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Ras-Dependent Recruitment of c-Myc for Transcriptional Activation of Nucleophosmin/B23 in Highly Malignant U1 Bladder Cancer Cells

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ABSTRACT

U1 bladder cancer cells of high malignancy exhibited higher proliferation capacity than U4 premalignant cells. Higher expression of Ras, c-Myc, and nucleophosmin/B23 and greater c-Myc transactivation and nucleophosmin/B23 promoter activities were detected in U1 cells compared with U4 cells. Moreover, c-Myc and nucleophosmin/B23 were increased in U1 but not in U4 cells upon serum stimulation from quiescence. Likewise, only in U1 cells could serum stimulate transcriptional activity of nucleophosmin/B23 promoter and c-Myc response element. The increase of nucleophosmin/B23 promoter activity could be abrogated by mitogen-activated protein kinase/extracellular signal-regulated kinase activating kinase inhibitor and was associated with recruitment of c-Myc to the promoter. U1 cells constitutively expressing dominant-negative Ras reduced the levels of Ras, nucleophosmin/B23, and p-ERK, and consequently abolished the serum-induced up-regulation of nucleophosmin/B23 promoter activity and c-Myc promoter recruitment. Our results indicate that Ras and c-Myc play important roles in the up-regulation of nucleophosmin/B23 during proliferation of cells associated with a high degree of malignancy, thus outlining a signaling cascade involving these factors in the cancer cells.

Transition cell carcinoma of the bladder is the second-most common malignancy of the genitourinary tract and the second-most common cause of death from genitourinary tumors (Raghaven et al., 1990; Konetry and Getzenberg, 2001). Depending on the depth of muscle invasion, bladder cancers tend to occur in two principal forms: low-grade superficial tumors, and high-grade invasive cancer. Superficial bladder cancer accounts for approximately 70 to 80% of all newly diagnosed bladder cancers. Because of the multifocal nature of urothelial cancers, patients who survive bladder cancer remain at risk of invasive disease. Most invasive tumors are nodular, metastatic during the early phase, and have a poor prognosis (Newling, 1996; Foresman and Messing, 1997).

The nucleophosmin/B23 gene (also known as B23) seems to be involved in the control of cell growth, cell differentiation, and programmed cell death (Hsu and Yung, 1998; Liu and Yung, 1998; You et al., 1999). Nucleophosmin/B23 has been known to have a multifunctional role in the cells. It has been implicated as an increased nucleolar activity that is necessary for cell proliferation, as a cytoplasmic/nuclear shuttle protein, in relieving transcription repression by YY1, in binding to nuclear and nucleolar localization signals on the human immunodeficiency virus type 1 Rev protein and the human T-cell leukemia virus-1-Rex protein, in binding to cell cycle-regulated nucleolar protein p120, and in inhibiting DNA-binding and transcriptional activity of interferon regulatory factor-1, which is a tumor suppressor (Feuerstein et al., 1988; Borer et al., 1989; Fankhauser et al., 1991; Adachi et al., 1993; Inouye and Seto, 1994; Tanaka et al., 1994; Valdez et al., 1994; Kondo et al., 1997). Nucleophosmin/B23 is significantly more abundant in tumor and proliferating cells than in normal resting cells. Nucleophosmin/B23 overexpression at the RNA and protein levels may contribute to the onset of cancer (Feuerstein et al., 1988; Chan et al., 1989;

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ABBREVIATIONS: ChIP, chromatin immunoprecipitation; Dn-Ras-U1, U1 cells constitutively expressing dominant-negative Ras; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase activating kinase; ERK, extracellular-signal regulated kinase; p-, phosphorylated; MAP, mitogen-activated protein; MAPK, mitogen activated protein kinase; PCR, polymerase chain reaction; PTA, thymidine kinase promoter; siRNA, small interfering RNA; PBS, phosphate-buffered saline; bp, base pair; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene diflouride; TBST, Tris-buffered saline/Tween 20; ECL, enhanced chemiluminescence; PD98059, 2′-amino-3′-methoxyflavone; PIPES, 1,4-piperazinediethanesulfonic acid; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal.

