THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 42, pp. 35678–35688, October 12, 2012

HOXC6 Is Deregulated in Human Head and Neck Squamous Cell Carcinoma and Modulates Bcl-2 Expression*[S]

Received for publication, March 13, 2012, and in revised form, August 10, 2012 Published, JBC Papers in Press, August 15, 2012, DOI 10.1074/jbc.M112.361675

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Background: HOXC6 is involved in malignant progression, yet its signaling pathways in HNSCC remain largely unknown.
Results: HOXC6 induces Bcl-2 expression by directly increasing promoter activity.
Conclusion: The role of HOXC6 in HNSCC is associated with the aberrant cell growth and anti-apoptotic role.
Significance: These studies provide a new mechanistic link between HOXC6 and Bcl-2 in HNSCC cell lines.

Homeobox C6 (*HOXC6*) genes belong to the homeoprotein family of transcription factors, which play an important role in morphogenesis and cellular differentiation during embryonic development. The aim of this study was to explore the role of HOXC6 in the regulation of Bcl-2 in human head and neck squamous cell carcinoma (HNSCC). The HOXC6 and Bcl-2 gene were identified as being overexpressed in HNSCC tissue and cell lines. Transfection assays demonstrated that HOXC6 increased the levels of Bcl-2 mRNA and protein. A luciferase reporter assay suggested that HOXC6 induced activity of the Bcl-2 promoter. A series of Bcl-2 promoter deletion mutants were examined and the minimal HOXC6-responsive region was identified to be in the TAAT motif (-420 bp) of the Bcl-2 promoter. Interestingly, the inhibition of HOXC6 using siRNA led to the repression of Bcl-2 expression and induced caspase-3-dependent apoptosis; overexpression of HOXC6 in HNSCC cells increased the resistance to paclitaxel-induced apoptosis. Together, our findings suggest that HOXC6 is an important mechanism of the anti-apoptotic pathway via regulation of Bcl-2 expression.

Head and neck squamous cell carcinoma (HNSCC)[2] is the sixth most common cancer worldwide with 600,000 cases diagnosed per year. HNSCC is responsible for high death rates worldwide every year (1–4). Currently, the 5-year survival rate for HNSCC is ~60%. Approximately two-thirds of HNSCC patients present with advanced stage disease at diagnosis, and 90% of head and neck cancers are diagnosed as squamous cell carcinoma (5).

Human Homeobox (HOX) genes identify transcription factors that act as major regulators of embryonic development; they are also involved in several processes such as cellular morphogenesis and differentiation (6–9). There are 39 different human HOX genes clustered into four different groups (HOXA, HOXB, HOXC, and HOXD) (6, 7). Recently, there has been a growing interest in investigating the relationship of altered HOX genes with carcinogenesis and malignant progression. HOX genes have been described as deregulated in several cancers including leukemia, colon, skin, prostate, breast, ovary, and, more recently, in oral cavity cancer (9–17). In addition, some studies observed the aberrant expression of various HOX genes in oral squamous cell carcinoma (OSCC) and esophageal squamous cell carcinoma (ESSC) (10, 18, 19). However, the specific mechanisms by which HOX genes contribute to the development of cancer have not been completely described.

The overexpression of Homeobox C6 (HOXC6) has been detected in several human carcinomas, including gastrointestinal, breast, leukemia, and lung cancers; high expression-levels of HOXC6 have also been associated with lymph node metastasis (9, 14–16, 20). It has been demonstrated that HOXC6 is expressed in response to hormonal signals (8). In a recent study, HOXC6 was shown to play an important role in several cellular events through the regulation of its functional biological targets such as bone morphogenic protein 7 (BMP7), fibroblast growth factor receptor 2 (FGFR2), and platelet-derived growth factor receptor α (PDGFRA); it was also shown to regulate the PI3K/Akt, Notch, and Wnt signaling pathways (21–25). Although HOXC6 is critical for various regulated cellular processes and is correlated with cancer progression, the function of HOXC6 in HNSCC is largely unknown.

The overexpression of anti-apoptotic members of the Bcl-2 protein family, including Bcl-XL and Bcl-2, appears to play an important role in conferring apoptosis resistance in many cancers, including HNSCC (26–28). Overexpression of Bcl-2 is observed in a majority of HNSCC and correlates with chemotherapy resistance (26). Bcl-2 knockdown in HNSCC cell lines has been shown to promote apoptosis and sensitize the cells to chemotherapeutic agents (28). Similarly, molecular targeting of Bcl-2 with small molecule inhibitors or short peptides derived

* This research was supported by National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology (No. R13-2008-010-00000-0; No. 2012-0002140).
[S] This article contains supplemental Figs. S1 and S2.
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[2] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; HOX, human homeobox; MSP, methylation-specific PCR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodine; pNA, *p*-nitroanilide; INOK, immortalized oral keratinocyte; Cyt C, cytochrome *c*; PARP, poly (ADP-ribose) polymerase.

from the BH3 domains of pro-apoptotic proteins promotes apoptosis and chemosensitivity in HNSCC cells (29, 30). Recently, it was shown that Bcl-2 proteins physically interact with other cellular proteins and that the relationship between these proteins is an important determinant of cellular homeostasis, cancer development, and apoptosis (31).

Therefore, we hypothesize that HOXC6 may regulate Bcl-2 expression in HNSCC cells. Here, our data provide novel evidence that HOXC6 induces expression and transcriptional activity of Bcl-2 in HNSCC cells. We have also identified that induction of HOXC6 using transfection strategies significantly attenuated paclitaxel-induced apoptosis through the induction of Bcl-2 expression. We provide the first evidence for HOXC6 controls Bcl-2 expression, suggesting a potential role of HOXC6 in coordinating carcinogenesis in HNSCC cell lines.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The human head and neck squamous cell carcinoma (HNSCC) cells (pharynx squamous cell carcinoma (SCC)) from the FaDu cell line were purchased from American Type Culture Collection (ATCC, Rockville, MD). The human oral SCC YD-8 and YD-10B cell lines and Larnynx SCC Hep2 and SNU-1076 cell lines were purchased from Korea Cell Line Bank (Seoul, Korea). FaDu and Hep2 cell lines were incubated in MEM medium and SNU-1076, YD-8, and YD-10B cell lines were incubated in RPMI medium containing 10% fetal bovine serum and 100 units/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA) at 37 °C in an atmosphere containing 5% CO_2. The INOK cells, human immortalized oral keratinocytes, were used as control cells.

RNA Isolation and RT-PCR Analysis—Total RNA was isolated from cell lines using Trizol (Invitrogen) according to the manufacturer's instruction and was reverse-transcribed using the Reverse Transcription System (Promega, Madison, WI). The reverse transcription reaction was performed sequentially for 10 min at 25 °C, for 60 min at 42 °C, and for 5 min at 95 °C. GAPDH was used as an internal control. PCR Master Mix reagent (Applied Biosystems) was used for quantitative PCR. Primers were designed by Applied Biosystems according to the complementary DNA sequences as follows: HOXC6 (5′-GAG AAT GTC GTG TTC AGT TC-3′, 5′-GAT CTG TCG CTC GGT CAG GCA A-3′), Bax (5′-CAG CTG ACA TGT TTT CTG ACG GC-3′, 5′-CTC CCG CCA CAA AGA TGG TCA CG-3′), and Bcl-2 (5′-AGT TCG CCG AGA TGT CCA GGC A-3′, 5′-ACT TGT GGC CCA GAT AGG CAC C-3′). The PCR products were analyzed using gel electrophoresis with 1.2% agarose gel.

