Further studies have supported the involvement of YB-1 in cell proliferation. A YB-1 knockout mutation in mice caused a marked decrease in cell proliferation rates, resulted in embryonic lethality.[13,14] Transgenic expression of YB-1 causes the development of breast carcinomas with various histological types[15] indicating that YB-1 is an oncogene. Nuclear activation of YB-1 is mediated by the essential cell growth signalling PI3 K/Akt pathway.[16–18] YB-1 knockdown by its cognate siRNA inhibited the cell proliferation of human breast cancer cells, prostate cancer cells and multiple myeloma cells in culture.[19–21] YB-1 knockdown also suppressed expression of various cell cycle- and DNA replication-related genes as well as growth factor genes.[17,22] YB-1 overexpression induces EGF/TGFα-independent cell growth and constitutive EGFR activation by human mammary cells in culture.[23] Activation of YB-1 was significantly associated with the expression of EGFR family proteins such as EGFR and HER2 in patients with breast cancer.[18,24] Taken together, these studies *in vitro* and *in vivo* as well as in cancer patients strongly suggest that YB-1 is closely involved in tumour growth and malignant progression of cancer. Recently, the possible role of YB-1 in epithelial-mesenchymal transition of breast epithelial cells in its close context with Snail gene has been reported.[25]

Furthermore, concerning the possible role of YB-1 in cell cycle and DNA replication, Jurchott and colleagues have reported that nuclear YB-1 expression is induced during G1-S transition of the cell cycle.[22] During S phase in cell cycle progression, replication of cellular DNA is initiated by formation of pre-replicative complexes composed of Orc1-6, Cdt1, CDC6 and minichromosome maintenance helicase at the replication origin.[26–28] Our previous study demonstrated that the expression of several cell cycle-related genes was specifically down-regulated by YB-1 knockdown in human cancer cells, and one representative gene that is suppressed is CDC6.[17] CDC6 plays a key role in loading the minichromosome maintenance complexes on the origin recognition complex bound at the replication origin.[29,30] CDC6 is a target for ubiquitin-mediated proteolysis by anaphase promoting complex in G1 phase.[31] Phosphorylation of the amino terminal domain of CDC6 by cyclin-dependent kinases (CDKs) protects CDC6 from the proteolysis for S phase entry.[26] These studies indicated that the essential role of CDC6 in the initiation of DNA replication. The high level of CDC6 was also associated with oncogenic activity in human cancer.[32,33]

In this study, we further examined whether CDC6 plays a role in the YB-1-promoted cell growth of human cancer cells, and how YB-1 controls expression of the CDC6 gene. The possible role of CDC6 in cell growth and cell cycle will be discussed in its close connection with YB-1 in human cancer cells.

2. Materials and methods

2.1. Cell lines and reagents

A549, EBC-1, MCF-7, T-47D, KPL-1 and MDA-MB231 were cultured in DMEM supplemented with 10% foetal bovine serum. PC-9 and QG56 were cultured in RPMI supplemented with 10% FBS v/v. Cell lines were maintained in a 5% CO_2 atmosphere at 37 °C. Anti-YB-1 was generated by immunization of a New Zealand white rabbit with synthetic peptides (C-terminal amino acids 299–313) as described previously.[4] The YB-1 antibody could detect both cytoplasmic and nuclear YB-1. Anti-CDC6 was obtained from Proteintech Group Inc. (Chicago, IL). Anti-cyclin A, cyclin B1, cyclin D1, cyclin E, CDK1, CDK2, CDK4, $p16^{INK4A}$ and $p21^{Cip1}$ antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from TREVIGEN Inc. (Gaithersburg, MD).

2.2. Quantitative real-time polymerase chain reaction (QRT-PCR)

Forty eight hours after small interfering RNA (siRNA) transfection, total RNA was isolated from cell culture using ISOGEN reagent (Nippon Gene Co. Ltd., Tokyo, Japan) according to the manufacture's instructions. RNA concentration was assessed by spectrophotometry at 260 nm. RNA was reverse-transcribed from random hexamers using AMV reverse transcriptase (Promega, Madison, WI). QRT-PCR was performed using the Real-Time PCR system 7300 (Applied Biosystems, Foster City, CA). In brief, the PCR amplification reaction mixtures (20 μl) contained cDNA, primer pairs, the dual-labelled fluorogenic probe and TaqMan Universal PCR Master Mix (Applied Biosystems). The primer pairs and probes were obtained from Applied Biosystems. The thermal cycle conditions included maintaining the reactions at 50 °C for 2 min and at 95 °C for 10 min, and then alternating for 40 cycles between 95 °C for 15 s and 60 °C for 1 min. The primer pairs and probes were obtained from Applied Biosystems. The relative gene expression for each sample was determined using the formula 2^(−delta Ct) = 2^(Ct(GAPDH) − Ct(target)), which reflected the target gene expression normalised to GAPDH levels.

2.3. Western blot analysis

Cells were rinsed with ice-cold PBS and lysed in a lysis buffer (pH 7.5) containing 50 mmol/l Tris–HCl, 0.1% NP-40 v/v, 350 mmol/l NaCl, 50 mmol/l NaF, 1 mmol/l Na_3VO_4, 5 mmol/l EDTA, 1 mmol/l PMSF and 10 μg/ml each of aprotinin and leupeptin. Lysates were subjected to SDS–PAGE and blotted onto Immobilon membrane (Millipore Corp., Billerica, MA). After transfer, the membrane was incubated with the primary antibody and visualised with secondary antibody coupled to horseradish peroxidase and Supersignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc.). Bands on Western blots were analysed densitometrically using Scion Image software (version 4.0.2; Scion Corp., Frederick, MD).

2.4. Transfection of small interfering RNA or CDC6 expression vector

SiRNA corresponding to nucleotide sequences of YB-1 (siYB-1; 5′-GGUUCCACCUUACUACAU-3′, siYB-1′; 5′-AGAAGGUCAU-CGCAACGAA-3′) and CDC6 (5′-UUUACACGAGGAGAACAG-GUUACGG-3′) were purchased from QIAGEN Inc. (Valencia, CA) and Invitrogen (Carlsbad, CA), respectively. SiRNA duplexes were transfected using Lipofectamine RNAiMAX and Opti-MEM

medium (Invitrogen) according to the manufacturer's recommendations. The expression vector for CDC6 (pCMV6/CDC6) was obtained from OriGene Technologies Inc. (Rockville, MD). Cells were transfected with the expression vector using Lipofectamine2000 and Opti-MEM medium (Invitrogen) according to the manufacturer's recommendations.

2.5. *Cell proliferation assay*

Cells (5×10^3) were seeded in 24-well plates and cell number in each well was counted by Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter Inc., Fullerton, CA) at 3 d (breast cancer cell lines) or 2, 4 and 6 d (lung cancer cell lines) after transfection of siRNA. Results are expressed as the mean ± SD of triplicate wells.

2.6. *Cell cycle analysis*

Cells were stained with propidium iodide (PI) using the Cycle Test Plus DNA Reagent kit (BD Biosciences, San Jose, CA) according to the manufacturer's recommendations. Cell distribution according to cell cycle phase was determined by measuring the DNA content using a BD FACSCalibur flow cytometer employing the Cell Quest Software. The percentage of cells in the G0/G1, S and G2/M phases was determined using Modifit LT software (Verity Software House Inc., Topsham, ME). Cells with hypodiploid DNA (content less than that of G0/G1-phase cells) were considered to be apoptotic (sub-G1).

2.7. *Chromatin immunoprecipitation (ChIP) assay*

ChIP assay was performed by using EZ ChIP kit (Millipore Corp.) according to the manufacturer's recommendations. Briefly, soluble chromatin from 1×10^6 cells was incubated with 1 μg of anti-YB-1 antibody. Purified DNA was dissolved in 50 μl of H_2O and 4 μl of DNA was used for PCR analysis (32 cycles) with the following primer pairs: CDC6#1, 5′-TCTACTGTAGTTCCCTCATTT-3′ (forward) and 5′-AGGGAAGACAGCTATATAGAA-3′ (reverse); CDC6#2, 5′-ATGATGCAAATGGGTACTTTA-3′ (forward) and 5′-AATCCGAATGGCCACAGCGTT-3′ (reverse). PCR products were then analysed on 2% agarose gels and stained with ethidium bromide.

2.8. *Electrophoretic mobility shift assay (EMSA)*

The sequences of the oligonucleotides used for the probes in EMSAs were as follows: human YB-1 oligo (WT oligo), 5′-GGTGAGGCTGATTGGCTGGGCAGGA-3′; human YB-1 mutant oligo (MT oligo), 5′-GGTGAGGCTGCTGCTCTGGGCAGGA-3′. Oligonucleotides were annealed with their complementary strands. The double-stranded products were end-labelled with α-^{32}P dCTP (GE Healthcare Bio-Science) using the Klenow fragment (Takara Bio, Shiga, Japan) and purified from gels. Then, 12 μg of nuclear extracts from A549 cells were incubated for 15 min at room temperature in a final volume of 20 μl containing 10 mmol/l Tris–HCl (pH 7.9), 20 mmol/l NaCl, 1 mmol/l EDTA, 10 mmol/l DTT, 0.1 mg/ml bovine serum albumin (BSA), 5% glycerol v/v, 0.05% NP40 v/v, 0.05 mg/ml poly (dI-dC) and ^{32}P-oligonucleotide probe. For competition assays, the appreciate competitor DNA fragments were added. The reaction mixtures were resolved by electrophoresis on a non-denaturing 4% polyacrylamide gel at 120 V for 120 min, at room temperature, in 0.5 × tris-borate-EDTA (TBE) buffer (45 mmol/l Tris base, 45 mmol/l boric acid and 1 mmol/l EDTA). Gels were dried and analysed using a bio-imaging analyser (BAS-2500; Fujifilm, Tokyo, Japan).