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Kondo et al., 1997). Blockage of nucleophosmin/B23 expression with its antisense oligonucleotides has shown that nucleophosmin/B23 is crucial for rendering cancer cells resistant to the induction of differentiation and apoptosis (Hsu and Yung, 1998; Liu and Yung, 1998; You et al., 1999). It thus seems that an excess of nucleophosmin/B23 may contribute to cancer progression.

Ras is among those oncoproteins whose structure and function are most thoroughly characterized. Although the immediate target of Ras protein is not clear, many cytoplasmic factors are known to function downstream of the Ras signaling pathway (Crews and Erikson, 1993; Mccormick, 1994). Earlier studies demonstrate that microinjection of the Ras protein into quiescent cells induces DNA synthesis (Mulcahy et al., 1985). Ras signaling pathway is linked directly to growth stimulation. Activation of Ras triggers uncontrolled proliferation and morphological alteration, contributing to the malignant phenotype of cancer cells. Ras interacts with effector proteins such as Raf kinase and phosphatidylinositol 3′-kinase proteins (Rodriguez-Viciana et al., 1997). Although these effector pathways exert essential roles in the signaling involved in Ras transformation, downstream factors play critical mediating roles in the neoplastic growth of cancers.

The c-*myc* gene, encoding a basic helix-loop-helix transcriptional factor, was first identified through its involvement in neoplasia in humans and animals (Cole, 1986). Several mechanisms, including gene amplification, point mutations, and chromosomal rearrangements, have been found to activate c-myc in human tumors. Myc mediates tumorigenesis through the activation of genes involved in cell metabolism, proliferation, and apoptosis and through the repression of genes that may promote cellular differentiation and cell cycle arrest.

Malignant progression is a complex and poorly understood process that seems to involve genetic and epigenetic factors. One possible factor in the progression of tumors is the changes in the expression of the associated cellular oncogenes, tumor suppressor genes, and transcription factors. Considerable effort devoted to elucidating the intracellular signaling pathways that control cellular proliferation has identified Ras and c-Myc as two critical components for the control of cellular growth. However, the precise manner in which these proteins might interact to synchronously regulate cell growth has been the subject of considerable interest and investigation.

The questions of whether nucleophosmin/B23 is functionally associated with oncogenes and how nucleophosmin/B23 is being elevated in tumor growth stimulation are presently unanswered. In this study, attempts were thus made to explore the possible interactions among Ras, c-Myc, and nucleophosmin/B23 in cancer cell growth. We first compared the cellular protein expressions of Ras, c-Myc, and nucleophosmin/B23 in U1 bladder cancer cells of high malignancy with those in the U4 premalignant cells. Next, using luciferase reporter and ChIP assays, we revealed that nucleophosmin/B23 transcription is activated by c-Myc in U1 but not in U4 cells upon serum stimulation. Furthermore, MEK inhibitor in Ras pathway could prevent the increase of nucleophosmin/B23 transcription in U1 cells upon serum stimulation. In U1 cells constitutively expressing dominant-negative Ras (Dn-Ras-U1), no increase of nucleophosmin/B23 transcription or c-Myc binding to the promoter was observed upon serum stimulation, suggesting a significant contribution of Ras to the recruitment of c-Myc to nucleophosmin/B23 promoter for transcriptional activation. Our results have demonstrated an important signaling pathway in tumor growth promotion with the linkages among Ras, c-Myc, and nucleophosmin/B23.

