lines were used in this study. The Reh line was obtained from C. Rosenfeld (INSERM, Villejuif, France). The five EU cell lines were established in our laboratory from pediatric BCP-ALL patients. Cultured cells resembled the primary leukemic cells, with Reh, EU-1, and EU-3 expressing wild-type p53, EU-2 expressing mutant p53, and EU-4 and EU-8 lacking p53 expression (30). Cell lines were grown in standard culture medium (RPMI 1640 containing 10% FBS, 2 mmol/liter l-glutamine, 50 units of penicillin and 50 μg/ml streptomycin) at 37 °C in 5% CO₂-air.

Plasmids—The expression plasmid containing KLF5 (pCDNA3.1-KLF5) was constructed in our laboratory as described previously (31). The expression plasmid containing KLF4 (pMT3-KLF4) was kindly provided by Dr. V. Yang (Emory University). The Sp1 expression plasmid (pPlacSp1) was provided by Dr. L. S. Phillips (Emory University). The wild-type p53 expression plasmid (pc53-SN3) was provided by Dr B. Vogelstein (Johns Hopkins University). The KLF6 expression plasmid was generated by inserting a cDNA fragment synthesized by reverse transcription-PCR into the pcDNA3.1+ vector (Invitrogen) at the HindIII and XhoI sites. Reverse transcription-PCR was performed using total RNA extracted from EU-1 cells and primer pair 5′-CCCGGACATGGGACGTG-3′ and 5′-GTTAATGGCGCG-3′ selected from upstream sequence of survivin core promoter (Promega). Constructs including different deleted or mutated fragments were then ligated to the pGL3 basic vector. DNA sequencing was performed to confirm that the sequence of the PCR products were correct as compared with the survivin promoter as published in the Human Genome data base.

Transfection and Luciferase Activity Assay—Gene transfections were performed to analyze the effect of KLF5 on survivin expression. EU-4 or EU-8 cells in exponential growth were transfected or co-transfected with plasmids as described above by electroporation at 280–300 V, 950 microfarads using a Gene Pulser II system (Bio-Rad). For stable transfection, the cells were seeded 48 h post-transfection into culture dishes

for the selection of G418-resistant colonies. For gene reporter assays, cells were transiently co-transfected by electroporation with the survivin promoter-luciferase constructs and different expression plasmids. Briefly, 1 × 10⁷ cells in exponential growth were mixed with the corresponding survivin promoter-luciferase constructs plus different doses of p53, KLF4, KLF5, KLF6, and Sp1 plasmids and electroporated as described above. Transfected cells were resuspended in 10 ml of RPMI containing 10% FBS. At 48 h post-transfection, cell extracts were prepared with 1 × lysis buffer, and then 20-μl aliquots of the supernatant were mixed with 100 μl of luciferase assay reagent (Promega) and anal-lyzed on a Microplate Luminometer (Turner Designs). Luciferase activ-ity was normalized to β-galactosidase activity as an internal control.

CHIP Assay—The CHIP assay as described previously (32) was performed to analyze the DNA binding activity of KLF5 protein. EU-4 cells, which express high level of KLF5, were used for CHIP assay for the in vivo binding of KLF5 to survivin promoter. First, formaldehyde was added at 1% to the culture media, and cells were incubated at room temperature for 10 min with mild shaking to cross-link KLF5 protein to the survivin promoter. Then, cells (1 × 10⁶) were washed twice with cold PBS and resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) with 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A. After a brief sonication, lysates were cleared by centrifugation and were diluted 10-fold with dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, and 167 mM NaCl) containing protease inhibitors as above. Anti-KLF5 or control antibodies were added at 4 °C overnight with rotation. Immunoprecipitated complexes were collected by protein A/G plus-agarose. Precipitants were sequentially washed with low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 20 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), and LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), once, respectively, followed by two washes with 1 × TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). After the final wash, 250 μl of elution buffer (1% SDS, 0.1 M NaHCO₃) was added and incubated at room temperature for 15 min with rotation. Then 5 μl NaCl was added to reverse the formaldehyde cross-linking by heating at 65 °C for 4 h. After precipitation with ethanol, the pellets were resuspended and treated with proteinase K. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Pellets were resuspended in TE buffer and subjected to PCR amplification using forward and reverse primers (′-GATTACGCGCTGACCCACT-3′ and −ATCTGGCG-GTTAATGGCCG-3′) selected from the survivin core promoter sequence (−253 to −234 and −11 to +10, respectively). A pair of primers (′-GGATATATCCAAGCTGGG-3′ and −GGTGTCCACCTGGAC-CG-3′) selected from upstream sequence of survivin core promoter (−2750 to −2733 and −2409 to −2392, respectively) served as control. The PCR product was separated by agarose gel electrophoresis.