2.9. *Immunohistochemistry*

Tissue needle core biopsy sections were taken from 93 breast cancer patients in the Department of Breast Oncology, National Kyushu Cancer Center, Japan, between 2002 and 2007. Tissue specimens were fixed in buffered 10% formaldehyde and embedded in paraffin. The specimens were sliced in 4 μm sections and deparaffinised using xylene. The sections were then subjected to heat-induced epitope unmasking. The endogenous peroxidase activity was blocked by incubation at room temperature with 3% hydrogen peroxide v/v in methanol for 30 min. Non-specific antibody binding was inhibited by incubating the sections in non-specific blocking reagent for 30 min (Protein Block, Dako, Carpinteria, CA). The sections were then incubated with diluted rabbit polyclonal anti-CDC6 antibody (1:100) and rabbit polyclonal anti-YB-1 antibody (1:2000) at 4 °C overnight. After washing, the sections were incubated with labelled polymer HRP, anti-rabbit (Envision kit, Dako), at room temperature for 60 min, and then with 3,3′-diaminobenzidine, counterstained with hematoxylin, and mounted. Negative controls omitting the primary antibody were included. All IHC studies were evaluated by two experienced observers who were blind to the conditions of the patients. We evaluated the proportion and intensity of the immunoreactive cells of invasive breast cancer cells following the protocol used to evaluate oestrogen/progesterone receptors in breast cancer, proposed by Harvey and colleagues.[34] Cases with a total score of >2 and >6 were regarded as YB-1 and CDC6 nuclear positive, respectively.

2.10. *Statistical analysis*

Association between YB-1 and CDC6 was tested by Fisher's exact test. Statistical analysis was performed with SPSS regression Models 11.0J (SPSS Inc., Chicago, IL).

3. Results

3.1. *Inhibition of cell proliferation with decreased expression of CDC6 by knockdown of YB-1*

Gene expression profiles in a breast cancer cell line comparing YB-1 siRNA-treated and control siRNA-treated cells using a high density oligonucleotide microarray showed down-regulation of a DNA replication related gene, CDC6.[18] Consequently, we first examined the effect of YB-1 knockdown on cell proliferation of various breast and lung cancer cell lines. Treatment with YB-1 siRNA decreased the expression of YB-1 mRNA in all four breast cancer cell lines tested (Fig. 1A). Proliferation of all four breast cancer cell lines was markedly suppressed by YB-1 knockdown (Fig. 1B). Expression of

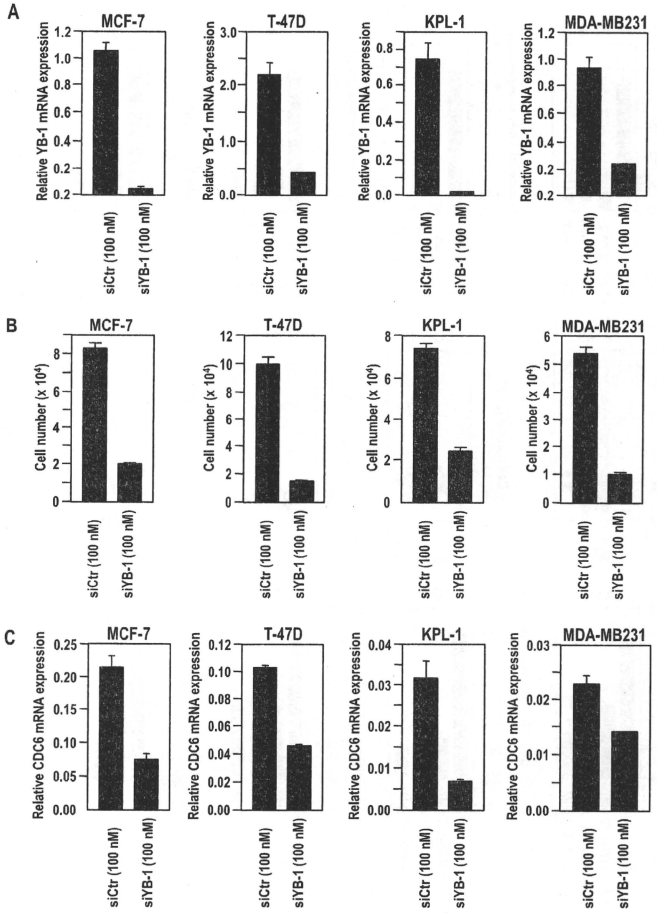

Fig. 1 – Effects of YB-1 siRNA on cell proliferation and CDC6 expression in breast and lung cancer cell lines. (A) Knockdown of YB-1 by treatment of YB-1 siRNA was determined by QRT-PCR in breast cancer cell lines. (B) Proliferation of breast cancer cell lines was suppressed by treatment of YB-1 siRNA. (C) Effect of YB-1 knockdown on expression of CDC6 in breast cancer cell lines. (D) Knockdown of YB-1 by treatment of YB-1 siRNA was determined by QRT-PCR in lung cancer cell lines. (E) Proliferation of lung cancer cell lines was suppressed by treatment of YB-1 siRNA. (F) Effect of YB-1 knockdown on expression of CDC6 in lung cancer cell lines. Data are expressed as the mean ± SD.

CDC6 mRNA was also decreased in YB-1 siRNA-treated breast cancer cells (Fig. 1C). We further examined whether cell proliferation and the expression of CDC6 mRNA were also affected by YB-1 knockdown in lung cancer cell lines. In four lung cancer cell lines, knockdown of YB-1 inhibited cell proliferation of all lung cancer cell lines (Fig. 1D and E). Exposure to YB-1 siRNA decreased expression of CDC6 mRNA to more than 50% of control siRNA in lung cancer cell lines (Fig. 1F).

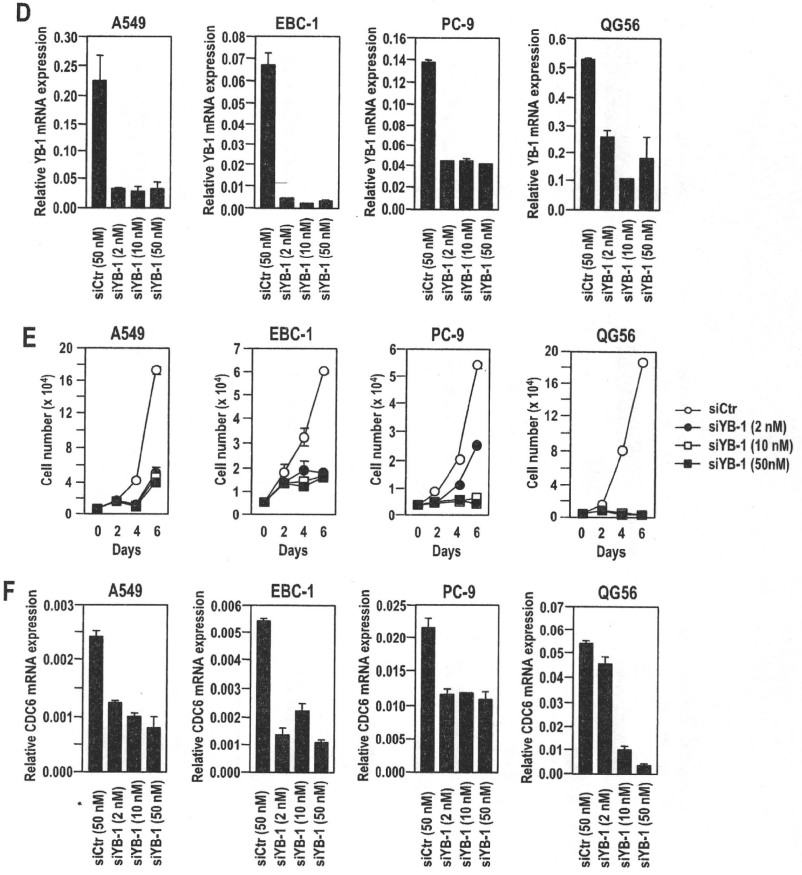

Fig 1. *(continued)*

3.2. *Cell cycle block at S phase and expression of cell cycle-related genes in siYB-1-treated cancer cells*

CDC6 is essential for the S phase entry in cell cycle.[26] Since YB-1 knockdown caused the reduction of CDC6 expression in cancer cells, we evaluated the effect of YB-1 siRNA on cell cycle by flowcytometric analysis. In breast cancer cell lines, YB-1 knockdown induced a marked decrease of S phase contents in all cancer cell lines tested (Fig. 2A). Decrease of the population of cells in S phase by YB-1 knockdown was also observed in all lung cell lines tested (Fig. 2B).