Materials and Methods

Cells and Reagents. MGH-U1 cell line (U1) was established from a grade III bladder transitional cell carcinoma (Kato et al., 1978). MGH-U4 cell line (U4) was derived from a male patient who had a bladder tumor of carcinoma in situ and severe atypia of the bladder (Lin et al., 1985). U1 or U4 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% antibiotics in 5% CO_2 humidified incubator at 37°C. The number of viable cells were determined by trypan blue exclusion and counted with a hemocytometer. G418 and PD98059 were from Sigma (St. Louis, MO). Anti-nucleophosmin/B23 monoclonal antibody was kindly provided by Dr. P. K. Chan (Department of Pharmacology, Baylor College of Medicine, Houston, TX). Anti-c-Myc polyclonal antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-Ras monoclonal antibody was from BD PharMingen (San Diego, CA). Anti-phospho-p44/p42 MAPK polyclonal antibody and anti-phospho-c-Myc (Thr58/Ser62) were from Cell Signaling Technology (Beverly, MA). Anti-p44/p42 MAPK polyclonal antibody, anti-β-Actin monoclonal antibody, and horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG were from Chemicon International Inc. (Temecula, CA). Cell viability was assessed by the exclusion of 0.2% trypan blue.

Plasmid. The 5′ region of nucleophosmin/B23 gene (−552/+2217) contains c-Myc binding site was cloned into luciferase reporter gene vector pGL3-control vector (pGL3-B23I1). The orientation of the promoter in pGL3 was determined by nucleotide sequencing using the sequence kit (GE Healthcare, Uppsala, Sweden). c-Myc binding site mutations (5′-E box mutation; 3′-E box mutation; 5′, 3′-E boxes mutations) were performed using site-specific mutagenesis polymerase chain reaction (PCR). The expression vectors (pcDNA3.1-H-RasS17N and pcDNA3.1-H-RasG12V) were from UMR cDNA Resource Center (Rolla, MO). The pSV-β-galactosidase vector was from Promega (Madison, WI).

The pMyc-TA-Luc vector contains six tandem copies of the E-box consensus sequence, located upstream of the minimal TA promoter, the TATA box from the herpes simplex virus thymidine kinase promoter (PTA). Located downstream of PTA is the firefly luciferase reporter gene (luc). After c-Myc proteins bind the E-box elements, transcription is induced, and the reporter gene is activated.

The sequence of siRNA for nucleophosmin/B23 is 5′-GGACAA-GAAUCCUUCAAGA-3′. It was synthesized and cloned into pSilencer 2.0-U6 plasmid as described in the user manual (Ambion Inc., Austin, TX). The control or siRNA-expressed plasmid was cotransfected with pcDNA3.1 to establish G418-resistant clones.

Cell Transfection and Establishment of Stable Clones. Transfections were performed using Lipofectamine reagent (Invitrogen, Carlsbad, CA) method. Before transfection, cells (5×10^5 to 1×10^6) were seeded in 6-cm dishes overnight. Plasmid DNA (2 μg) and Lipofectamine Reagent (20 μg) were each diluted in serum-free medium (500 μl). DNA and Lipofectamine reagent were then mixed and incubated for 30 min at room temperature to allow DNA-Lipofectamine complexes formation. Cells were rinsed twice with PBS, and replaced with serum-free medium (1.5 ml) and then overlaid with DNA-liposome complexes. After 6 h of incubation at 37°C in CO_2 incubator, the DNA-containing medium was replaced by fresh medium containing 10% serum. For establishment of stable clones, the transfected cells were distributed in 10-cm dishes at a number of 5×10^5, and 0.5 mg/ml G418 (Calbiochem, San Diego, CA) was added to the cell culture 48 h after transfection. After selection with G418 for 3 weeks, individual clones were expanded to mass cultures and

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subsequently assayed for c-H-Ras expression. The transfectants were maintained in culture medium supplemented with 0.5 mg/ml G418.

Soft Agar Assay. Cells were cultured in six-well plates (Nalge Nunc International, Rochester, NY). Cell suspensions in RPMI containing 0.35% agar and 10% fetal calf serum were layered over the 0.5% base layer to a final cell density of 2×10^3 cells/well. Four weeks after seeding, clones were stained with 0.5% crystal violet (in 70% methanol) for visualization, and the clones whose diameter was greater than 1 mm were counted. The survival percentage was expressed as the relative seeding efficiency of U1 versus U4 cultures.