Quantitative Real-time RT-PCR—Quantitative real-time PCR was performed using SYBR® Green. PCR runs and fluorescence detection were performed in a Rotor-Gene 6000 Real-Time PCR system (Corbett Research, Sydney, Australia). In brief, the reaction mixture contained 10 ng of cDNA diluted in 2.5 μl of DEPC-treated water, 5 μl of Power SYBR® Green PCR Master Mix (2×; Applied Biosystems), and 2 μl of gene-specific primers (final concentration 50 nM each), in a final reaction volume of 10 μl. The sequences of the real-time PCR primers were as follows: HOXC6: forward 5′-CACCGCCTATGATC-CAGTGAGGCA-3′ and reverse 5′-GCTGGAACTGAACAC-GACATTCTC-3′, Bcl-2: forward 5′-GACTGTCAGCTGCT-GTCTGGGCAA-3′ and reverse 5′-GCCAAGACCTCTT-CAGCTACTGC-3′. The GAPDH real-time PCR primers were forward 5′-AGCCAAAAGGGTCATCATCTCTGC-3′ and reverse 5′-GCATTGCTGATGATCTTGAGGCTG-3′. The cycling conditions were as follow: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s, 58 °C for 20 s, and 70 °C for 20 s.

Immunohistochemistry—Immunohistochemistry was performed using the Envision System with diaminobenzidine (DAKO Cytomation, Glostrup, Denmark) according to the manufacturer's protocol. In brief, antigen retrieval was performed with citrate buffer (pH 6.0) by heating in a microwave at a controlled final temperature of 121 °C for 15 min. Sections were incubated overnight at 4 °C with the primary antibody against HOXC6 (diluted 1:200) or Bcl-2 (diluted 1:200), and then incubated with the secondary antibody for 30 min at room temperature. After rinsing with PBS three times each for 10 min, the sections were incubated with 3,3-diaminobenzidine (DAB) liquid for 1 min, counterstained with Mayer hematoxylin, dehydrated, and then mounted.

Bisulfite Treatment of Genomic DNA and Methylation-specific PCR (MSP)—Genomic DNA sequences were taken from the Ensembl database and analyzed for promoter CpG islands. CpG islands were predicted using MethPrimer online software. 5 μg of genomic DNA from FaDu, SNU1076, and YD-10B cells were treated with sodium bisulfite and analyzed by methylation-specific PCR to assay the methylation status of CpG islands located in the Bcl-2 promoter regions. We used the following MSP primers: 1) for methylated Bcl-2 primer sequence: 5′-TAT ACG GTT AGA AAG GGT TTA GGC-3′ and 5′-GAA CGA ACG ACG AAA TAC GA-3′; 2) for ummethylated Bcl-2 primer sequence: 5′-ATA TGG TTA GAA AGG GTT TAG GTG G-3′ and 5′-AAC CAA ACA AAC AAC AAA ATA CAA A-3′. Methylation-specific PCR was carried out with 20 μl of 1 units of Hot start Taq polymerase (Takara) per reaction. The PCR conditions are as follows: 1 cycle of 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s; and 1 cycle of 72 °C for 5 min.

Preparation of Cell Lysates and Western Blot Analysis—The cell pellet was dissolved in a 1% cell lysate buffer and centrifuged for 10 min at 12,000 rpm. Protein concentrations were determined using a BCA Protein Assay Kit (Pierce). The proteins were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane followed by Western blot analysis. A solution of 5% nonfat dried milk in TBS containing 0.1% Tween-20 was used to block nonspecific binding. The membrane was subsequently incubated with an anti-HOXC6 mouse monoclonal antibody (Novus Biologicals, LLC) or an anti-Bcl-2 antibody (Santa Cruz Biotechnology). After incubation, the blots were extensively washed in TBS containing 0.1% Tween-20. For detection, an ECL kit (Amersham Biosciences Life Sciences) was used according to the manufacturer's instructions.

Plasmid Construction—The putative promoter of the human Bcl-2 gene was PCR amplified (from −1.180 bp to +474 bp) and cloned into the NheI and HinD III sites of the pGL3-basic luciferase reporter vector (Promega). This reporter was designated

as wild-type Bcl-2-Luc (pGL3-Bcl-2-B1) in this study. Several deletion mutants were generated from the 1.2 kb Bcl-2 promoter. The nucleotide sequences of the PCR forward primers used for generation of the Bcl-2 promoter deletion plasmids were as follows: pGL3-Bcl-2-B1 (−1.18 kb from the start codon), 5′-GAG AAC TTC GTA GCA GTC ATC CTT-3′; pGL3-Bcl-2-B2 (−758 bp), 5′-CTC GAG CTC TTG AGA TCT C-3′; pGL3-Bcl-2-B3 (−543 bp), 5′-CTT GAC AGA GGA TCA TGC TG-3′; pGL3-Bcl-2-B4 (−445 bp), 5′-TAC TTA AAG TGC ATT CGA G-3′; pGL3-Bcl-2-B5 (−414 bp), 5′-CAG GCA GCT TAA TAC ATT CT-3′; pGL3-Bcl-2-B6 (−353 bp), 5′-ACT CAG TGT GTA CAG GGA AAC-3′; and pGL3-Bcl-2-B7 (−172 bp), 5′-GGA AAC ACC AGA ATC AAG TG-3′. The same reverse primer (5′-ACA CAT GAC CCC ACC GAA CTC AAA GAA-3′) was used for all deletion plasmid generation steps.

siRNA Interference Assay—siRNA constructs for HOXC6 were obtained in the form of Silencer® select validated siRNA (Applied Biosystems, Foster City, CA). The sense sequence of the HOXC6 siRNA was 5′-CUC GUU CUC GGC UUG UCU A (dTdT)-3′ and the antisense sequence was 5′-UAG ACA AGC CGA GAA CGA G (dTdT)-3′. Cells were transfected with siRNA (20 nM) using X-tremeGENE siRNA Transfection Reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. The cells were harvested 24 h after transfection. Total cell lysates were separated by SDS-PAGE and analyzed by Western blot as described above.

MTT Assay—FaDu and YD-10B cells were plated in 12-well plates at a density of 5×10^4 cells per well plate 12 h prior to transfection. A total of 100-μl of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT reagent (Sigma-Aldrich) was added to each well. After 4 h of incubation at 37 °C, the supernatant was aspirated, and formazan crystals were dissolved in 500 μl of DMSO at 37 °C for 15 min under gentle agitation. The absorbance per well was measured at 570 nm using a VERS Amax Microplate Reader (Molecular Devices Corp., Sunnyvale, CA).

Chromatin Immunoprecipitation (ChIP) Assay—To crosslink proteins to DNA, formaldehyde (final concentration 1%) was added to the culture medium and incubated for 10 min at room temperature. Cells were scraped and collected and then lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris at pH 8.1) containing protease inhibitors. Aliquots of cell lysates were sonicated to shear DNA into 0.2–1.0 kb fragments, and the cellular debris was removed. Chromatin aliquots were precleared with salmon sperm DNA/protein A-agarose-50% Slurry (Millipore Corp., Billerica, MA). Samples were then incubated with 2 μg of specific HOXC6 antibody overnight at 4 °C with rotation. Immunocomplexes were mixed with salmon sperm DNA/protein A-agarose-50% slurry followed by incubation for 1 h at 4 °C with rotation. Beads were collected by brief centrifugation, and the immunocomplexes were eluted using freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$). Chromatin was then de-crosslinked for 4 h at 65 °C. After proteinase K treatment for 1 h at 65 °C, DNA was phenol/chloroform-extracted and ethanol-precipitated. PCR amplification was used to detect the Bcl-2 promoter region containing HRE bound to HOXC6. The sequences of the ChIP-PCR primers were as follows: 480 TAAT: forward 5′-TGGAGAGTGCT-GAAGATTG-3′ and reverse 5′-CCATTGAAAGTTACAT-TAAACC-3′, 420 TAAT: forward 5′-GTGCATTCGAGTA-AATTTAATTTCC-3′ and reverse 5′-ACTTCCTCTGTGA-TGCTGAAAGG-3′. In addition, the normal and tumor tissues were also cross-linked using 1% formaldehyde, and the chromatin was subjected to immunoprecipitation using the indicated antibodies. Isotype-specific IgG was used as a control. DNA was resuspended and used for PCR amplification using the gene-specific primers. The primers for Bcl-2 promoter regions were: 5′- GTGCATTCGAGTAAATTTAATTTCC-3′ (sense) and 5′ ACTTCCTCTGTGATGCTGAAAGG-3′ (antisense).