EMSA—Sequence-specific DNA binding activity of KLF5 to survivin promoter was assayed by EMSA. Nuclear protein extraction was prepared using a kit (NE-PER™ from Pierce). Nuclear protein (5 μg) from EU-4 cells, which express high level of KLF5, was incubated for 15 min in a binding buffer (10 mM Tri-HCl, 50 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 7.5 mM MgCl₂) plus 0.1 μg of poly(dI-dC) carrier, and 32P-labeled oligonucleotide probe 5′-GGCGGGGTGGTTGGCTTAAACCATTAATTGAG-3′ spanning the −61/−40 of survivin promoter that contains the third GT box. A mutated probe with changes of the GGGTG core consensus from GT to CA served as control. Anti-KLF5 and rabbit IgG antibodies as additional controls were used to supershift the specific complexes of interest by pretreating the extract for 1 h at
4 °C with antibodies. The samples were electrophoresed on a 5% polyacrylamide gel, dried, and developed with intensifier screen at −70 °C.

**KLF5 siRNA and Transfection**—The siRNA was designed to target KLF5 mRNA. Its target sequence was 5′-AAAGTATAGCCAGAGACGTGCG-3′. A scrambled siRNA was used as a control. EU-8 cells were transfected with 50–200 nM of chemically synthesized siRNA (Invitrogen) in 12-well plates. Briefly, EU-4 cells were seeded at 1 × 10^5 per well and cultured for overnight. The siRNA solution was then mixed with Lipofectamine™ Reagent in Opti-MEM I media for 20 min following the manufacturer’s protocol and was added to EU-4 cells in a total volume of 0.5 ml. After 4-h incubation, 1 ml of normal medium was added to the cell culture.

**Northern Blot Assay**—Total RNA (10 μg per lane) was electrophoresed using a 1% agarose gel and transferred to a nylon filter. Probes were prepared by a randomized-labeling approach using [α-32P]dCTP. Hybridization was performed in 50% (v/v) formamide, 5 × SSC, 1% SDS, 5 × Denhardt’s solution, 20% dextran sulfate, 100 μg/ml sheared salmon sperm DNA at 42 °C for 16 h. A final wash was carried out in 1 × SSC, 0.1% SDS at 65 °C for 30 min. After washing, the filter was autoradiographed for 24 h.

**Western Blot Assay**—Cells were lysed in a buffer composed of 150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 25 μg/ml leupeptin for 30 min at 4 °C. Equal amounts of protein extracts (10 μg) were resolved by SDS-PAGE and transferred to nitrocellulose filter. After blocking with buffer containing 20 mM Tris-HCl, pH 7.5, and 500 mM NaCl, 5% nonfat milk for 1 h at room temperature, the filter was incubated with specific antibodies for 3 h at room temperature. After washing, the filter was incubated with horseradish peroxidase-labeled secondary antibody for 1 h. Blots were developed using a chemiluminescent detection system (ECL, Amersham Biosciences, Buckinghamshire, UK).

**Co-immunoprecipitation**—Cells were lysed in a buffer composed of 50 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 10 mM sodium phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. After centrifugation, 30 μg of the clarified cell lysate was incubated with 15 μl protein G plus/protein A-agarose and 1 μg of p53, KLF5, or MDM2 antibodies, respectively. After 24-h incubation, the agarose was centrifuged, washed four times with ice-cold lysis buffer, suspended in electrophoresis sample buffer, and boiled for 5 min. The immunoprecipitated protein was further analyzed by Western blotting as described above.