Reverse Transcriptase-Polymerase Chain Reaction. Confluent U1 and U4 Cells in 10-cm dishes were harvested, and total RNA was isolated by the TRIzol reagent (Invitrogen). A weight of 2 μg of total RNA was reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random hexamer by incubating the reaction mixture (20 μl) at 37°C for 50 min. The PCR was performed in a final volume of 25 μl of solution containing 1 μl of reverse-transcribed cDNA, 75 mM Tris-HCl, pH 8.8, 0.01% Tween 20, 2 mM $MgCl_2$, 200 μM concentrations of each dNTP, 0.5 μM concentrations of each primer (B23, forward: 5′-GAATTCTTGTTGAAGCAGAGGCAATG-3′; reverse: 5′-AAGCTTACTTCCTCCACTGCCAGAGA-3′; β-actin, forward: 5′-GAATTCAGAAAATCTGGCACCACACC-3′; reverse: 5′-AAGCTTCCATCTCTTGCTCGAAGTCC-3′), and 1 U of *Taq* DNA polymerase (GENEMARK Technology, Taipei, Taiwan). After an initial denaturation for 7 min at 95°C, 22 cycles of amplification (95°C for 1 min, 60°C for 1 min, and 72°C for 1 min) were performed followed by a 7-min extension at 72°C. Five microliters of PCR products was electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining (400-bp β-actin and 700-bp B23 fragment).

Western Blotting. Cells were harvested and washed twice in ice-cold PBS, and then lysed in RIPA buffer (1% Triton X-100, 1% SDS, 20 mM Na_2HPO_4, 100 mM NaCl, and 0.2 mM phenylmethylsulfonyl fluoride). Lysates were boiled in SDS sample buffer [62.5 mM Tris, pH 6.8, 5% β-mercaptoethanol (Merck and Co., Inc. Whitehouse Station, NJ), 10% glycerol, 2% SDS, and 0.001% bromphenol blue], and then was fractionated by 12% SDS-PAGE. Separated proteins in SDS-PAGE were electrotransferred to Hybond-PVDF membrane (GE Healthcare). The PVDF membrane was then soaked in a blocking solution containing 5% (w/v) nonfat milk in TBST [20 mM Tris, pH 7.5, 0.5 M NaCl, and 0.1% (v/v) Tween 20] for 1 h at room temperature. Antibody against the indicated protein was diluted in TBST. The soaked PVDF membrane was then incubated with antibody overnight at 4°C and then washed with TBST three times for 15 min each and incubated in horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG at room temperature for 1 h. The membrane was washed three times with TBST buffer for 15 min each. Immunobands were detected by the enhanced chemiluminescence reaction (ECL; GE Healthcare) and exposed to the X-ray film (Fuji Photo Film Corporation, Tokyo, Japan).

Luciferase and β-Galactosidase Activity Assays. Cells (5×10^5 to 1×10^6) were plated in the 6-cm dishes on day 0. The cells were transfected with pGL3-B23I1 and pSV-β-galactosidase in the serum-free RPMI on day 1. On day 4, cells were treated with or without serum and incubated for 6 h. Cells were washed with PBS twice and lysed in Reporter Lysis Buffer (Promega). Twenty microliters of cell lysate was mixed with 50 μl of the luciferase assay substrate (Promega). Luciferase activity was quantified in an LMax II microplate reader (Molecular Devices, Sunnyvale, CA). For β-galactosidase activity assay, 100 μl of cell lysate was mixed with 100 μl of the 2× reaction buffer 200 mM sodium phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, and 1.33 mg/ml *o*-nitrophenyl-β-D-galactopyranoside) and was incubated at 37°C for 30 min. Na_2CO_3 (500 μl) was added to the reaction mixture, and the optical density at a wavelength of 420 nm was recorded. Luciferase activity was normalized to the β-galactosidase activity of the same sample.