Reporter Gene Construct and Reporter Gene Assay—The cells were transfected in serum-free medium with three plasmids (pRL-TK, Bcl-2 promoter-luciferase, and/or pCDNA3.1-HOXC6) in triplicate using Fugene 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's protocol. Forty hours after transfection, cells were washed once with PBS and lysed by incubation with 500 μl of cell lysis buffer for 15 min at room temperature. A 20-μl aliquot of cell lysate was then assayed for luciferase activity using the Dual Luciferase Reporter (DLR) assay system (Promega) according to the supplier's recommendations. For each experiment, the luciferase activity (experimental reporter) was normalized to Renilla luciferase (control reporter) activity. The change in normalized luciferase activity was calculated relative to that for cells that were transfected with vehicle alone.

Flow Cytometric Cell Cycle Analysis and Annexin V-FITC/PI Double Staining—The cells were fixed in chilled 75% methanol and stained with a propidium iodine (PI) solution (100 μg/ml RNase and 10 μg/ml PI in PBS) for cell cycle analysis. The cells were stained using the Vybrant® apoptosis assay kit (Molecular Proves) followed by labeling with Alexa Fluor® 488 Annexin V and PI for apoptosis analysis. Data acquisition and analysis were carried out using the Cell Lab Quanta™ SC flow cytometer and software (Beckman Coulter Inc., Miami, FL).

Measurement of Caspase Activities—Caspase-9 and caspase-8 activities were evaluated using caspase-9 and caspase-8 colorimetric assay kits (BioVision, Inc.). The assay is based on spectrophotometric detection of chromophore *p*-nitroanilide (pNA) after cleavage from the labeled caspase-9 substrate LEHD-pNA and caspase-8 substrate IETD-pNA. The absorbance at 405 nm of the released pNA was monitored in a spectrophotometer. The fluorometric assay for caspase-3 activity was performed according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI). A specific caspase-3 substrate, *N*-Ac-DEVD-N′-MC-R110, which opens cleavage by active caspase-3 and generates highly fluorescent products, was measured with a fluorescence spectrophotometer with an excitation wavelength of 485 nm and emission at 535 nm.

Statistical Analysis—Data are expressed as the mean ± S.E. of at least three individual experiments. Statistical comparisons between groups were performed using two-tailed Student's *t* test (Excel, Microsoft). Statistical significance was set at *, $p < 0.05$; **, $p < 0.01$.

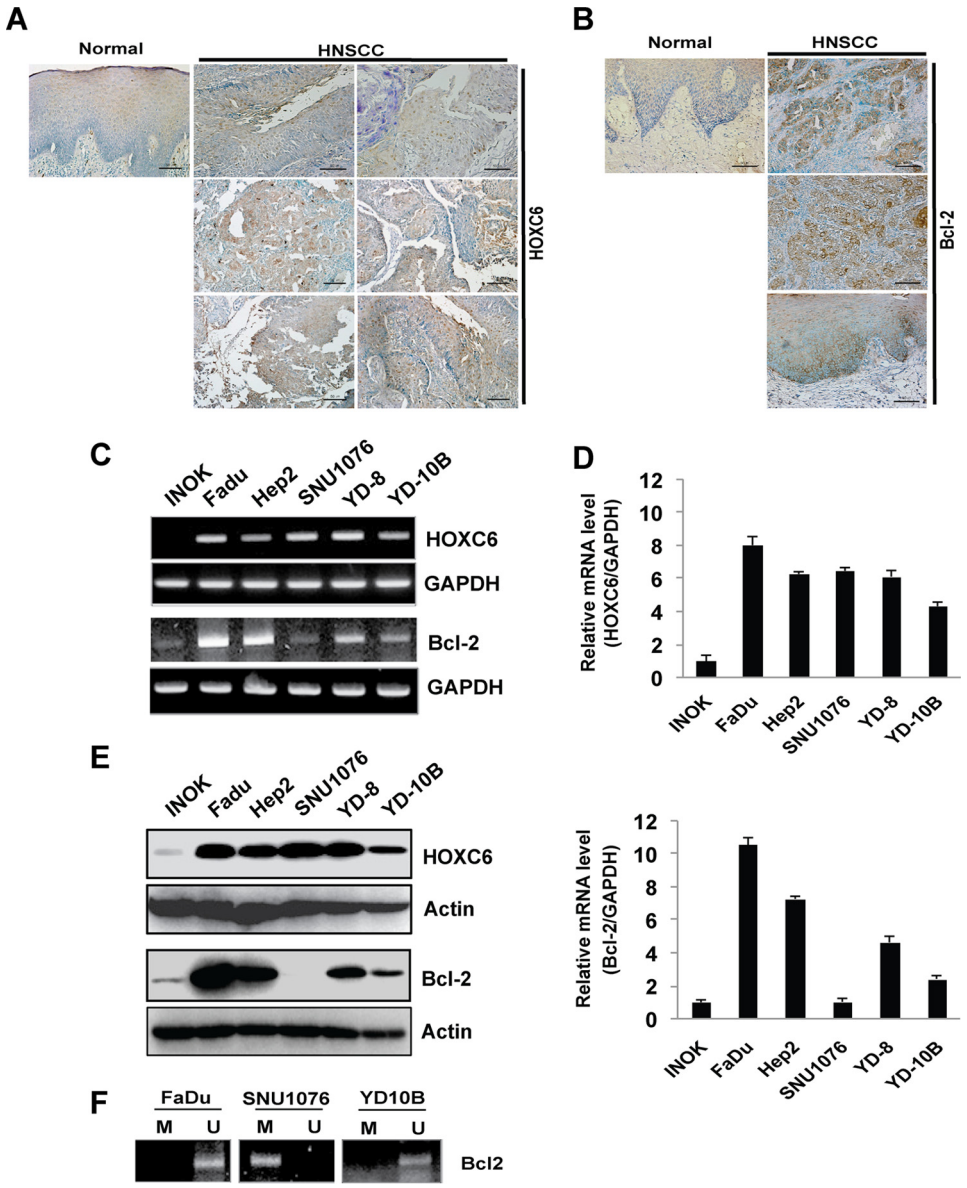

FIGURE 1. **Evaluation of mRNA and protein expression of HOXC6 and Bcl-2 in HNSCC tissues and HNSCC derived cell lines.** *A* and *B*, immunohistochemical staining of HOXC6 and Bcl-2 in HNSCC and normal tissue. Strong expression of HOXC6 and Bcl-2 was shown in HNSCC tissue. Normal tissue showed only weak expression of Bcl-2. All photomicrographs were taken at a magnification ×200. *C* and *D*, HOXC6 and Bcl-2 mRNA levels were analyzed in HNSCC-derived cell lines (FaDu, Hep2, SNU1076, YD-8, and YD-10B) by RT-PCR analysis and real-time quantitative RT-PCR and normalized with GAPDH. *E*, Western blot was performed for the expression of HOXC6 and Bcl-2 protein levels. Whole-cell lysates (40 μg/lane) were subjected to immunoblotting for the indicated proteins. Probing with β-actin was used to show equal protein loading. INOK cells, human immortalized oral keratinocytes, were used as control cells. *F*, methylation status of the Bcl-2 promoter was evaluated with methylation-specific PCR (MSP) in FaDu, SNU1076, and YD-10B cells. Primer sets used for amplification are designated as unmethylated (*U*) and methylated (*M*).