Next, we investigated the effects of YB-1 knockdown on expression profiles of genes related to the cell cycle. Treatment with YB-1 siRNA resulted in decrease of S phase contents in a dose-dependent manner (data not shown). Entry into S phase was strongly inhibited by 50 nmol/l of YB-1 siRNA to approximately 20% of control siRNA-treated cells. YB-1 knockdown by 50 nmol/l YB-1 siRNA decreased the expression of cyclin D1 and CDK2 which promote both G1 and S phase in A549 cells (Fig. 3A). Expression of $p21^{Cip1}$, a cell cycle inhibitor protein, was up-regulated in siYB-1-treatd cells. Expression of cyclin A, cyclin E, CDK1, CDK4 was not affected

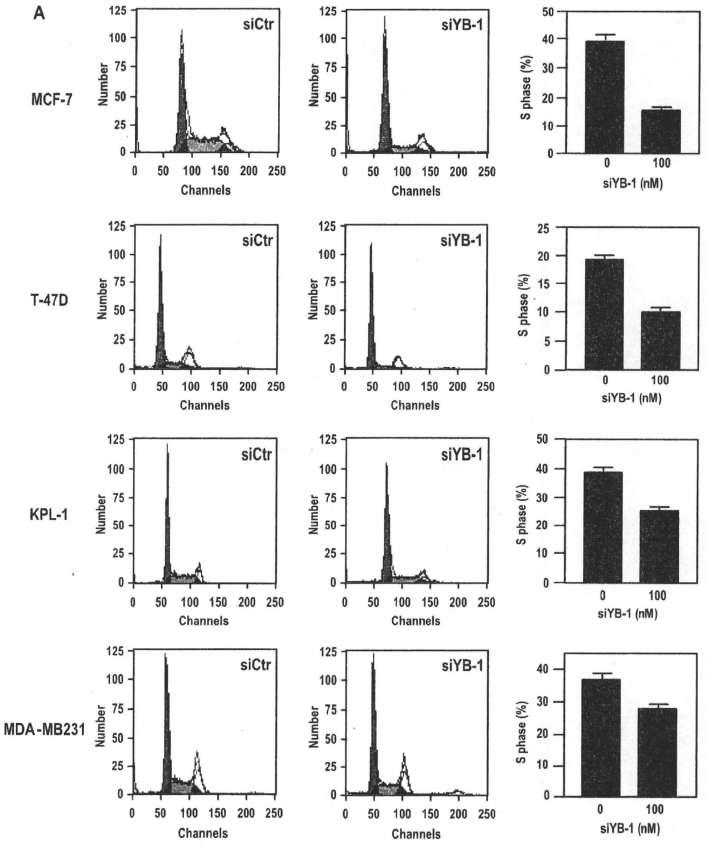

Fig. 2 – Decrease of S phase contents by YB-1 knockdown in breast (A) and lung (B) cancer cell lines. Cells were treated with siRNA for 48 h, and then detached from substratum by limited trypsin digestion and single cell suspension was used for propidium iodide staining. DNA-content in single cells was measured using the BD FACSCaliber flow cytometer. Data are expressed as the mean ± SD.

by YB-1 knockdown, while there was no apparent expression of $p16^{INK4A}$ or cyclin B1. Furthermore, expression of CDC6 was markedly decreased when exposed to YB-1 siRNA (Fig. 3A). We further examined the effects of YB-1 knockdown on expression profiles of genes related to the cell cycle in breast cancer cells. Expression of both $p21^{Cip1}$ and $p16^{INK4A}$ was up-regulated in siYB-1-treatd MCF-7 cells (Fig. 3B). Expression of CDK1 was decreased by YB-1 knockdown.

Knockdown of YB-1 decreased of CDC6 expression and suppressed S phase entry. We next examined whether both proliferation and S phase entry were inhibited by decreased expression of CDC6. Expression of CDC6 was decreased by treatment with CDC6 siRNA (Fig. 3C). Cell proliferation was susceptible to growth inhibition by CDC6 siRNA (Fig. 3D). Flowcytometric analysis showed decreased S phase contents when treated with CDC6 siRNA (Fig. 3E). We further examined

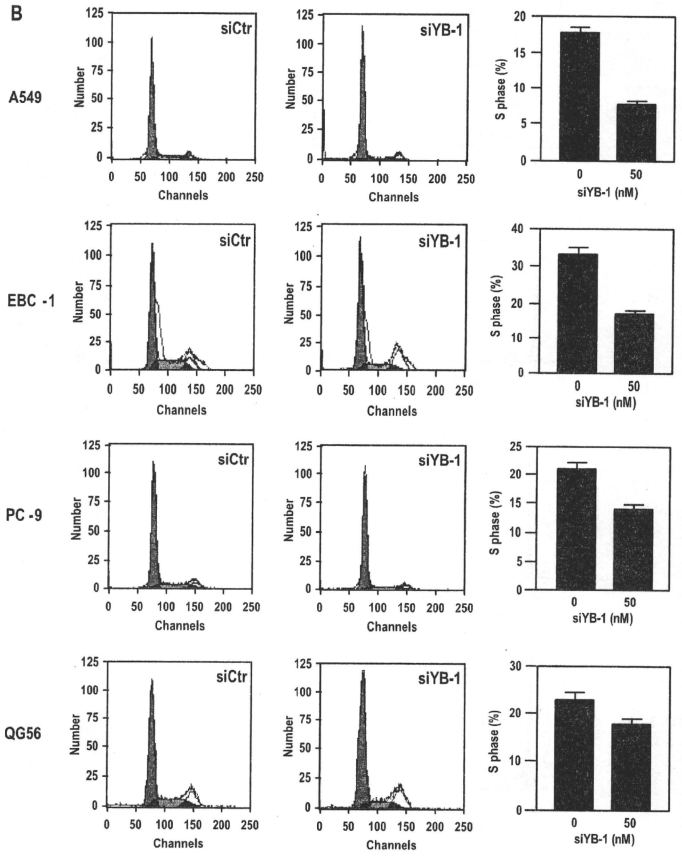

Fig 2. *(continued)*

the effects of different siRNAs for YB-1 (siYB-1 and siYB-1′) on regulation of CDC6 expression. Since expression of CDC6 was decreased by both siRNAs, we concluded that decreased expression of CDC6 was induced by target-specific silencing of YB-1 (Fig. 3F).

3.3. *Exogenous introduction of CDC6 gene abrogates YB-1 siRNA-induced inhibition of cell proliferation*

To determine whether CDC6 is directly involved in inhibition of both cell proliferation and S phase entry induced by YB-1 knockdown, we performed double transfection experiments of YB-1 siRNA and a CDC6 expression vector. Fig. 4A shows down-regulation of YB-1 expression by treatment with YB-1 siRNA, and up-regulation of CDC6 by transfection with the CDC6 expression vector. Inhibition of both cell proliferation and S phase entry by YB-1 knockdown was reversed by over-expression of CDC6 (Fig. 4B and C). We also confirmed if YB-1 interacts with 5′-flanking regulatory region of CDC6 gene which contains a Y-box (inverted CCAAT) element by ChIP assay. The ChIP assay confirmed that YB-1 was observed in the promoter region of CDC6 gene in vivo (Fig. 4D). To confirm whether YB-1 could bind to Y-box, nuclear extracts prepared from A549 cells was hybridized with labelled oligonucleotide probe containing Y-box sequence. Fig. 4E shows binding of YB-1 to Y-box element by electrophoretic mobility shift assay.

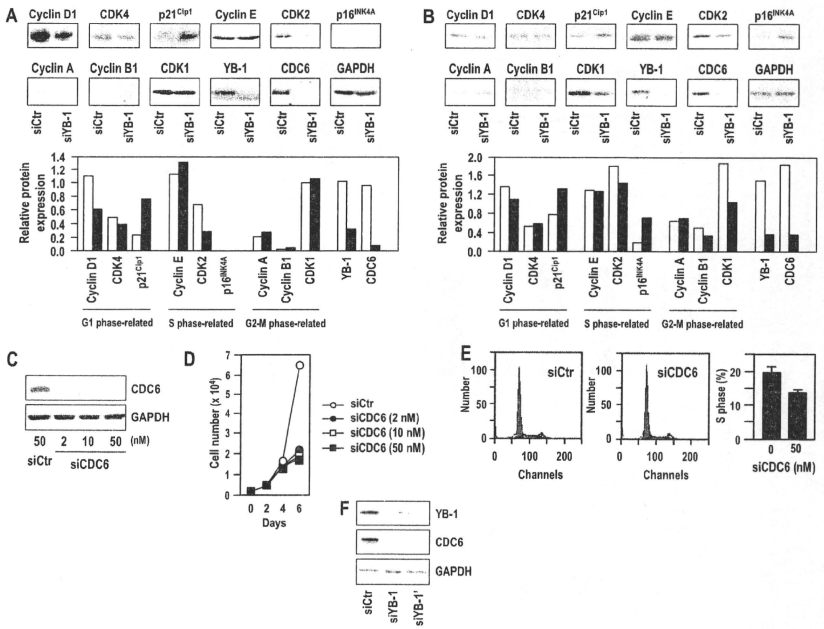

Fig. 3 – Effect of YB-1 knockdown on expression profiles of genes related to cell cycle and inhibition of both cell proliferation by CDC6 siRNA. (A) and (B) Effect of YB-1 knockdown on expression of cyclin D1, CDK4, $p21^{Cip1}$, cyclin E, CDK2, $p16^{INK4A}$, cyclin A, cyclin B1 and CDK1 was analysed by immunoblotting. Cells were incubated with 50 nmol/l siRNA for 48 h, and lysates were prepared. Levels of selected gene expression were measured densitometrically (A; A549 cells, B; MCF-7 cells). (C) Knockdown of CDC6 by treatment of CDC6 siRNA was determined by immunoblotting. Cells were incubated with 50 nmol/l siRNA for 48 h, and lysates were prepared. (D) Effect of CDC6 siRNA on proliferation of A549 cells. (E) Decrease of S phase contents by CDC6 knockdown in lung cancer cells. Data are expressed as the mean ± SD. (F) Effects of different siRNAs for YB-1 (siYB-1 and siYB-1') on CDC6 expression in A549 cells. Cells were incubated with 50 nmol/l siRNA for 48 h, and lysates were prepared.