Chromatin Immunoprecipitation. Cells were cross-linked by adding formaldehyde (final concentration, 1%) directly to the cells in a culture dish for 10 min at room temperature. Glycine (final concentration, 125 mM) was added to stop the cross-link for 5 min at room temperature. Cells were washed twice with ice-cold PBS and harvested in cell lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) and incubated on ice for 10 min. Samples were pelleted by centrifugation (1600 rpm for 2 min). The pellet was resuspended in nuclear lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) and incubated on ice for 10 min. Nucleus were sonicated and centrifuged with full speed at 4°C. The supernatant was precleared with salmon sperm DNA/protein A agarose/50% slurry for 1 h at 4°C with agitation. Protein concentration of precleared supernatant was determined, and 300 μg of protein was incubated with 4 μg of anti-c-Myc polyclonal antibody (Upstate Biotechnology) or with control IgG and rotated at 4°C overnight. Immunoprecipitation, washing, and elution of immune complexes with 1% SDS, 0.1 M $NaHCO_3$ were carried out. Cross-links were reversed by the addition of NaCl to a final concentration of 200 mM, and RNA was removed by the addition of 10 μg of RNase A per sample followed by incubation at 65°C for 5 h. The samples were then mixed with 10 μl of 0.5 mM EDTA, 20 μl of 1 M Tris-HCl, pH 6.5, and 2 μl of 10 mg/ml Proteinase K and incubated for 1 h at 45°C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and resuspended in 20 μl of H_2O. Total input samples were resuspended in 100 μl of H_2O before PCR. PCR reactions contained 1 μl of immunoprecipitate, 75 mM Tris-HCl, pH 8.8, 0.01% Tween 20, 2 mM $MgCl_2$, 200 μM concentrations of each dNTP, 0.5 μM concentrations of each primer and 1 U of *Taq* DNA polymerase (GENEMARK Technology) in a total volume of 25 μl. The forward primer for human B23 promoter was 5′-AAGCATGGGCCTGCTTGTTG-3′ and reverse primer was 5′-GAGAGCTGCCATCACAGTAC-3′. The product size was 351 bp. These primers were designed from human genomic DNA sequence (GenBank accession number AC091980). The control primers for human vimentin promoter designed from human genomic sequence (GenBank accession number AL133415) were 5′-CTGAAGTAACGGGACCATGC-3′ and 5′-AGAAGAGGCGAACGAGGG-3′. The amplicon size was 284 bp. After 30 cycles of amplification, PCR products were run on a 1.5% agarose gel and analyzed by ethidium bromide staining.

In Vitro p44/p42 MAP Kinase Assay. The assay was performed as stated in the user manual of p44/p42 MAP kinase assay kit (Cell Signaling Technology). In brief, cells were washed twice by ice-cold PBS and harvested in the ice-cold cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Cell lysate was subjected to immunoprecipitation with immobilized phosphor-p44/p42 MAP kinase (Thr202/Tyr204) monoclonal antibody for overnight rotation at 4°C. The immunoprecipitates were incubated with 2 μg of Elk-1 fusion protein and 200 μM ATP in the kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) for 30 min at 30°C. The labeled Elk-1 fusion protein was subjected to SDS-PAGE and transferred to the PVDF membrane. The membranes were incubated with anti-phosphoserine monoclonal antibody overnight at 4°C followed by horseradish peroxidase-conjugated goat anti-mouse IgG incubation. Immunobands were detected by the enhanced chemiluminescence reaction (ECL, Amersham Pharmacia Biotech) and exposed to the X-ray film (FujiFilm, Tokyo, Japan).

Results

Ras, c-Myc, and Nucleophosmin/B23 Were Highly Expressed in U1 Cells. U1 cells exhibited higher proliferation capacity than U4 cells (Fig. 1A). Compared with U4 cells, U1 cells grew more rapidly in the medium with or without serum. The efficiency of colony formation in soft agar of U1 cells