RESULTS

HOXC6 and Bcl-2 Expression in HNSSC Tissues and Cell Lines—Recent studies have shown that deregulated HOXC6 expression is related to carcinogenesis. We hypothesized that the abnormal expression of HOXC6 was associated with development and malignant progression of HNSCC. To examine altered HOXC6 protein expression we performed immunohistochemical staining on tissue sections derived from the HNSCC patient. Non-malignant normal tissues were used as controls. The HOXC6 expression levels were importantly higher in the examined six HNSCC tissues compared with normal oral tissues. This expression was mainly cytoplasmic, with very few cells showing weak nuclear expression. Non-malignant oral tissues presented either negative or very low HOXC6 staining (Fig. 1*A*). Analysis of the correlation of anti-apoptotic protein Bcl-2 with HNSCC was also performed by immunohistochemical staining and similar results were obtained with HOXC6. The immunodetection of Bcl-2 disclosed a strong cytoplasm staining in HNSCC tissues, while normal oral tissues expressed weak HOXC6 staining (Fig. 1*B*).

To confirm the abnormal expression of HOXC6 and Bcl-2 in human HNSCC cell lines, we performed real time RT-PCR and Western blot analysis. Five human HNSCC cell lines (FaDu, Hep-2, SNU-1067, YD-8, and YD-10B) and the control keratinocyte cell line (INOK) were used in an initial screen to identify

potential associations between HOXC6 and Bcl-2. Most HNSCC cell lines had elevated HOXC6 mRNA and protein levels compared with the control INOK cells (Fig. 1, *C* and *E*). The real time RT-PCR analysis confirmed that HOXC6 and Bcl-2 were significantly up-regulated in 5 HNSCC cell lines (Fig. 1*D*). The ratio of HOXC6 mRNA expression was >4-fold in all HNSCC cell lines, and the highest ratio was a 7-fold increase, as determined using real time RT-PCR. Similarly, Bcl-2 expression was increased in these cell lines, with the exception of the SNU1076 cells.

Aberrant methylation of DNA has been recognized as an important mechanism for the regulation of the expression of genes (32). To investigate whether DNA methylation is related to the suppression of Bcl-2 in SNU1076 cells, the methylation status of the Bcl-2 promoter was evaluated using MSP. No methylation of the Bcl-2 promoter was observed in FaDu and YD-10B cells, whereas methylation of the Bcl-2 promoter was showed in SNU1076 cells, regardless of HOXC6 expression; unmethylation was observed in FaDu and YD-10B, but was not observed in SNU1076 (Fig. 1*E*). Our results showed that the increased DNA methylation of this CpG region of the Bcl-2 promoter is a possible mechanism for gene silencing in the SNU1076 cells. These results suggest that aberrant expression of HOXC6 and Bcl-2 protein may be associated with the development of HNSCC. Hence, FaDu and YD-10B cells were chosen for this approach.

HOXC6 Regulates the Expression of Bcl-2 and Cell Proliferation in HNSCC Cells—We then examined whether the overexpression of HOXC6 in FaDu and YD-10B cells affects the expression of Bcl-2. Correlations between HOXC6 levels and Bcl-2 expression were assessed following the measurement of transcripts using RT-PCR and Western blotting. Notably, the overexpression of HOXC6 increased expression of Bcl-2 mRNA and protein in the FaDu and YD-10B cells (Fig. 2, *A* and *B*). However, the mRNA and protein levels of Bax, a pro-apoptotic protein, in HOXC6-induced FaDu and YD-10B cells were not affected. As expected, the overexpression of HOXC6 caused increased activity of Bcl-2 promoters in FaDu and YD-10B cells (Fig. 2*C*). In addition, the MTT assay showed that cells transfected with HOXC6 had significantly higher cell viability levels than their controls (Fig. 2*D*). These results indicate that expression of HOXC6 induces expression of Bcl-2 and increase the cell viability.

We used siRNA transfection to knock down HOXC6 expression in FaDu and YD-10B cells to determine the impact on Bcl-2 expression. RT-PCR and Western blots were performed to analyze mRNA and protein expression levels, respectively. The decrease in Bcl-2 mRNA levels occurred with knock down of HOXC6 by siRNA as shown in Fig. 2*E*. When HOXC6 protein expression was decreased using siRNA against HOXC6, Bcl-2 protein expression was also repressed by 70% as shown in Fig. 2*F*. Therefore, these results suggest that HOXC6 positively regulates Bcl-2 in both cell lines.

HOXC6 Directly Regulates Bcl-2 Promoter Activity—To test the effects of HOXC6 expression on Bcl-2 promoter activity, we generated a luciferase reporter construct driven by the 1.2 kb Bcl-2 promoter. INOK, FaDu, and YD-10B cells were transiently transfected with the Bcl-2 promoter-luciferase reporter plasmids. As shown in Fig. 3*A*, the ratio of Bcl-2 activity was >3-fold in FaDu and YD-10B cells compared with INOK cells (Fig. 3*A*).

We next attempted to define the binding sites of HOXC6 in the Bcl-2 promoter by serially deleting regions within the promoter. As depicted in Fig. 3*B*, the deletion constructs pGL3-Bcl-2-B1, -B2, -B3, and -B4 did not have drastic effects on the luciferase activity. However, the deletion construct pGL3-Bcl-2-B5 dramatically decreased the ability to be activated by HOXC6 (Fig. 3*B*). These data suggest that there may be critical binding sites for HOXC6 in the pGL3-Bcl-2-B4 promoter region.

It was demonstrated in the previous report that HOXC6 belongs to the HOX family of transcription factors that bind to the TAAT sequences of target gene promoters to regulate gene expression (14). According to the promoter sequence analysis for pGL3-Bcl-2-B4, there is one tentative TAAT element binding site (−420 bp) for HOXC6 (Fig. 3*C*).

To confirm binding of HOXC6 to the Bcl-2 promoter, we performed a chromatin immunoprecipitation (ChIP) assay. The crosslinked extracts in FaDu and YD-10B cells were immunoprecipitated with either antibodies against HOXC6 or control anti-IgG (Fig. 3*D*). The crosslinked DNA was analyzed by PCR using primers designed to amplify the HOXC6-responsive region that covers the TAAT motif (−420 bp) of the pGL3-Bcl-2-B4 promoter. Compared with the IgG control group, HOXC6 was determined to be associated with the Bcl-2 promoter region containing the TAAT motif. In addition, a PCR fragment (147 bp) corresponding to −609 to −463 (containing −480 TAAT motif from start codon) of Bcl-2 promoter was used as HOXC6 binding negative control. This fragment was not detected when HOXC6 antibody was used for the pull-down assay (Fig. 3*D*).

To examine the binding of HOXC6 to the Bcl-2 promoter in tumor, we performed a ChIP assay in HNSCC tissues. We found that Bcl-2 promoter-specific PCR primers amplified this promoter region from DNA that was immunoprecipitated with the anti-HOXC6 antibody in tumor tissue but not in normal tissue, demonstrating that the interaction between HOXC6 protein and the Bcl-2 promoter was specific in tumor. (supplemental Fig. S1).

To confirm whether the −420 bp TAAT motif was involved in HOXC6-mediated regulation of Bcl-2 promoter activity, the −420 bp TAAT binding site within the Bcl-2 promoter region was mutated and the activity of the mutated promoter was evaluated in FaDu and YD-10B cells without HOXC6 overexpression. As shown in Fig. 3*E*, the introduction of point mutation at HOXC6 (−420 bp TAAT) binding site of the Bcl-2 promoter attenuated Bcl-2 promoter activity compared with that of the wild-type control. These results indicate that HOXC6 directly binds the −420 TAAT elements on Bcl-2 promoter region and regulates its gene expression.