3.4. *Immunostaining of YB-1 and CDC6 in human breast cancers*

To examine whether nuclear expression of YB-1 is associated with CDC6 expression in human breast cancers, immunohistochemical analysis was performed. Clinical and pathological characteristics at diagnosis are summarised in Table 1. Representative images of immunohistochemical staining showed a case with the presence of nuclear YB-1 and CDC6, and another case with the absence of nuclear YB-1 and CDC6 (Fig. 5). Nuclear expressions of YB-1 was detected in 41 of 93 patients (44%; nuclear YB-1 positive), and that of CDC6 was in 26 of 93 patients (28%; nuclear CDC6 positive), respectively. There was significant correlation between the expression of YB-1 and CDC6 in nucleus of cancer cells ($P = 0.012$, Table 2).

4. Discussion

In our present study, we observed a marked inhibition of cell proliferation of almost all cancer cell lines tested by knockdown of YB-1, consistent with previous studies of human prostate, breast and multiple myeloma cell lines.[19–21] YB-1 is thus a potent mitogenic biomarker of cell growth of cancer cells. Furthermore, we demonstrated specifically decreased population at S phase of cancer cells by knockdown of YB-1. Of various cell cycle-related genes, down-regulation of cyclin D1 and up-regulation of $p21^{Cip1}$ both of which are related to the G1 phase were observed by YB-1 knockdown. Chatterjee and colleagues have reported a relevant study that cyclin D1 is specifically down-regulated by YB-1 knockdown in human multiple myeloma cells, resulting in a marked decrease of viable cells, suggesting that YB-1 knockdown-induced cell

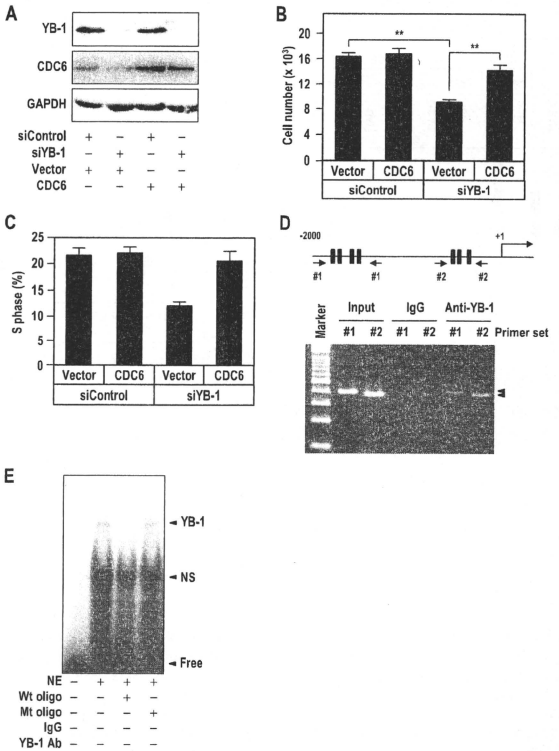

Fig. 4 – CDC6 cancelled YB-1 siRNA-induced suppression of both proliferation and S phase entry. (A) A549 cells were transfected with indicated siRNA (50 nmol/l) and plasmid (2 μg) for 48 h, and lysates were prepared. Expression of YB-1 and CDC6 was analysed by immunoblotting. (B) Effect of CDC6 over expression of YB-1 knockdown-induced inhibition of cell proliferation. (C) Effect of CDC6 over expression on S phase entry. Conditions of cell harvesting and staining as in Fig. 2. Data are expressed as the mean ± SD. (D) The *in vivo* YB-1 binding to the CDC6 promoter was analysed by ChIP assay. Schematic representation of the promoter region of CDC6 gene. Black boxes and arrows indicate CCAAT box and PCR primer regions, respectively. Arrowheads indicate PCR products (393 bp and 383 bp). (E) Nuclear extracts from A549 cells was hybridized with radioactive labelled Y-box oligonucleotide probes, 5′-TCGACTTCCCAAGAACAGCA-3′. The samples were separated by non-denaturing PAGE and the bands were detected by autoradiography.

growth arrest is due to decreased expression of cyclin D1.[21] On the other hand, overexpression of YB-1 facilitated expression of both cyclin A and cyclin B1 in breast cancer cells.[22] Our previous study demonstrated up-regulation of cyclin A and down-regulation of cyclin B1 in breast cancer cells treated with YB-1 siRNA,[18] and also up-regulation of cyclin B1 and B2 in ovarian cancer cells by YB-1 siRNA.[17] However, expression of both cyclin A and cyclin B1 was not affected by YB-1 knockdown in lung cancer cells (this study), suggesting that regulatory role of YB-1 in expression of cyclin A and/or cyclin B is dependent on cell types and/or cell lines.

Up-regulation of $p21^{Cip1}$, an inhibitor of cyclin D/CDK4 complexes, was also accompanied by down-regulation of cyclin D1 in YB-1 siRNA-treated cells. In our present study, however, there was no change in expression levels of CDK4 by YB-1 knockdown. Of CDK family proteins, expression of S phase-related gene, such as CDK2, was down-regulated by YB-1 siRNA. Cell cycle progression is controlled by an

Table 1 – Clinical and pathological characteristics of 93 patients with breast cancers.

Characteristic	N
Age (years)	18–73 (mean: 48.2)
Histological grade	
I, II	61
III	32
Menopausal status	
Pre	59
Post	34
Tumor size	
≤5	63
>5	30
Lymph node metastasis	
Absent	31
Present	62
Nuclear grade	
I, II	61
III	32

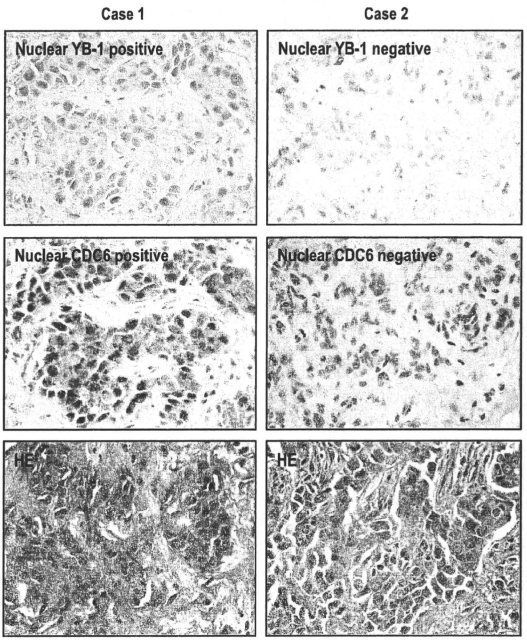

Fig. 5 – Immunohistochemical analysis of YB-1 and CDC6 in breast cancer patients. Two clinical samples of patients with YB-1 positive specimens (case1) and YB-1 negative specimens (case 2). Expression of both YB-1 and CDC6 was recognised in two patterns: nuclear positive or negative.

Table 2 – Correlation between nuclear YB-1 expression and CDC6 expression in human breast cancers.

YB-1	CDC6	
	Positive ($N = 26$)	Negative ($N = 67$)
Positive ($N = 41$)	17 (41.5%)	24 (58.5%)
Negative ($N = 52$)	9 (17.3%)	43 (82.7%)

Fisher's exact $P = 0.012$.

interplay of cyclins and CDKs, and activity of CDKs is positively and negatively regulated by cyclins and CDK inhibitors, respectively.[35] Cyclin D1 is induced in response to mitogenic signals and assembles with its catalytic partner, CDK4, as cell cycle progress through G1 phase.[36] Furthermore, it is well known that down-regulation of CDK4 activity and up-regulation of CDK inhibitors causes G1 phase arrest.[37,38] Activation of cyclin E/CDK2 and cyclin A/CDK2 was observed in G1-S transition phase. Thus, the alteration in expression profile of certain genes related to cell cycle-related by knockdown of YB-1 also indicated that YB-1 may play a key role in S phase entry during cell cycle progression.