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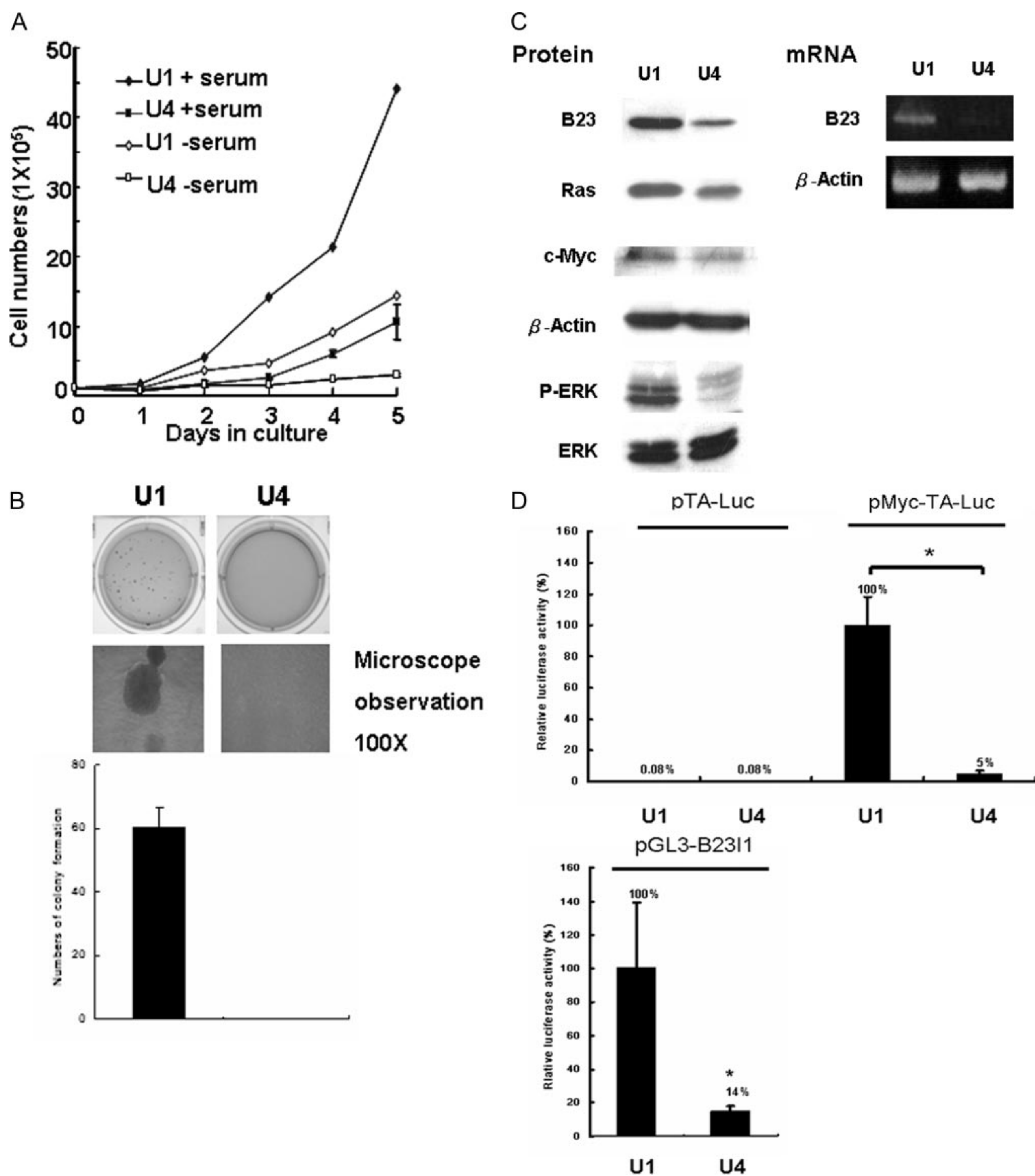