Knockdown of HOXC6 Induces Cell Death in HNSCC Cell Lines—To determine whether depletion of HOXC6 by siRNA can induce apoptosis followed by cell growth inhibition, we performed MTT assay and flow cytometry analysis using Annexin V/PI double staining. The MTT assay indicated that expression of siHOXC6 significantly inhibited the growth of

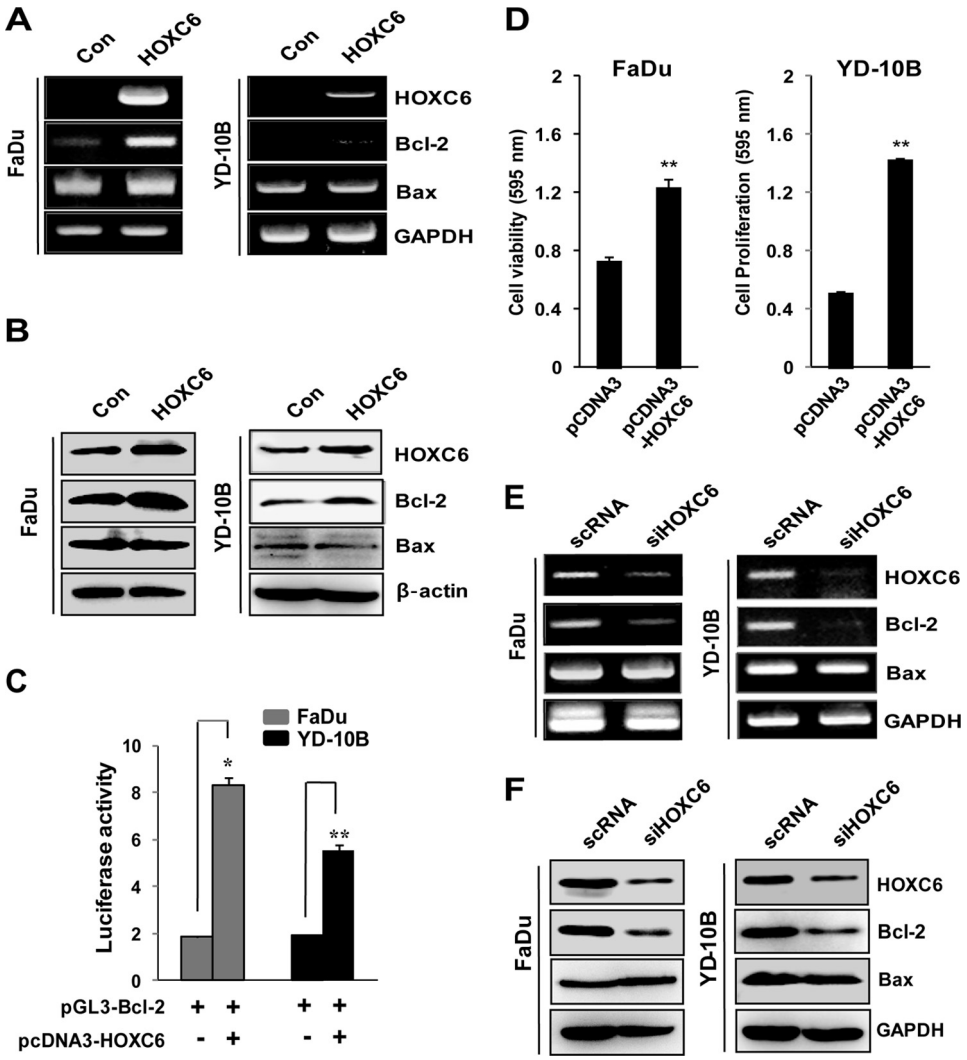

FIGURE 2. **HOXC6 regulates the expression of Bcl-2 at the transcriptional level as well as cell proliferation.** FaDu and YD-10B cells were transfected with pcDNA3-HOXC6 plasmid as described under "Experimental Procedures." *A*, after 48 h transfection, mRNA was determined by RT-PCR assays, and (*B*) HOXC6, Bcl-2, and Bax protein levels were determined by Western blot analysis. *C*, FaDu and YD-10B cells transiently transfected with pCDNA3-HOXC6. At 24 h post-transfection, cells were co-transfected pGL3-Bcl-2 promoter region together with pRL-TK for 24 h (which carried the *Renilla* luciferase gene controlled by HSV-tk promoter as internal control). Cell lysates were subjected to luciferase activity assays using the dual luciferase reporter assay system, and the luciferase activity was normalized to pRL-TK activity (mean ± S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$). *D*, FaDu and YD-10B cells were transfected for 48 h with either pcDNA3-HOXC6 or control vector. After transfection, the cell viability was analyzed using the MTT assay. **, $p < 0.01$ (*E*) RT-PCR quantitation of Bcl-2 mRNA in FaDu and YD-10B cells transiently transfected with scRNA or HOXC6 siRNA. After 48 h of transfection, mRNA level of Bcl-2 was determined by RT-PCR analysis. *F*, Western blots of HOXC6 and Bcl-2 expression in cells co-transfected with scRNA or HOXC6 siRNA. Whole cell lysates were obtained post-transfection and the samples were separated by 12% SDS/PAGE and probed for HOXC6, Bcl-2, Bax, and actin as a loading control.

FaDu and YD-10B cells, whereas down-regulation of HOXC6 did not affect the growth of INOK control cells (Fig. 4*A*). As shown in Fig. 4*B*, siHOXC6 induced the cell death compared with the untreated control cells. In the vehicle-treated cells, 3.0% were positive for Annexin V-FITC staining while siHOXC6 treatment resulted in increases of 13.7% and 13.2% in FaDu and YD-10B cells, respectively. We also found that siHOXC6 inhibited the expression level of Bcl-2 and increased the level of cleaved PARP and cytochrome *c* release in both cell lines (Fig. 4*C*). In addition, activation of caspase-3 was observed in siHOXC6-treated cells (Fig. 4*D*). These results suggest that siHOXC6 induces caspase-3-dependent apoptosis in FaDu and YD-10B cells.

HOXC6 Inhibits Paclitaxel-induced Apoptosis in HNSCC Cells—To examine whether the cytotoxic effect of paclitaxel on HNSCC cells would be affected by HOXC6 expression, we compared the apoptosis related protein levels in FaDu and YD-10B cells cell treated with paclitaxel alone or with the combination of paclitaxel and HOXC6 transfection. RT-PCR and Western blot analysis revealed that the expression of Bcl-2 decreased when FaDu and YD-10B cells were exposed to paclitaxel. Remarkably, overexpression of HOXC6 recovered the decreased expression levels of Bcl-2 mRNA and protein with paclitaxel treatment (Fig. 5, *A* and *B*). We further examined the effect of HOXC6 on the level of cytosolic cytochrome *c* (Cyt C) and poly (ADP-ribose) polymerase (PARP), a component of the signaling pathway of apoptosis. Western blot analysis revealed that paclitaxel caused an increased Cyt C and cleaved PARP levels in FaDu and YD-10B cells. HOXC6-transfected cells strongly inhibited the levels of cleaved PARP and Cyt C by paclitaxel treatment, suggesting that they inhibit paclitaxel-induced apoptosis (Fig. 5*B*). An MTT assay was performed to evaluate the