**Determination of Cell Growth Rate**—KLF5 siRNA-transfected EU-4 cells and control cells (EU-4 transfected with irrelevant siRNA) were cultured in RPMI 1640 containing 10% FBS at an initial concentration of 10^5/ml. Cells were then counted using a hemocytometer under light microscopy. Triplicate flasks were counted for each time point to determine the growth rate.

**Cytotoxicity and Apoptosis Assays**—The effect of KLF5 siRNA on doxorubicin-induced cytotoxicity in EU-4 cells was determined by the WST assay. Briefly, cells were cultured in 96-well microtiter plates with different concentrations of doxorubicin for 44 h. WST (25 μg/well) was then added, and cells were incubated for an additional 4 h. The OD of the wells was then read with a microplate reader at a test wavelength of 450 nm and a reference wavelength of 620 nm. Appropriate controls lacking cells were included to determine background absorbance.

An annexin-V assay (Oncogene, San Diego, CA) was used to quantify apoptotic cells. Briefly, cells with or without treatment were washed once with PBS and stained with fluorescein isothiocyanate-annexin-V and PI for 30 min according to the manufacturer’s instructions. Stained cells were detected using the FACScan (BD Biosciences) and analyzed using WinList software (Verity Software House Inc).

**RESULTS**

**KLF5 Is Widely Expressed in ALL, and Its Expression Is Associated with Survivin Expression**—Although KLF5 is involved in cell proliferation and cancer, the expression of this gene has not been studied in many human cancers including leukemia. We examine the expression of KLF5 in primary ALL cells and ALL cell lines derived from pediatric patients by northern blotting. KLF5 mRNA was expressed in all 11 freshly isolated ALL specimens, and five of six ALL cell lines, although the levels of expression varied among different samples and cell lines (Fig. 1A). Interestingly, the expression of KLF5 appeared related to the level of expression varied among different samples and cell lines (Fig. 1A). Interestingly, the expression of KLF5 appeared related to the level of expression varied among different samples and cell lines (Fig. 1A). Interestingly, the expression of KLF5 appeared related to the level of expression varied among different samples and cell lines (Fig. 1A). 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**KLF5 Is a Positive Regulator of Survivin Promoter Activity**—Since the core promoter of survivin contains multiple GT-boxes (GGGTG or CACCC, Fig. 2) that are preferentially used for binding and responding to KLF proteins, we determined the role of GT-boxes within the core promoter of survivin in regulating promoter activity in ALL cells. We generated a series of 5'-3' deleted and mutated constructs of this region and performed transfection and gene reporter assays in EU-4 cells, which express high level of KLF5. Our results showed that GT-boxes mediated the majority of the promoter activity. When the three GT-boxes were mutated, the activity of the promoter construct pLuc-269 containing the core survivin promoter was only about one-fourth of the wild-type promoter activity (Fig. 3, pLuc-269m versus pLuc-269). Significantly, the third GT-box (−56/−52) regulated approximately half of the total promoter activity in EU-4 as shown in Fig. 3, in which the activity of construct pLuc-110 containing only the third GT-box was ~50% of total promoter (pLuc-689 and pLuc-269) activity, and the construct pLuc-96 with deletion of the only third GT-box showed almost no promoter activity as compared with control. Regulation of the survivin promoter by the third GT-box was further confirmed by mutation assay, in which the promoter construct pLuc-110m containing a mutation in the third GT-box expressed no promoter activity.

Because the GT-box is the response element for Sp/KLF family proteins, we tested whether KLF5 but not other members of this family specifically induces survivin promoter activity in ALL cells. We performed co-transfection of the deleted survivin promoter construct pLuc-110 with the expression plasmids KLF5 and several Sp/KLF members Sp1, KLF4, and KLF6 in EU-8 line. The expression plasmid of p53 was also included in this study. As shown in Fig. 4A, co-transfection of KLF5 significantly (up to 4-fold) increased the activity of pLuc-110 promoter construct containing only the third GT box. Co-transfection of KLF4, KLF6, and Sp1 neither increased nor decreased luciferase activity of the pLuc-110 construct, although a slight increase and decrease of...
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pLuc-110 activity were observed following Sp1 and KLF4 transfection, respectively. Consistent with our previous results, p53 repressed survivin promoter activity in a dose-dependent manner (Fig. 4B). As also shown in Fig. 4B, the p53 repression of pLuc-110 activity was abrogated by co-transfection with KLF5.