Fig. 1. Ras, c-Myc, and nucleophosmin/B23 were highly expressed in U1 cells. A, U1 or U4 cells (1×10^5 cells/dish) were seeded onto 10-cm dishes. The cells were grown in RPMI containing (+serum) or not containing (−serum) 10% fetal bovine serum. Cells were harvested at the indicated times. Viable cells were determined by tryphan blue exclusion method, and cell numbers were obtained by counting with a hemocytometer. Points, means of triplicates ± S.D. B, U1 or U4 cells were cultured in six-well plates. Cell suspensions in RPMI containing 0.35% agar and 10% fetal calf serum were layered over the 0.5% base layer to a final cell density of 2×10^3 cells/well. The colonies were stained (top) and scored (bottom) 28 days after seeding. Values were expressed as the means ± S.D. for three determinations. C, U1 and U4 cells were grown in RPMI containing 10% fetal bovine serum for 24 h. Cells were harvested, washed, and lysed. Equal amounts (20 μg) of proteins were separated by 12% SDS-PAGE and blotted onto PVDF membrane. Ras, c-Myc, nucleophosmin/B23 (B23), ERK, phosphorylated-ERK (p-ERK), and β-actin were detected by Western blot using their specific antibodies and ECL reaction. Total RNA was also prepared. PCR analysis was performed with 2 μg of total RNA for each sample. The detection of β-actin mRNA was used as a control for the amount of RNA loaded. D, the absolute c-myc transactivation and nucleophosmin/B23 (B23) promoter activities were higher in U1 than in U4 cells. U1 and U4 cells (5×10^5 of U1 and 1×10^6 of U4) were transiently transfected with 1 μg of pMyc-TA-Luc, pTA-Luc, 1 μg of nucleophosmin/B23 promoter plasmid (pGL3-B23I1), and 1 μg of pSV-β-galactosidase (as an internal control) for 48 h. Cells were harvested and lysed by reporter lysis buffer. The cell extracts were analyzed for luciferase and β-galactosidase activities. Luciferase activities were normalized to the corresponding β-galactosidase values. Bars, means of triplicates ± S.D. *, $P < 0.05$ compared with U1 cells.

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was found to be significantly higher than U4 cells, consistent with a known important feature of such transformed cells (Fig. 1B). It is interesting that immunoblotting experiments showed that Ras, c-Myc, nucleophosmin/B23, and p-ERK were highly expressed in U1 cells (Fig. 1C). On the other hand, Ras, c-Myc, and nucleophosmin/B23 were present at much lower levels in the U4 cells. The mRNA level of nucleophosmin/B23 was also found to be significantly greater in U1 compared with that in U4 cells (Fig. 1C). Furthermore, it was noteworthy that the absolute c-Myc transactivation and nucleophosmin/B23 promoter activities were higher in U1 cells than in U4 cells (Fig. 1D). Using the vectors (pTA-Luc and pGL3-control) as negative controls, our results showed that there were virtually no differences of the measured activities in those vector-transfected U1 and U4 cells (Fig. 1D).

Up-Regulation of c-Myc and Nucleophosmin/B23 in U1 Cells Upon Serum Stimulation From Quiescence. Previous studies indicate that nucleophosmin/B23 is intimately associated with growth stimulation in tumor cells (Feuerstein et al., 1988). Therefore, we next wanted to compare the protein expressions of nucleophosmin/B23, c-myc, and p-ERK during serum stimulation in U1 and U4 cells. Cells were brought to the quiescence by serum starvation and then stimulated by the addition of fresh serum to promote synchronous entry to the cell cycle. Cells were harvested at quiescence or at various times (0.5, 1, 3, 6, or 12 h) after serum addition, and lysates were subjected to immunoblotting analysis. Level of nucleophosmin/B23 was found to be increased at 1 h and peaked at 6 h after serum stimulation in U1 cells. Our results further showed that *p*-ERK, c-Myc, and nucleophosmin/B23 were up-regulated in U1 cells but not in U4 cells upon serum stimulation (Fig. 2A). It was also noted that U4 cells had considerably lower levels of c-Myc compared with even the serum-deprived U1 cells (Fig. 2A). To elucidate the differential regulation of nucleophosmin/B23 gene expression, we then compared the activities of nucleophosmin/B23 promoter-reporter construct (pGL3-B23I1) during serum stimulation in U1 and U4 cells. Measurement of transcription from the pGL3-B23I1 promoter showed a significant increase (~53%) in the promoter activity at 6 h after resupplementation of serum to the U1 culture (Fig. 2B). Conversely, virtually no increase of nucleophosmin/B23 promoter activity was observed in U4 cells under the same circumstance (Fig. 2B).