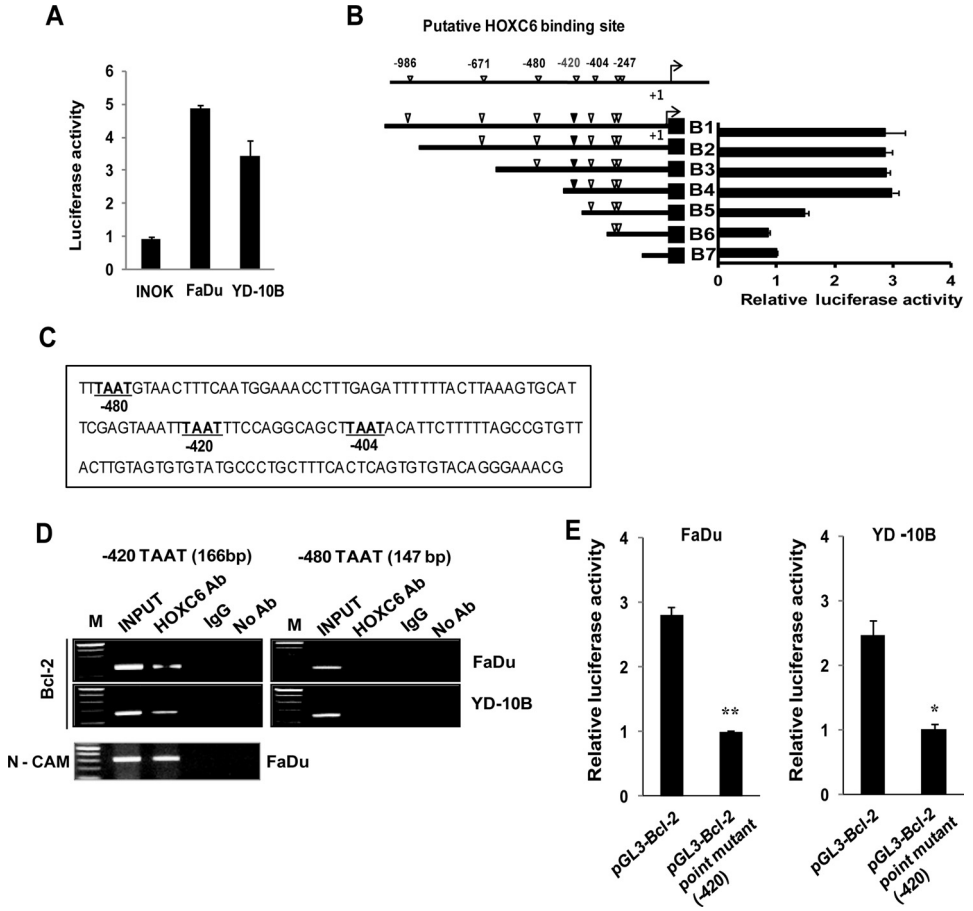

FIGURE 3. **Identification of the HOXC6 binding site in the promoter of Bcl-2 gene.** *A*, INOK, FaDu, and YD-10B cells were plated in 12-well cell culture dishes. Each group of cells was transiently transfected with pCDNA3-HOXC6. At 24 h post-transfection, cells were co-transfected pGL3-Bcl-2 promoter region together with pRL-TK. Cell lysates were subjected to luciferase activity assays using the dual luciferase reporter assay system, and the luciferase activity was normalized to pRL-TK activity. *B*, deletion analysis of the Bcl-2 promoter-reporter constructs (pGL3-Bcl-2-B1 to pGL3-Bcl-2-B7). Dual-reporter gene assay were performed with expression constructs carrying various lengths of Bcl-2 promoter. FaDu cells were co-transfected with one of seven constructs with a pRL-TK. After 48 h of transfection, cell extracts were prepared and analyzed for luciferase activity and normalized to *Renilla* luciferase activity. *C*, pGL3-Bcl-2-B4 promoter sequence containing one putative HOXC6 binding sites (TAAT motif, −420 bp). A diagram of the proximal 150 bp region of the pGL3-Bcl-2-B4 promoter. *D*, demonstration of HOXC6-binding site (TAAT motif, −420 bp) on the Bcl-2 promoter by ChIP assay. Chromatin lysates from FaDu, and YD-10B cells were immunoprecipitated (*IP*) with antibody against HOXC6. Nonimmune IgG was used as negative control for immunoprecipitation. Samples were processed as described under "Experimental Procedures." The *left panel* shows that PCR using chromatin (*Input*) pulled down by anti-HOXC6 antibody (HOXC6 Ab) as template yielded the Bcl-2 promoter, which was verified the Bcl-2 fragment (−420 TAAT containing, positive primer) of Bcl-2 promoter. When negative control IgG or no antibody (*No Ab*) was used for pull-down assay, no PCR product was observed. The *right panel* shows that immunoprecipitated DNA was amplified using primers representing a site upstream of the −420 TAAT HOXC6-binding site (−480 TAAT containing, negative primer) on the Bcl-2 promoter. No PCR product was observed pull-down of HOXC6 antibody. N-CAM promoter used as HOXC6 binding positive control using ChIP assay. *E*, pGL3-Bcl-2 promoter assay with TAAT motif (−420 bp) point mutation constructs in FaDu and YD-10B cells. FaDu and YD-10B cells were transfected with wild type pGL3-Bcl-2 promoter or point-mutated pGL3-MDR-1 (TAAT→TGGT). pRL(*Renilla* luciferase) plasmid was co-transfected as an internal control. The cells were harvested 48 h after transfection. The promoter activity of each preparation was normalized to the *Renilla* value. The relative promoter activity is averaged from at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

effects of HOXC6 on paclitaxel sensitivity of the cells. The cell viability of the paclitaxel-treated FaDu cells was significantly decreased compared with the untreated cells, while overexpression of HOXC6 increased cell viability in the FaDu cells compared with the vehicle-transfected cells (Fig. 5*C*). Interestingly, paclitaxel treated HOXC6 overexpressing cells significantly experienced increased cell viability compared with paclitaxel alone. These results strongly support that HOXC6 has the cell protection effect on paclitaxel-mediated cell sensitivity.

We investigated the effect of HOXC6 on paclitaxel-induced apoptosis in FaDu and YD-10B cells using flow cytometry analysis. We found that Annexin-V positive cells were induced by paclitaxel treatment in both cells. In contrast, HOXC6-expressing cells treated with paclitaxel displayed inhibited late apoptosis compared with the cells treated with paclitaxel alone (Fig. 5, *E* and *F*).

To support these results, we investigated caspase activity after the cells were treated with paclitaxel and/or HOXC6. The colorimetric assay revealed that caspase-3 and -9 activities were remarkably increased in the paclitaxel-treated FaDu cells, whereas the caspase-3 and -9 activities in HOXC6 overexpressing FaDu cells treated with paclitaxel were significantly lower than those in the FaDu/paclitaxel cells (Fig. 5, *E* and *F*). Caspase-8 activity was not affected by paclitaxel treatment and HOXC6 expression (Fig. 5*G*).

DISCUSSION

HOX genes constitute a family of transcription factors that are key to developmental processes, embryonic morphogenesis, and differentiation (33, 34). They are also expressed in various human cancers including esophagus, prostate, and colon,