To further investigate the role of the GT boxes in the survivin promoter in regulating the promoter activity in response to KLF5, we performed EMSA as well as CHIP analyses to evaluate the binding capacity of KLF5 to survivin promoter. Results from both CHIP (Fig. 5A) and EMSA (Fig. 5B) indicated that KLF5 induced survivin promoter activity by directly binding to the GT boxes in the promoter.

KLF5 Interacts with p53 in Regulating Survivin Expression—Results shown in Fig. 4B, in which KLF5 abrogated p53-repressed survivin promoter activity, suggest that a possible interaction between p53 and KLF5 mediates survivin expression. Therefore, we performed a co-immunoprecipitation assay to analyze binding between p53 and KLF5. From the data presented in Fig. 6A, it can be seen that p53 and KLF5 were able to bind each other in Reh cells expressing endogenous p53 and KLF5 and in EU-8 cells expressing transfected p53 and KLF5. The binding of p53 to MDM2 in Reh cells was detected in the control, whereas binding of KLF5 to MDM2 was not detected.

To further confirm that the physical interaction between p53 and KLF5 functions to regulate survivin promoter activity, we co-transfected p53 and the survivin promoter construct pLuc-269 containing three GT-boxes and the p53 consensus sequence into p53-null EU-4 cells that express high level of KLF5. Co-transfection of p53 and KLF5 in EU-8 cells, which do not express endogenous p53 and KLF5, respectively. Consistent with our previous results, p53 repressed survivin promoter activity to p5Luc-110 activity were observed following Sp1 and KLF4 transfection, respectively. Consistent with our previous results, p53 repressed survivin promoter activity in a dose-dependent manner (Fig. 4B). As also shown in Fig. 4B, the p53 repression of pLuc-110 activity was abrogated by co-transfection with KLF5.

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**FIGURE 6. Interaction between p53 and KLF5 in regulating survivin transcription.** A, physical interaction of KLF5 and p53 examined by immunoprecipitation (IP)-Western blot assays. Cell lysates from Reh cells treated with 10-gray IR for 3 h to induce p53 and from EU-8 cells co-transfected with p53 and KLF5 plasmids were precipitated with antibodies as indicated. Normal mouse or rabbit IgG served as control (Con). Proteins in immune complexes were separated on denaturing gels, transferred to filters, and detected by Western blotting against anti-KLF5, p53, and MDM2 (positive control for p53 binding). Antibodies for Western blotting were from different species than those used in immunoprecipitation. B, effect of p53 on KLF5-dependent survivin promoter activity. EU-4 cells were co-transfected with 5 μg of either pLuc-269 or pLuc-269m (with mutations of three KLF5-binding sites) plus increasing amounts (2.5, 5, and 10 μg) of p53 expression plasmid (lanes 2, 6, 2, and 7, and 4 and 8, respectively) or without addition of p53 (lanes 1 and 3). The total amount of plasmid was adjusted to 15 μg/ transfection using an empty vector. Transfection and luciferase activity assay were performed as described in the legend to Fig. 3.

269m containing mutations in the three GT-boxes served as a control. As shown in Fig. 6B, transfected p53 down-regulated pLuc-269 but not pLuc-269m luciferase activity in a dose-dependent manner, although the basal luciferase activity of pLuc-269m construct was significantly reduced by mutations of the GT-boxes.

**KLF5 siRNA Inhibits Survivin Expression and Sensitizes ALL Cells to Chemotherapeutic Drugs**—By gene transfection of KLF5, we have demonstrated that enforced expression of KLF5 induced survivin expression. We next examined the ability of silencing KLF5 in KLF5-overexpressing cells by siRNA to inhibit survivin expression. We chemically synthesized a specific KLF5 siRNA based on the sequence that has been shown to effectively inhibit KLF5 expression as reported previously (33). As seen from Fig. 7A, transfection of KLF5 siRNA into EU-4 cells down-regulated KLF5 expression in a dose-dependent manner. Consequently, the expression of survivin in KLF5 siRNA-treated EU-4 cells was decreased to the reduction of KLF5 level.