Concerning the possible mechanism underlying YB-1 knockdown-induced inhibition of cell proliferation, we first observed marked inhibition of CDC6 expression by YB-1 knockdown in lung and breast cancer cells. In our previously report, there was no decrease expression of CDC6 in YB-1 siRNA-treated ovarian cancer cells assayed by DNA microarray, suggesting that YB-1-induced regulation of CDC6 gene expression depends upon cancer cell types and/or types of stimuli. CDC6, which is stabilized by phosphorylation of its NH_2-terminal domain, is known to recruit minichromosome maintenance helicase at the DNA replication origin,[26–28] and CDC6 knockdown inhibits cell proliferation with a concomitant decrease with cell population at S phase.[39] Decreased accumulation of cells at S phase as well as inhibition of cell proliferation was most prominent in one cancer cell line (A549) (see Fig. 3D and E) as compared with other cell lines when treated with CDC6 siRNA (data not shown). S phase entry could be more dependent upon CDC6 in this cell line than other cell lines examined, suggesting involvement of other factor(s) that may compensate for the decrease in CDC6. Exogenous introduction of CDC6 gene resulted in significant increases of viable cells and the fraction of the cell population at S phase when treated with YB-1 siRNA. Furthermore, we found that YB-1 was bound to its consensus Y-box sequence in the promoter region for CDC6 gene. We thus favour an idea that cell cycle G1/S and cell proliferation are at least, in part, due to CDC6 in the cancer cell lines examined.

Nuclear expression and/or activation of YB-1 is significantly correlated with poor prognosis of cancer patients with ovarian cancer,[7,11] soft tissue tumours such as synovial sarcoma and rhabdomyosarcoma,[6,8] lung cancer,[40,41] breast cancer,[5,18] paediatric glioblastoma[42] and multiple myeloma.[21] Although nuclear YB-1 expression is well known to be closely associated with ABCB1-mediated drug resistance in various human malignancies,[2,12] it remains unknown if ABCB1-mediated drug resistance is involved in the close correlation between YB-1 and poor prognosis. In this study, nuclear expression of YB-1 was significantly correlated with CDC6 expression in breast cancer. Further study should be required to understand whether CDC6 expression could play a role in YB-1-triggered poor prognosis in various human malignancies.

In conclusion, expression of a key protein of G1/S phase, CDC6, is under control by YB-1 in cancer cells, and we present evidence that expression of CDC6 plays an essential role in the cell proliferation and cell cycle G1/S induced by YB-1. The YB-1/CDC6 axis will provide novel molecular pathway for therapeutic treatment of human malignant tumours.

Conflict of interest statement

None declared.

Acknowledgments

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DNA Damage and Cellular Stress Responses

Molecular Cancer Research

Enhanced Expression of PCAF Endows Apoptosis Resistance in Cisplatin-Resistant Cells

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Abstract

Histone acetyltransferase (HAT) regulates transcription. We have previously shown that two HAT genes, *Clock* and *Tip60*, are overexpressed, and upregulate glutathione biosynthesis and the expression of DNA repair genes in cisplatin-resistant cells. To better understand the mechanism of HAT-related drug resistance, we investigated the role of another HAT gene, *p300/CBP-associated factor* (*PCAF*), and found that *PCAF* was also overexpressed in cisplatin-resistant cells and endowed an antiapoptotic phenotype through enhanced E2F1 expression. PCAF-overexpressing cells showed enhanced expression of E2F1 and conferred cell resistance to chemotherapeutic agents. Downregulation of PCAF decreased E2F1 expression and sensitized cells to chemotherapeutic agents. Moreover, knockdown of PCAF induced G_1 arrest and apoptosis. These results suggest that PCAF is one of pleiotropic factors for drug resistance and seems to be critical for cancer cell growth. *Mol Cancer Res; 8(6); 864–72. ©2010 AACR.*

Introduction

Drug and apoptosis resistance are two sides of the same coin. The treatment of cancer cells with chemotherapeutic agents might activate signal transduction pathways and modulate the expression of genes that are involved in antiapoptotic or apoptotic functions. However, the molecular mechanisms of apoptosis resistance have not been studied in cisplatin-resistant cells. We have extensively studied mechanisms involved in cisplatin resistance (1, 2). Several mechanisms are involved in the acquisition of cisplatin resistance (3), which include reduced drug accumulation (4, 5), increased production of cellular thiol (6, 7) and augmented DNA repair activity (8, 9). We have recently reported that two histone acetyltransferases (HAT), Clock and Tip60, are overexpressed in cisplatin-resistant cells (10, 11). Clock regulates glutathione biosynthesis by activating ATF4 and induces multidrug resistance (10). Tip60 is a Clock target gene and modulates the expression of DNA repair genes (11). Thus, these HATs are directly involved in drug resistance. To better understand the mechanism of HAT-dependent drug resistance, we focused on another HAT gene, *p300/CBP-associated factor* (*PCAF*).

PCAF is a transcription cofactor with intrinsic HAT activity (12). In addition to acetylating histones, PCAF can interact with and acetylate many other proteins involved in transcription. It has been reported that PCAF has a dual function in cell viability. PCAF induced cell cycle arrest and/or apoptosis by regulating the function of tumor suppressor p53 (13) and apoptosis mediators p73 (14) and Bax (15). Interestingly, a transcriptional factor, E2F1, is acetylated and stabilized by PCAF in response to DNA damage (16). Therefore, we investigated the expression of E2F1 as an important mediator of PCAF-regulating cell viability or apoptosis resistance and observed the overexpression of E2F1 in cisplatin-resistant cells. To our knowledge, this is the first report of the involvement of PCAF/E2F1 pathway in drug resistance.

Materials and Methods

Cell culture

Human prostate cancer PC3, epidermoid cancer HeLa, and bladder cancer T24 cells were cultured in Eagle's MEM. Human lung cancer A549 cells were cultured in RPMI 1640. Media were purchased from Nissui Seiyaku and contained 10% fetal bovine serum. The cisplatin-resistant P/CDP6 and HeLa/CP4 cells were derived from PC3 and HeLa cells, as previously described (17), and were 63- and 23-fold, respectively, more resistant to cisplatin than their parental cells (18). All cell lines were maintained in a 5% CO_2 atmosphere at 37°C.

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Antibodies and drugs

Antibodies against PCAF, E2F1, Survivin, and HAT1 were purchased from Santa Cruz Biotechnology. Antibodies against caspase-3, cleaved caspase-3, caspase-7, cleaved caspase-7, and cleaved poly ADP ribose polymerase were purchased from Cell Signaling Technology. Antibodies against SirT1 were purchased from Epitomics. Anti-p73α antibody was purchased from Wako Pure Chemical Industries, Ltd. Anti–β-actin antibody was purchased from Sigma. Cisplatin and 5-fluorouracil were purchased from Sigma. Adriamycin was a kind gift from Kyowa Hakko Kogyo Co., Ltd. Oxaliplatin and 7-ethyl- 10-hydroxycamptothecin (SN-38) were kind gifts from Yakult Co. Ltd. H_2O_2 was purchased from Nacalai Tesque.

Plasmid construction

To obtain the full-length complementary DNA (cDNA) for human PCAF, PCR was carried out on a SuperScript cDNA library (Invitrogen) using the following primer pairs (single underlining indicates the start codon and stop codon): ATGTCCGAGGCTGGCGGGGC and TCACTTGTCAATTAATCCAGCTTCC for PCAF. This PCR product was cloned into the pGEM-T easy vector (Promega). To construct a plasmid expressing hemagglutinin (HA)-tagged PCAF, NH_2-terminal HA-tagged PCAF cDNA was ligated into a pcDNA3.1 vector (Invitrogen).

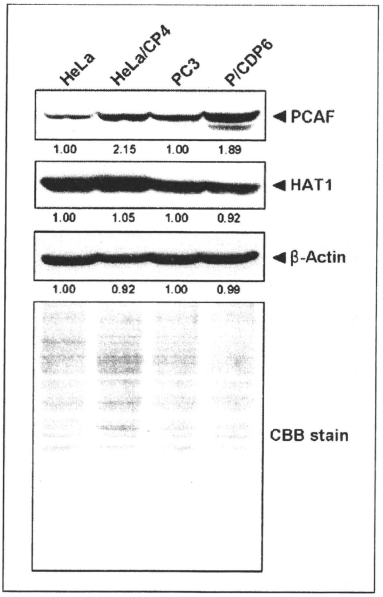

FIGURE 1. Cellular expression of PCAF in cisplatin-resistant cell lines. Whole-cell lysates (100 μg) of cisplatin-resistant cell lines (HeLa/CP4 or P/CDP6) or parent cell lines (HeLa or PC3) were subjected to SDS-PAGE, and Western blotting was done using the indicated antibodies. Immunoblotting of β-actin is shown as a loading control. The relative intensity is shown under each blot. Gel staining with Coomassie Brilliant Blue (CBB) is also shown.