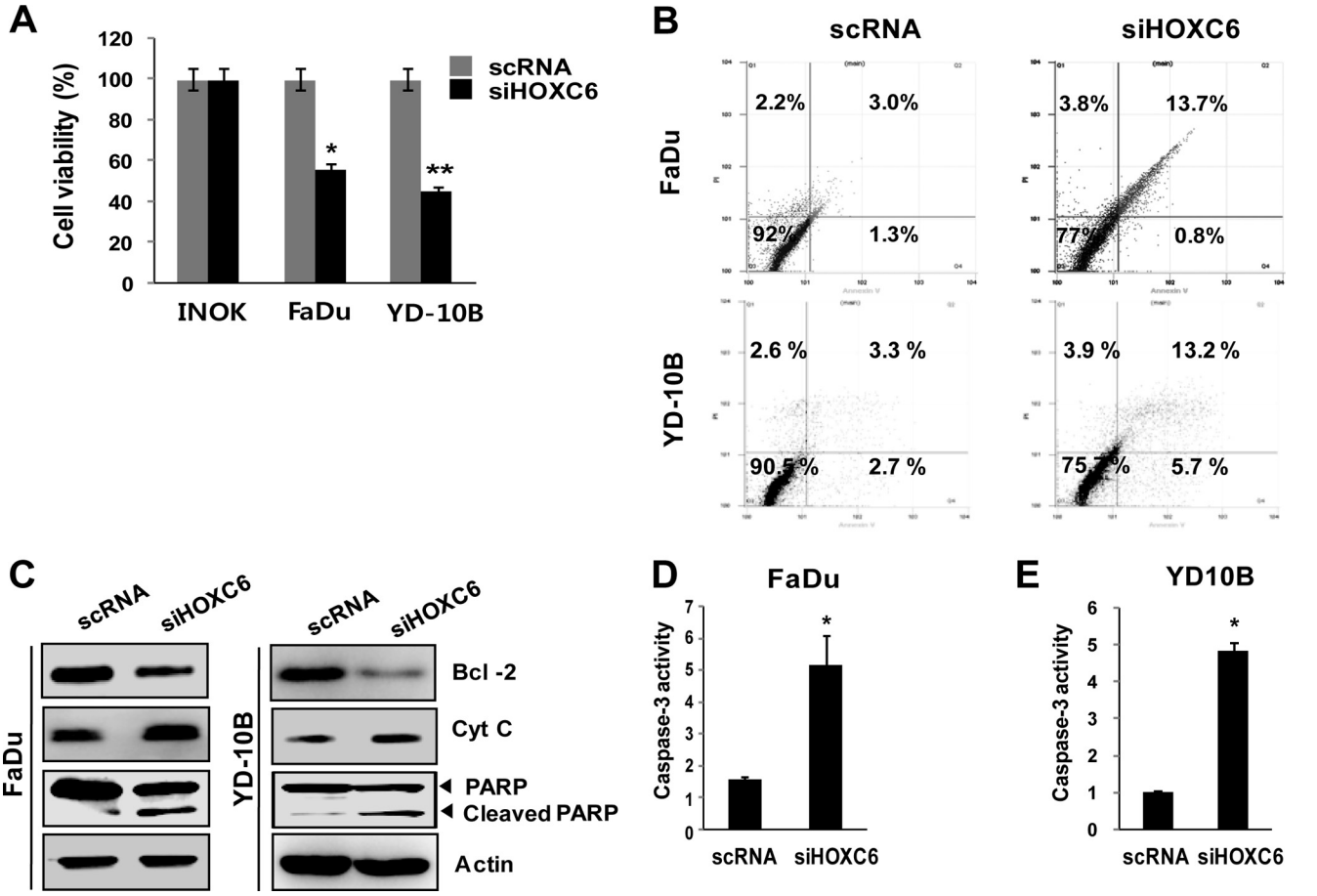

FIGURE 4. **Silencing of HOXC6 caused caspase 3-dependent apoptosis.** *A*, FaDu and YD-10B cells were transfected for 48 h with either HOXC6 siRNA or scrambled siRNA (*scRNA*). After 48 h, cell viability was analyzed using the MTT assay. The results of three experiments ($n = 3$) are summarized. *, $p < 0.05$; **, $p < 0.01$. *B*, representative of cell apoptosis with flow cytometry analysis. After siHOXC6 transfection, cells were stained with Annexin V/PI to analyze apoptotic cell populations. *C*, Western blotting of Bcl-2, cytochrome *c*, and cleaved PARP. Whole cell lysates were obtained after 48 h of transfection, and the samples were separated by 12% SDS/PAGE and probed for cleaved PARP, Bcl-2, Cyt C, and actin as a loading control. *D*, FaDu and YD-10B cells were plated in 96-well plates and treated with scRNA or SiHOXC6 as described under "Experimental Procedures." Caspase-Glo3 reagent was added to wells and luminescence was measured after 2 h of incubation. Data were collected from three independent experiments. *, $p < 0.05$.

suggesting a potential role in carcinogenesis (13, 14). Recently, a study described the expression of HOX genes in oral dysplasia and oral squamous cell carcinoma (OSCC), suggesting that overexpression of particular HOX genes is implicated in the development of oral dysplasia and oral SCC (19, 35). Moreover, another study using human salivary glands showed the possibility that HOX genes play a role in salivary gland carcinogenesis (36). Among them, aberrant expression of HOXC6 genes was observed in oral SCC and esophageal SCC (10, 19). There was a tendency for increased expression of HOXC6 in SCC with lymph node metastasis (20); this finding suggests that HOXC6 may play a key role in the acquisition of metastatic phenotypes of OSSC. However, the current study is insufficient in establishing its function, the specific cellular signaling to which it contributes, or its relationships with the HNSCC phenotype.

In this study, we identified that the levels of HOXC6 expression experience, importantly, an increase in expression by HNSCC cell lines compared with immortalized oral keratinocyte (INOK) cells when assessed by measuring mRNA. The levels of Bcl-2 mRNA were also significantly higher in HNSCC cell lines than in control INOK cells. Consistently, the analysis of immunoblotting demonstrated a statistically significant correlation between expression of HOXC6 and Bcl-2 in HNSCC cell lines. In SNU1076 cells, the level of Bcl-2 expression was lower than other HNSCC cell lines, although HOXC6 was increased in SNU1076 cells compared with INOK cells. Recently, in HOXC6 expressed tumors cell, target genes of HOXC6, including FGFR2, CD44, and WIF, have been shown to be silenced by hypermethylation (37–39). It was still not clear whether that the expression of Bcl-2 is mediated through methylation of specific CpG sites on the Bcl-2 promoter in SNU1076 cells. Nguyen *et al.* provided evidence that a 417 bp CpG island of the *Bcl-2* gene was identified as hypermethylated in all co-twins exhibiting autistic traits (40). Some studies reported Bcl-2 exon 2 methylation status in cancer (41). It was reported that Bcl-2 promoter is hypermethylated in colorectal cancer tissue (42). They suggest that aberrant methylation of Bcl-2 promoter may be involved in the expression of Bcl-2. This possibility was examined, for bisulfite-treated DNA from SNU601 cells, by nested methylation-specific PCR (MSP). We found that Bcl-2 promoter from SNU1076 cells yielded a PCR product only with methylation-specific primers, whereas DNA from FaDu and YD-10B cells yielded a PCR product only with unmethylation-specific primers (Fig. 1*F*). Our results suggested that the