As we reported previously, EU-4 cells with a high level of survivin expression were resistant to apoptosis induced by the chemotherapeutic drug doxorubicin (10, 34). In this study, we tested the effect of down-regulation of KLF5 and survivin by KLF5 siRNA on sensitivity of EU-4 cells to doxorubicin as well as on cell growth. As shown in Fig. 7B, transfection of KLF5 siRNA did not inhibit EU-4 growth as compared with control siRNA. However, transfection of KLF5 siRNA sensitized EU-4 cells to doxorubicin, as demonstrated in Fig. 7C. A significant difference was noted in mean cell survival after 48-h treatment between doxorubicin plus control siRNA and doxorubicin plus KLF5 siRNA at doxorubicin concentrations of 0.5 μM and greater (p < 0.01). Consistent with these observations, a flow cytometric apoptosis assay showed that a increased percentage of EU-4 cells treated with the combination of doxorubicin and KLF5 siRNA (from ~7 to 63%) were annexin-V-positive at 48 h post-treatment as compared with doxorubicin plus control siRNA (from ~7 to 21%) (Fig. 7D).

**DISCUSSION**

In this study, we demonstrated that the transcription factor KLF5 is widely expressed in childhood ALL and is a positive regulator inducing the expression of the anti-apoptotic protein survivin. Previous studies including those from our group have shown that the expression of survivin is repressed by p53 (8–10), and we have characterized a critical role of p53-regulated survivin expression in determining sensitivity of ALL cells to chemotherapy (10). In the present study, we further found that p53-mediated repression of survivin is regulated by KLF5. In ALL cells lacking p53 expression, the KLF5-regulated survivin expression may play a role in regulating apoptosis induced by chemotherapeutic drugs, since silencing of KLF5 by siRNA in KLF5-expressing ALL cells sensitized these cells to doxorubicin.

It is well known that the KLF family proteins are transcription factors regulating expression of genes linked to either positive or negative regulation of cell proliferation and growth. Therefore, it is not surprising that members of this family have also been implicated in the pathological process of cancers. Although the majority of KLF family members have not been characterized for their cellular function, several KLF members have recently been linked to human cancer. For example, KLF6 has been reported to act as a tumor suppressor and induce apoptosis in cancer cells (35, 36), whereas KLF5 acts as a positive regulator of cell proliferation and mediates cell survival, transformation, and angiogenesis (26–29, 37), although several previous reports (31, 38) claimed a tumor suppressor function of KLF5 in prostate and breast cancer due to frequent deletion of KLF5 gene in these tumors.

However, the underlying molecular mechanism for KLF5 in the pathophysiology of cancer remains largely unclear. In the present study, we examined the expression of KLF5 in ALL and its interaction with p53 in regulating survivin expression, hoping to gain insight into the properties of KLF5 in regulating sensitivity of ALL cells to chemotherapeutic-induced apoptosis. We found that KLF5 is widely expressed in both ALL cell lines and primary samples, with the majority of primary ALL specimens overexpressing this gene. However, it is apparent that the level of KLF5 expression in ALL cells is not a determinant for cell proliferation and sensitivity to apoptosis induced by chemotherapy, because some KLF5-overexpressing ALL lines did not show a growth advantage and resistance to chemotherapeutic agents (data not shown).
We have previously identified a p53-survivin signaling pathway in regulating sensitivity of ALL cells to apoptosis induced by the DNA-damaging agent doxorubicin (10). Although all of the ALL lines tested express similar levels of survivin, lines expressing wild-type p53 were sensitive to doxorubicin due to down-regulation of survivin following repression by doxorubicin-induced p53. In contrast, ALL cells expressing mutant p53 were resistant to doxorubicin due to mutant p53-mediated up-regulation of survivin. The p53-null ALL cells (i.e. EU-4) were also resistant to doxorubicin, although the mechanism for the resistance was not elucidated in the previous study. In the present study, we compared the EU-4 ALL line with another p53-null ALL line (EU-8) that lacks KLF5 expression in terms of sensitivity to doxorubicin. The KLF5-overexpressing line EU-4 was resistant to doxorubicin, whereas the KLF5-negative line EU-8 was sensitive to doxorubicin (data not shown). Therefore, we hypothesized that KLF5 in the absence of p53 may be critical in determining sensitivity to doxorubicin. We performed the present study to evaluate the cellular consequences of knock-out of KLF5 by siRNA in EU-4 cells. Our results show that silencing KLF5 by siRNA sensitizes EU-4 cells to doxorubicin.