Cloning of stable transfectants

The cloning of stable transfectant was done as previously described (19). Briefly, PC3 cells were transfected with pcDNA3.1-HA PCAF with Superfect reagent and cultured with 500 μg/mL hygromycin for 15 to 20 days. The resulting colonies were isolated, and the cellular expression level of HA-PCAF in each clone was investigated by Western blotting with the anti-HA antibody.

Western blotting

Whole-cell lysates were prepared as previously described (10, 20). The indicated amounts of whole-cell lysates or nuclear extract were separated by SDS-PAGE and transferred to polyvinylidene difluoride microporous membranes (Millipore) using a semidry blotter. The blotted membranes were treated with 5% (w/v) skimmed milk in 10 mmol/L Tris, 150 mmol/L NaCl, and 0.2% (v/v) Tween 20, and incubated for 1 hour at room temperature with the primary antibody. The antibodies and dilutions used were as follows: 1:1,000 dilution of anti-PCAF, 1:5,000 dilution of anti-HAT1, 1:1,000 dilution of anti–caspase-3, 1:1,000 dilution of anti–cleaved caspase-3, 1:1,000 dilution of anti–caspase-7, 1:1,000 dilution of anti–cleaved caspase-7, 1:1,000 dilution of anti–cleaved poly ADP ribose polymerase, 1:1,000 dilution of anti-E2F1, 1:1,000 dilution of anti-Survivin, 1:5,000 dilution of anti-SirT1, 1:1,000 dilution of anti-p73α, 1:5,000 dilution of anti-HA, and 1:5,000 dilution of anti–β-actin. The membranes were then incubated for 45 minutes at room temperature with a peroxidase-conjugated secondary antibody, visualized using an enhanced chemiluminescence kit (GE Healthcare Bio-Science), and the images were obtained by image analyzer (LAS-4000 mini, Fujifilm, Tokyo, Japan).

Knockdown analysis using small interfering RNAs

The following double-stranded RNA 25-bp oligonucleotides were commercially generated as follows (Invitrogen): 5′-UUUCCAAAGAGCCUUCAACCACAGG-3′ (sense) and 5′-CCUGUGGUUGAAGGCUCUUUGGAAA-3′ (antisense); PCAF small interfering RNA (siRNA) #1, 5′-UUUAGCUCACAUCCCAUUAAAGUGG-3′ (sense) and 5′-CCACUUUAAUGGGAUGUGAGCUAAA-3′ (antisense); PCAF siRNA #2, 5′-AUAUCCUGGAGCUUCUGUUCUCUUC-3′ (sense) and 5′-GAAGAGAACAGAAGCUCCAGGAUAU-3′ (antisense); PCAF siRNA #3, 5′-AAAGUUCUCCGAAGAGUCCACGGCU-3′ (sense) and 5′-AGCCGUGGACUCUUCGGAGAACUUU-3′ (antisense); E2F1 siRNA. Transfection of siRNA was done as

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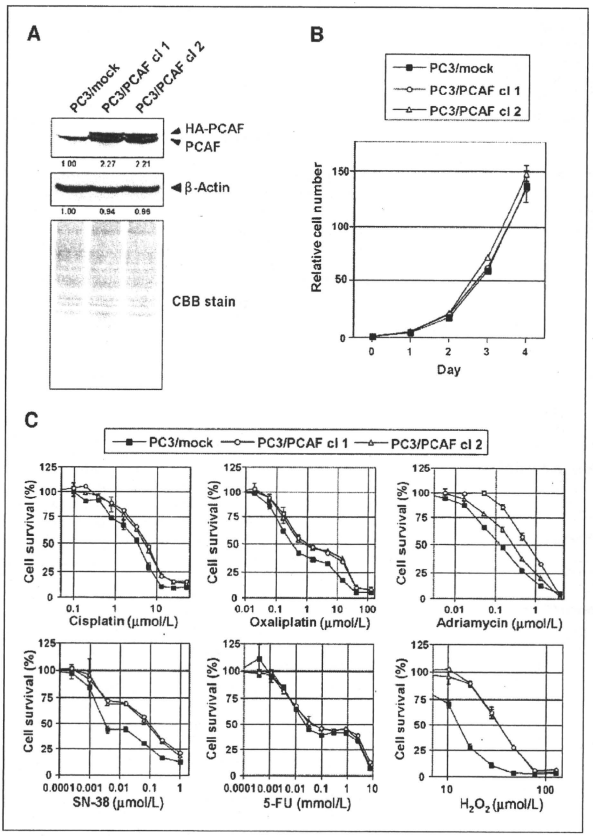

FIGURE 2. PCAF overexpression induces multidrug resistance. A, whole-cell lysates (100 μg) of PCAF-overexpressing cell lines (PC3/PCAF cl 1 and cl 2) or a control cell line (PC3/mock) were subjected to SDS-PAGE, and Western blotting was done using the indicated antibodies. Immunoblotting of β-actin is shown as a loading control. The relative intensity is shown under each blot. Gel staining with CBB is also shown. B, PC3, PC3/PCAF cl 1, or cl 2 cells (2.0×10^4 cells) were seeded onto 12-well plates and were counted daily. The cell number on day 0 corresponded to 1. Points, mean of at least three independent experiments; bars, SD. C, 1.0×10^3 PCAF-overexpressing cell lines or a control cell line were seeded onto 96-well plates. The next day, the indicated concentrations of the drugs were applied. After 48 h, cell survival was analyzed with a WST-8 assay. Cell survival in the absence of drugs corresponded to 100%. Points, mean of at least three independent experiments; bars, SD.

previously described (10, 20). Briefly, 250 pmol of siRNA and 5 μL of lipofectamine mixture were combined with 3.0×10^5 PC3 cells in 500 μL of culture medium and incubated for 20 minutes at room temperature. All cells were seeded into 35-mm dishes containing 2 mL culture medium and were harvested after culture for 72 hours for Western blotting, as described above. PC3 cells (1.0×10^3) were used in the WST-8 assay; 1.0 to 4.0×10^2 PC3 cells were used in colony formation assay; and 2.0×10^4 PC3, A549, or T24 cells were used in the cell proliferation assay as described below.

Cytotoxicity analysis

For the water-soluble tetrazolium salt (WST-8) assay, 1.0×10^3 PC3/mock, PC3/PCAF cl 1, cl 2, or PC3 cells transfected with siRNA, as described above were seeded onto 96-well plates. The next day, the indicated concentrations of the drugs were applied. After 48 hours, the surviving cells were stained with TetraColor ONE (Seikagaku Corp.) for 1 to 2 hours at 37°C. The absorbance was then measured at 450 nm. For the colony formation assay, 1.0×10^2 PC3 cells transfected with control siRNA, 2.0×10^2 PC3 cells transfected with PCAF siRNA #1, or 4.0×10^2 PC3 cells

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transfected with PCAF siRNA #2, as described above were seeded onto 35-mm dishes with 2 mL of culture medium. The next day, the indicated concentrations of the drugs were applied. After 7 days, the number of colonies was counted.

Cell proliferation assay

PC3, A549, or T24 cells (2.0×10^4 cells) transfected with siRNA, as described above, were seeded onto 12-well plates. The cells were harvested with trypsin and counted daily with a Coulter-type cell size analyzer (CDA-500, Sysmex). PC3 cells (2.0×10^4 cells) transfected with the dilution series of siRNA, as described above, were counted on the second day.

Flow cytometry

PC3 cells (3.0×10^5) transfected with siRNA, as described above, were seeded onto six-well plates and cultured for 48 hours. The cells were harvested, washed twice with ice-cold PBS supplemented with 0.1% bovine serum albumin, and fixed in 70% ethanol. After washing twice with ice-cold PBS, the cells were resuspended in PBS with 0.1% bovine serum albumin, incubated with RNase (Sigma), and stained with propidium iodide (Sigma). The cells were analyzed using an EpicsXL-MCL flow cytometer (Beckman Coulter).

Statistical analysis

The protein expression levels were assessed numerically using a Multi Gauge Version 3.0 (Fujifilm, Tokyo, Japan). Pearson's correlation was used for statistical analysis, and significance was set at the 5% level.