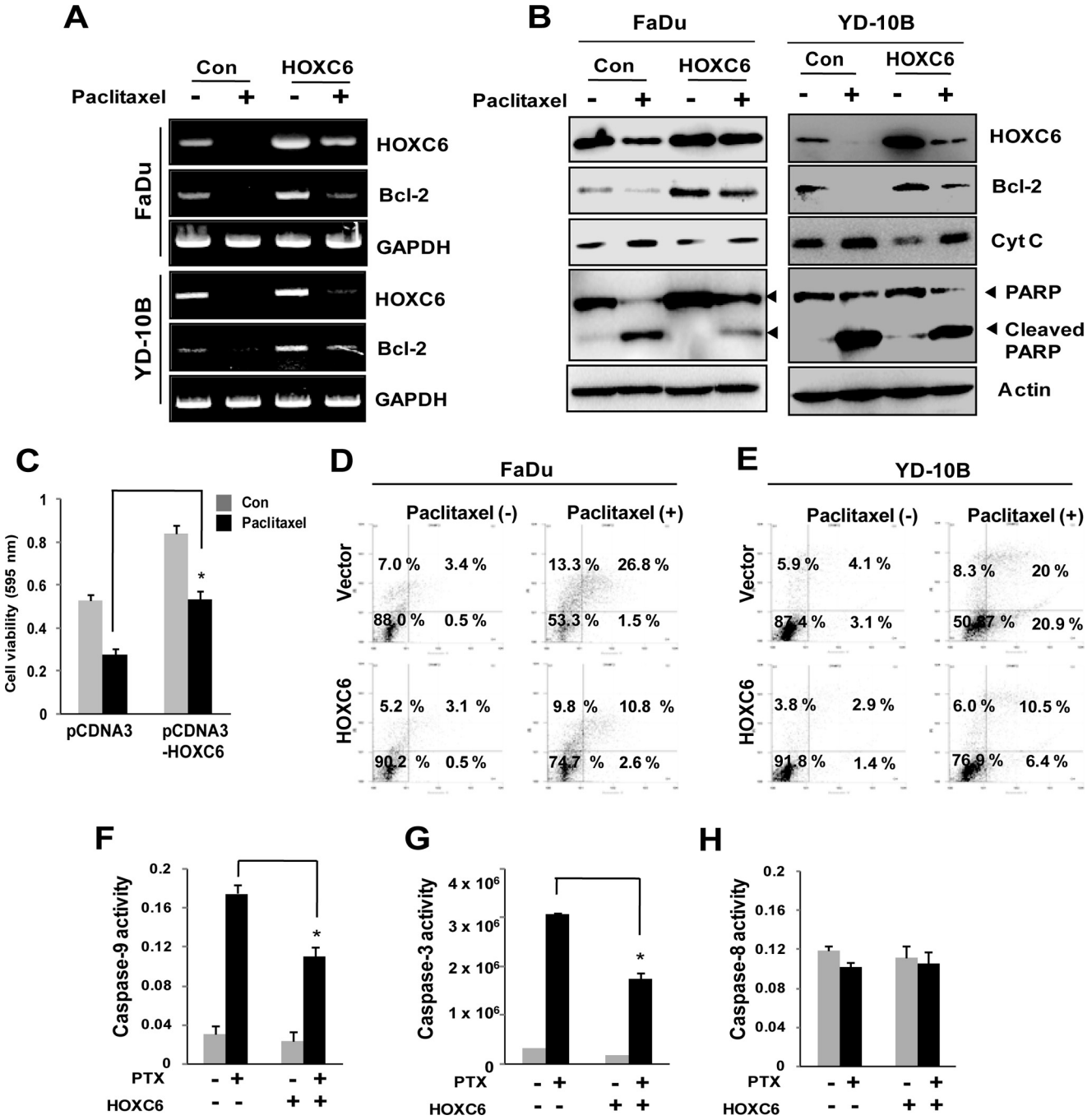

FIGURE 5. **HOXC6 prevents paclitaxel-induced apoptosis.** *A*, FaDu and YD-10B cells were pre-transfected with HOXC6 expression plasmids for 24 h before treatment with paclitaxel at 20 nM for 24 h. Whole-cell lysates were subjected to RT-PCR analysis using specific primers. *B*, FaDu and YD-10B cells were transfected with HOXC6 and then treated with paclitaxel (20 nM, 24 h). The cell lysates prepared from untreated and treated cells were analyzed for the expression of native PARP, Cyt C, and Bcl-2 by Western blotting. Immunoblots showing native (116 kDa) and cleaved (89 kDa) PARP protein bands. *C*, cell viability as assessed by an MTT assay in the presence of paclitaxel (20 nM) in FaDu cells transfected with HOXC6 expression plasmids. Values are presented as the means ± S.E. ($n = 3$). *, $p < 0.05$. *D* and *E*, flow cytometry analysis in the presence of paclitaxel (20 nM) in FaDu and YD-10B cells transfected with HOXC6 expression plasmids. FaDu and YD-10B cells were incubated with Annexin V-FITC reagent and PI for 30 min at room temperature. Annexin V-FITC-binding was assessed by flow cytometry. *F–H*, caspase-9, -3, and -8 activities of cells treated with the combination of pcDNA3-HOXC6 and paclitaxel were measured. FaDu cells were transfected with pcDNA3-HOXC6 before the addition of paclitaxel (20 nM). Untreated cells were used as the control. The caspase-3, -8, and -9 activities were measured 24 h after paclitaxel treatment. Values are presented as the means ± S.E. of three independent experiments. *, $p < 0.05$.

increased DNA methylation of this CpG region of Bcl-2 promoter is a possible mechanism for increased gene silencing and decreased protein abundance of Bcl-2 in the SNU1076 cells. These findings also suggest that epigenetic hypermethylation of Bcl-2 genes via alternative mechanisms may likely prevent their expression, although HOXC6 is overexpressed in SNU1076 cells. In addition, we examined the paclitaxel sensitivity of SNU1076 cells with and without HOX6 knockdown. Paclitaxel strongly inhibited cell proliferation in HOXc6 siRNA or scramble siRNA treated SNU1067 cells (supplemental Fig. S2). These results support that Bcl-2 exerts its effect on paclitaxel-mediated drug responsiveness in SNU1076 cells.

Although the exact action mechanism of HOXC6 in SNU1076 cells is unknown, many different molecules and signaling pathways have been demonstrated in various cancer cell lines. Because different types of tumor cells have different genetic alterations and characteristics, the molecular targets of HOXC6 could be different in SNU1076 cell lines.

HOXC6 is a HOX family member that presumably functions by binding directly to DNA promoter elements via its homeodomain, which is a DNA binding domain located within its N terminus (43). The DNA core sequence of the homeodomain binding sites (HBSs) frequently contains the sequence TAAT. Previous studies showed that HOXC6 binds to similar elements on the neural cell adhesion molecule (N-CAM) promoter (44). In our study, the results of the promoter deletion mutants confirm that HOXC6 directly binds to the specific TAAT, a sequence motif in the Bcl-2 gene promoter. In addition, ChIP approaches clearly demonstrated that HOXC6 was associated with the TAAT motif (−420 bp from start codon) in Bcl-2 promoter. These studies support the new HOXC6 mechanism that involves direct binding of HOXC6 to specific promoter elements to activate Bcl-2 expression in HNSCC cells. Moreover, siRNA directed against HOXC6 resulted in decreased Bcl-2 mRNA levels and reduced luciferase reporter activity in FaDu and YD-10B cells. Therefore, we believe that HOXC6 induces Bcl-2 expression in HNSCC cells by directly increasing its promoter activity.

Generally, cells harboring multiple pathphysiologic alterations are normally eliminated by apoptosis. In normal cells, diminished apoptosis plays a critical role in tumor initiation, progression, and drug resistance (26). Several proteins that inhibit apoptosis have been identified, including Bcl-2 family members Bcl-2 and Bcl-XL. Particularly, Bcl-2 acts as a key regulator of cellular apoptosis and is an important determinant of cellular sensitivity or resistance to chemotherapy drugs (31). Overexpression of Bcl-2 is commonly observed in HNSCC and may play an important role in overcoming apoptosis in HNSCC (45, 46).

We found evidence that the presence of HOXC6 in HNSCC is strongly associated with Bcl-2 expression. Small-interfering RNA knock-down of HOXC6 expression induces apoptosis, and overexpression of HOXC6 increases the growth/proliferation of FaDu and YD-10B cells. In this study, the overexpression of HOXC6 was investigated to understand the effect of HOXC6 upon apoptosis induced by the chemotherapeutic agent paclitaxel using the MTT test and a flow cytometry approach. One of the interesting findings of this study was that the expression of HOXC6 was significantly associated with the inhibition of paclitaxel-induced apoptosis, thereby confirming the anti-apoptotic role of HOXC6. Paclitaxel decreased expression of the anti-apoptotic protein Bcl-2 and increased caspase-3-dependent cleaved PARP. The reduced mRNA and protein levels of Bcl-2 were recovered by HOXC6 expression in FaDu and YD-10B cells. In addition, overexpression of HOXC6 inhibits the activity of paclitaxel-induced caspase-3 and -9 and the arrest of cell growth, suggesting that HOXC6-mediated Bcl-2 expression plays a protective role in enhanced paclitaxel cytotoxicity. The overexpression of HOXC6 in HNSCC may block this apoptotic pathway and allow aberrant cell growth through mitosis.

Previous studies suggested that the PI3K/Akt pro-proliferative and survival pathway are influenced by several HOXC6 direct targets such as BMP7, IGFBP3, and PDGFRA (22–24). It is possible that another mechanism by which HOXC6 exerts its pro-survival function may be the PI3K/Akt pathway.

In conclusion, the evidence presented in this report indicates that HOXC6 is highly expressed in HNSCC cell lines and induces proliferative activity. Our results also provide novel information about the function of HOXC6 in the regulation of Bcl-2 gene expression and its potential anti-apoptotic role.

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