We further demonstrate that the role of KLF5 in regulating doxorubicin resistance is through regulation of survivin. First, we note a correlation between KLF5 and survivin expression in ALL. EU-8 cells lacking KLF5 expression express very low level of survivin, and enforced KLF5 expression in this line induces survivin expression. In contrast, inhibition of KLF5 in EU-4 by siRNA is accompanied by down-regulation of survivin. Furthermore, we found that the third GT-box within the survivin promoter acts as a major response element for promoter activity, and KLF5 specifically and strongly induces survivin promoter activity through this GT-box.

Another finding of the present study is that p53 is involved in KLF5-mediated regulation of survivin. Previous studies have demonstrated that p53 is able to bind several KLF members such as KLF4 and KLF6, which regulate the expression of p21/WAF-1 and IGF-IR genes, respectively (39, 40). In this study, we found that p53 can also bind KLF5 and down-regulate its effect on survivin expression. It is known that transcriptional repression mediated by p53 is either via direct DNA binding or through interactions with other transcription factors (41–43). However, with regard to the regulation of survivin by p53, two independent studies have reported different results. Data from Hoffman et al. (8) demonstrated that p53 binds to the p53-binding sites in the survivin promoter, whereas studies from Mirza et al. (9) showed that p53 does not bind the survivin promoter and the putative p53-consensus

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**FIGURE 7.** The effects of KLF5 siRNA on expression of endogenous KLF5 and survivin and on cell growth and apoptosis induced by doxorubicin (Dox). A, EU-4 cells were treated with different concentrations of either KLF5 siRNA or control siRNA for 24 h. The expression of endogenous KLF5, survivin, and actin was detected by Western blot analysis. The upper bands in the KLF5 blots (two upper panels) were nonspecific bands that served as control for equal protein loading. B, time course of EU-4 growth in suspended culture with KLF5 siRNA. Cells were cultured with 200 nM KLF5 siRNA and control siRNA, respectively, in 10 ml of RPMI 1640 medium supplemented with 10% FBS at an initial concentration of 10^6/ml. Cells were fed every 3 days with addition of same concentration of siRNA. Cells were counted daily. Data for the total number of cells (mean ± S.D. for triplicate cultures) is shown. C, EU-4 cells were treated with different concentrations of KLF5 siRNA and doxorubicin alone or with fixed amount (150 nM) of KLF5 siRNA or control siRNA and different doses of doxorubicin as indicated. Cells were incubated for 48 h, and cell viability was determined by WST assay. D, time course of apoptosis induced by doxorubicin in combination with either KLF5 siRNA or control siRNA in EU-4 cells. Cells were treated with doxorubicin (1.5 μM) plus siRNA (15 nM) for the indicated time, and apoptotic cells were detected by annexin-V staining. Data represent the mean percentage of annexin-V-positive cells from three independent experiments; bars, ± S.D.
KLF5 and p53 Regulate Survivin Expression

sequence within survivin promoter is not required for transcription repression of survivin. Our results support the notion that the putative p53-binding site is not necessary for transcriptional regulation as shown by the inability of p53 to repress a survivin-promoter construct containing a p53 response element but mutated KLF5-binding sites. Indeed, our results indicate that p53 represses survivin through interaction with KLF5.

The interaction between p53 and KLF5 in regulating survivin expression may have possible clinical significance. The majority of pediatric ALL patients whose leukemic cells express normal p53 respond well to chemotherapeutic treatment. In those patients whose leukemic cells have an inactivated p53, KLF5 strongly up-regulates survivin expression, which may be important in conferring therapeutic resistance. Targeting the KLF5-survivin pathway using siRNA against either KLF5 or survivin may be a treatment approach for ALL and other cancers with inactivated p53.

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