Results

Enhanced expression of PCAF in cisplatin-resistant cells

We have previously shown that two HATs, Clock and Tip60, are overexpressed in cisplatin-resistant cells and are involved in drug resistance (10, 11). HATs are categorized into two families, MYST (MOZ, YBF2/SAS3, SAS2, and Tip60) and GNAT (Gcn5-related N-acetyltransferases). Clock and Tip60 are both members of the MYST family. Therefore, we investigated the expression of the GNAT family in cisplatin-resistant cells. As shown in Fig. 1, cellular PCAF, but not HAT1, was overexpressed

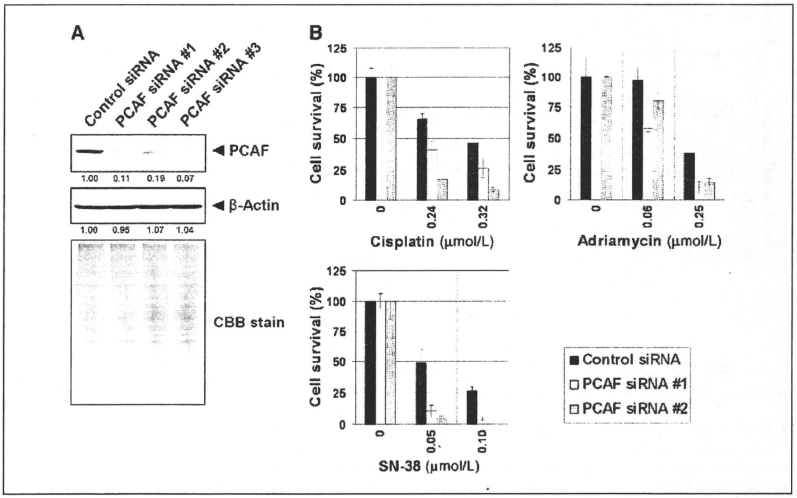

FIGURE 3. Downregulation of PCAF sensitized cells to chemotherapeutic agents. A, PC3 cells were transiently transfected with 250 pmol of control siRNA, or PCAF siRNA #1, #2, or #3. After 48 h, whole-cell lysates (100 μg) were subjected to SDS-PAGE, and Western blotting was done using the indicated antibodies. Immunoblotting of β-actin is shown as a loading control. The relative intensity is shown under each blot. Gel staining with CBB is also shown. B, PC3 cells transfected with control siRNA, or PCAF siRNA #1 or #2 were seeded onto 35-mm dishes. The next day, the indicated concentrations of the drugs were applied. After 7 d, the number of colonies was counted. Cell survival in the absence of drugs corresponded to 100%. Columns, mean of at least three independent experiments; bars, SD.

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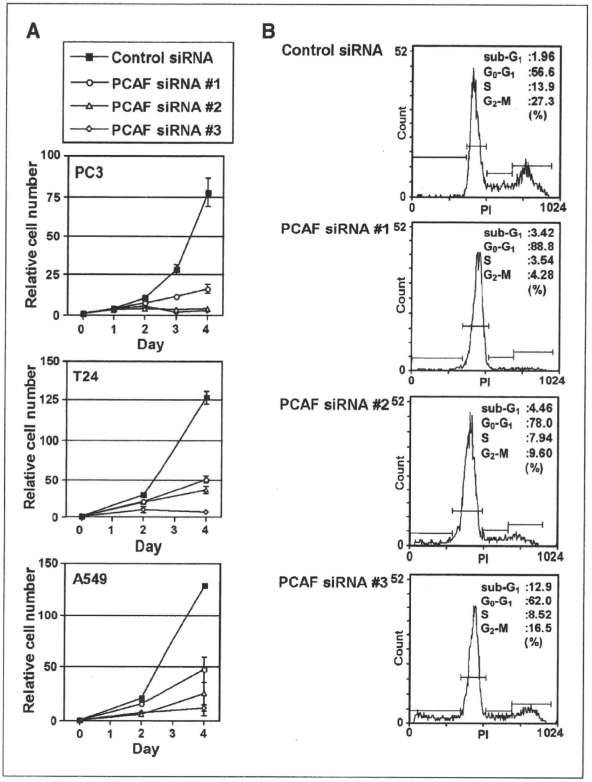

FIGURE 4. PCAF expression is indispensable for cancer growth. A, PC3, A549, or T24 cells, transfected with 250 pmol of control siRNA, or PCAF siRNA #1, #2, or #3 were seeded onto 24-well plates and counted daily or every 2 d. The cell number on day 0 corresponded to 1. Points, means of at least three independent experiments; bars, SD. B, PC3 cells were transfected as in A. After 48 h, the cells were stained with propidium iodide and analyzed by fluorescence-activated cell sorting. The cell cycle fraction is shown at the top right of each graph.

in two independent cisplatin-resistant cells, HeLa/CP4 and P/CDP6, in comparison with their parental cells.

PCAF-overexpressing cells showed multidrug resistance

To investigate whether PCAF expression is involved in drug resistance, we first established stable transfectants to overexpress PCAF in human prostate cancer PC3 cells. Two stable transfectants, PC3/PCAF cl 1 and cl 2, showed ~2.2-fold higher expression of PCAF than that of the vector-alone transfectant, PC3/mock (Fig. 2A). The growth rates of these cells were almost similar to PC3/mock (Fig. 2B). The PCAF-overexpressing cell lines showed increased resistance to cisplatin, oxaliplatin, Adriamycin, SN-38, and hydrogen peroxide (H_2O_2), but not to 5-fluorouracil (Fig. 2C). These results indicate that PCAF-overexpressing cell lines showed multidrug resistance or apoptosis resistance.

Downregulation of PCAF-sensitized cells to chemotherapeutic agents

To access whether PCAF is involved in the apoptosis-resistant phenotype, PCAF-knockdown cells were treated with anticancer agents and analyzed for cell death by the WST-8 assay. Unexpectedly, knockdown of PCAF expression did not decrease the survival rate (data not shown). One possible explanation is that knockdown of PCAF

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expression take a time to change cellular sensitivity to drugs. Then, colony formation assay was done. As shown in Fig. 3, downregulation of PCAF decreased survival rates of colonies against cisplatin, Adriamycin, and SN-38.

Induction of G_1 arrest and apoptosis by PCAF downregulation

Next, we analyzed the effect of PCAF downregulation on cell growth using p53-null PC3 cells, lung cancer A549 cells with wild-type p53, and bladder cancer T24 cells with mutant p53. As shown in Fig. 4A, knockdown of PCAF expression significantly induced cell growth retardation in these cell lines. Knockdown of PCAF expression also induced growth retardation of P/CDP6, HeLa, and HeLa/CP4 cells (data not shown). Furthermore, the cell cycle profile showed that knockdown of PCAF expression in PC3 cells induced either G_1 arrest or apoptosis (Fig. 4B). Both siRNA #1 and #2 mainly induced G_1 arrest, and siRNA #3 induced apoptosis. To confirm these results, we examined whether reducing PCAF expression can induce the activation or execution of apoptosis using siRNA #3. Knockdown of PCAF expression significantly activated and cleaved caspase-3 and caspase-7, and poly ADP ribose polymerase in PC3 cells (Fig. 5). Similar results were observed when HeLa cells were used (data not shown).

PCAF regulates E2F1 expression

It has been shown that PCAF is required for the stabilization and accumulation of E2F1 and is involved in E2F1-dependent apoptosis in response to DNA damage (16). Therefore, we analyzed E2F1 expression in cisplatin-resistant cells and PCAF-overexpressing cells. E2F1 expression was significantly upregulated in cisplatin-resistant cells (Fig. 6A). Moreover, stable transfectants overexpressing PCAF showed a 12-fold higher expression of E2F1 protein compared with control cells (Fig. 6B). Knockdown of PCAF expression also reduced the level of E2F1 protein (Figs. 5B and 6C). Similarly, an antiapoptotic gene, *Survivin*, was upregulated in cisplatin-resistant cells and PCAF-overexpressing cells, but a proapoptotic gene, *p73*, was not (Fig. 6A and B). The deacetylase SirT1, binds to E2F1 and inhibits the PCAF-E2F1-p73 apoptotic pathway (21, 22), and was upregulated in PCAF-overexpressing cells (Fig. 6B). To confirm our hypothesis that PCAF regulates antiapoptotic function in cancer via E2F1, we investigated whether E2F1 certainly involved in drug sensitivity. Downregulation of E2F1 sensitized cells to cisplatin, Adriamycin, and SN-38 (Fig. 7).

Discussion

In this study, we provide insight into the function of PCAF in drug resistance. Here, we found that PCAF expression was upregulated in cisplatin-resistant cells (Fig. 1). Interestingly, PCAF-overexpressing cells showed resistance not only to cisplatin but also to other chemotherapeutic agents, suggesting that PCAF overexpression is not specific to cisplatin-resistant cells (Fig. 2C). Furthermore, downregulation of PCAF sensitized cancer cells to chemotherapeutic agents (Fig. 3). To rule out the multidrug resistance phenotype, we investigated the expression of several ABC transporters. However, we did not observe enhanced expression of the ABC transporters in PCAF-overexpressing cells (data not shown). To understand the molecular mechanism of PCAF-dependent apoptosis resistance, we investigated genes related to tumor cell survival in cisplatin-resistant cells and PCAF-overexpressing cells. E2F1 has an antiapoptotic function (23-25) and is acetylated and stabilized by PCAF (16). As expected, E2F1 expression was upregulated in cisplatin-resistant cells and

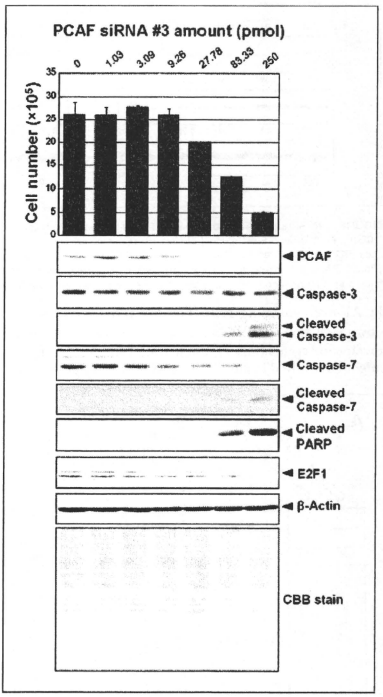

FIGURE 5. Downregulation of PCAF induced apoptosis. PC3 cells were transiently transfected with the dilution series of PCAF siRNA #3. After 48 h, the cells were counted; whole-cell lysates (100 μg) were subjected to SDS-PAGE; and Western blotting was done using the indicated antibodies. Immunoblotting of β-actin is shown as a loading control. The relative intensity is shown under each blot. Gel staining with CBB is also shown.

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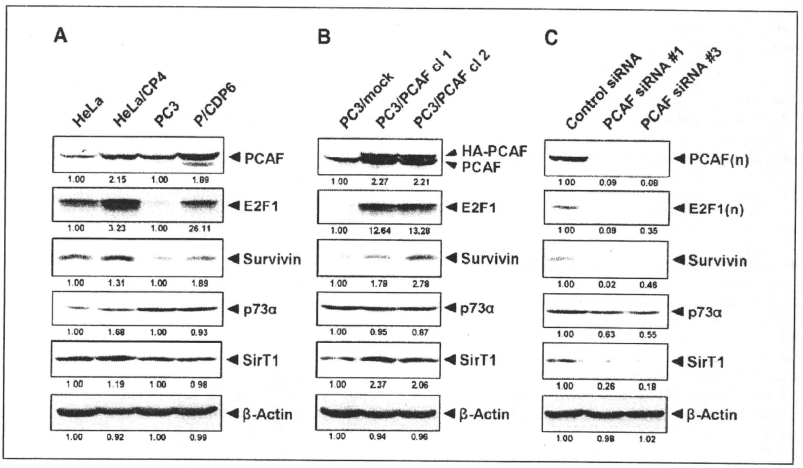

FIGURE 6. PCAF expression induced antiapoptosis genes by regulating E2F1 expression. A to C, whole-cell lysates (100 μg) and nuclear extracts [100 μg, PCAF(n) and E2F1(n) in C] of cisplatin-resistant cell lines (HeLa/CP4 or P/CDP6), parent cell lines (HeLa or PC3), PCAF-overexpressing cell lines (PC3/PCAF cl 1 and cl 2), a control cell line (PC3/mock) or PC3 cells transfected with control siRNA, or PCAF siRNA #1 or #3 as described in (Figs. 1, 2A, and 4A)respectively were subjected to SDS-PAGE, and Western blotting was done using the indicated antibodies. Immunoblotting of β-actin is shown as a loading control. The relative intensity is shown under each blot.

PCAF-overexpressing cells (Fig. 6A and B). Moreover, downregulation of E2F1 sensitized cancer cells to chemotherapeutic agents (Fig. 7). These results suggest that E2F1 might be involved in antiapoptosis. Survivin is a prosurvival gene inhibiting apoptosis, and it has been shown that E2F1 bound to the Survivin promoter and induced its transcription in rat embryonic fibroblasts (26). The expression of Survivin was upregulated in cisplatin-resistant cells

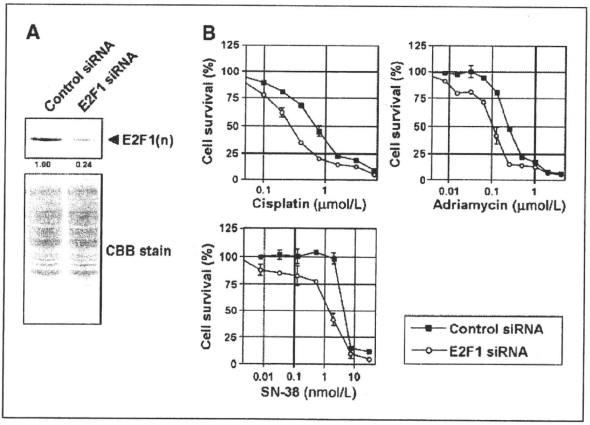

FIGURE 7. Downregulation of E2F1 sensitized cells to chemotherapeutic agents. A, PC3 cells were transiently transfected with 250 pmol of control siRNA or E2F1 siRNA. After 48 h, nuclear extracts (100 μg) were subjected to SDS-PAGE, and Western blotting was done using the indicated antibodies. The relative intensity is shown under each blot. Gel staining with CBB is also shown. B, 1.0×10^3 PC3 cells, transfected as in A, were seeded onto 96-well plates. The next day, the indicated concentrations of the drugs were applied. After 48 h, cell survival was analyzed with a WST-8 assay. Cell survival in the absence of drugs corresponded to 100%. Points, mean of at least three independent experiments; bars, SD.

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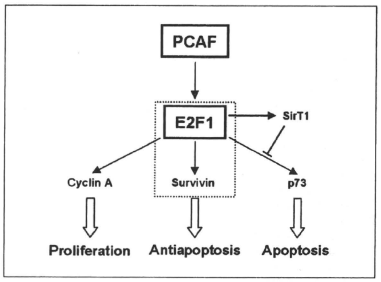

FIGURE 8. PCAF/E2F1 pathways enable cancer cells to acquire apoptosis resistance. PCAF stabilizes E2F1. E2F1 may contribute to apoptosis resistance by transcriptional induction of target genes such as cyclin A, Survivin and SirT1.

and PCAF-overexpressing cells (Fig. 6A and B). These findings indicate that PCAF might induce Survivin expression through E2F1 and induce an antiapoptotic phenotype.

Downregulation of PCAF induced cell growth retardation due to cell cycle arrest and apoptosis (Figs. 4 and 5), indicating that PCAF is a potential target for cancer treatment. These results are consistent with the reports that knockdown of PCAF inhibited tumorigenesis and tumor progression in nude mice (27), and inhibitors of PCAF HAT activity decreased cell proliferation (28, 29). In addition, E2F1 expression was decreased by the downregulation of PCAF (Fig. 6C). Because PCAF can stabilize the E2F1 protein (16), downregulation of PCAF might lead to the destabilization of the E2F1 protein. E2F1 has a well-known function in promoting cell cycle progression by regulating pRB (30, 31). Thus, downregulation of E2F1 might induce cell cycle arrest. Furthermore, it is consistent with the function that cyclin A, which promotes the cell cycle G_1-S and G_2-M transitions and is regulated by E2F1 (31), was decreased in addition to decreased E2F1 expression (data not shown). Although downregulation of PCAF also induced apoptosis, we could not determine whether cell cycle arrest resulted in apoptosis or not. Because both PCAF and E2F1 have proapoptotic functions, they should not be involved in this process directly.

Downregulation of PCAF induced cell growth retardation in p53-null, p53-mutated, and wild-type p53 cells (Fig. 4A), suggesting that PCAF reduced cell viability in a p53-independent manner. Although it has been shown that PCAF induces apoptosis in a p53-dependent/independent manner, the growth rate of PCAF-overexpressing cells is broadly similar to that of control cells (Fig. 2B). Because PCAF-overexpressing cells were established from p53-null PC3 cells, apoptosis might not be induced. Alternatively, Survivin or other genes might negatively regulate apoptosis.

The growth of cisplatin-resistant cells is very slow but viable, although PCAF expression is upregulated. P/CDP6 cells are p53-null cells, but HeLa/CP4 cells are mutant-type p53 cells. This suggests that, in cisplatin-resistant cells and PCAF-overexpressing cells, the PCAF-inducing apoptosis system may be abolished, regardless of p53 expression, or suppressed by an antiapoptotic gene such as Survivin. It has been shown that E2F1 can activate the expression of proapoptotic genes (30, 32-36). However, we did not observe increased expression of the proapoptotic gene *p73*, which is an E2F1 target gene, in either cisplatin-resistant cells or PCAF-overexpressing cells (Fig. 6A and B). It has been reported that SirT1 represses E2F1-dependent p73 promoter activity and apoptosis (21, 22). SirT1 expression was upregulated in PCAF-overexpressing cells (Fig. 6B), suggesting that SirT1 might negatively regulate the E2F1-dependent p73-apoptotic pathway. In cisplatin-resistant cells, SirT1 expression was not increased (Fig. 6A), indicating that E2F1-dependent apoptosis system might be out of control of SirT1 and regulated by other anti-apoptotic genes such as Survivin. These results suggest that PCAF/E2F1 pathways enable cancer cells to acquire apoptosis resistance as shown in Fig. 8.

The functional coordination of HATs and histone deacetylase (HDAC) is important for gene expression, DNA repair, cell cycle, and apoptosis. HDAC inhibitors can induce cell cycle arrest, differentiation, and apoptosis. Inhibitors of HDAC are a promising target for cancer chemotherapy. Because PCAF downregulation induces cell cycle arrest and apoptosis, we believe that HAT inhibitors are also promising agents for cancer chemotherapy. PCAF downregulation and HDAC inhibitors can both induce apoptosis in cancer cells, indicating that inducing an imbalance in cellular acetylation of proteins might be critical for apoptosis induction.

Collectively, this study is the first report of PCAF-dependent antiapoptosis in relation to drug resistance. Furthermore, PCAF seems to be an indispensable component of cancer cell survival. PCAF is one of pleiotropic factors for drug resistance and might be a potential target of cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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