Because ERK was highly activated (phosphorylated) in U1 cells (Fig. 1C), we then determined the possible linkage between MAPK/ERK signaling pathway to nucleophosmin/B23 expression. Our results showed that treatment of U1 cells with an MEK inhibitor, PD98059, depressed the nucleophosmin/B23 promoter activity in minus serum condition and during the serum stimulation (Fig. 2C). The expressions of c-Myc and nucleophosmin/B23 decreased in U1 cells treated with PD98059 (Fig. 2D). Recent study by Sears et al. (1999) has shown that activation of the Ras pathway extends the half-life of the Myc protein. Phosphorylation of residues at Thr58 and Ser62 is associated with degradation of Myc. Using antibody that recognizes c-Myc that is doubly phosphorylated at Thr58 and Ser62, we examined c-Myc and its phosphorylation under PD98059 treatment and in the presence of proteasome inhibitor. As shown in Fig. 2E, proteasome inhibitor MG132 blocked the PD98059-mediated down-regulation of c-Myc. Under such circumstance, c-Myc was highly phosphorylated compared with the control. Our results have demonstrated that MAPK/ERK and phosphorylation of c-Myc are critical for controlling c-Myc protein accumulation.

c-Myc Is Recruited to Nucleophosmin/B23 Promoter in U1 but Not in U4 Cells upon Serum Stimulation from Quiescence. To implicate the role of c-Myc in transcriptional activation of nucleophosmin/B23 in U1 cells, we first determined the c-Myc transcriptional activities during serum stimulation in U1 and U4 cells. Our results showed that a significant increase (~51%) in the c-Myc transcriptional activity at 6 h after serum resupplementation to the U1 cells (Fig. 3A). In contrast, virtually no increase of c-Myc transcriptional activity was observed in U4 cells upon serum stimulation (Fig. 3A). Previous characterization of nucleophosmin/B23 as a c-Myc target by ChIP analysis has revealed that the fragment containing the two canonical E boxes of intron 1 is the predominant binding site of c-Myc in the nucleophosmin/B23 promoter (Zeller et al., 2001). We therefore wanted to perform reporter assays with nucleophosmin/B23 promoter luciferase constructs and studied the effect of point mutations in the two canonical E boxes (B23 5′- and 3′-E boxes) of intron 1 sequences (Zeller et al., 2001). Figure 3B shows the schematic diagram for the construct containing nucleophosmin/B23 promoter and the c-Myc-binding point mutation sites. Mutations in the two canonical boxes (5′-E box, 3′-E box, or 5′-3′-E box) not only resulted in some loss of basal nucleophosmin/B23 transcription but also abrogated much of the transcription activation upon serum stimulation in U1 cells (Fig. 3C). To further examine the role of c-Myc in transcriptional activation of nucleophosmin/B23 in cancer cells, we undertook chromatin immunoprecipitation assays to assess the nucleophosmin/B23 promoter occupancy by c-Myc in U1 and U4 cells upon serum stimulation. Chromatin was collected 6 h after resupplementation of serum, and ChIP analysis was subsequently performed using an antibody against c-Myc (Fig. 3D). Our results demonstrated that c-Myc binding to the nucleophosmin/B23 promoter site was significantly elevated in U1 cells upon serum stimulation (Fig. 3D). However, the degree of c-Myc binding to the nucleophosmin/B23 promoter did not change in U4 cells upon serum stimulation (Fig. 3D). As a control, promoter fragment corresponding to the vimentin gene, which is not regulated by c-Myc, was not detected in the immunoprecipitate (Fig. 3D).

Ras-Dependent Regulation of Nucleophosmin/B23. To examine whether the increasing the levels of Ras in U4 cells could result in the up-regulation of c-Myc and nucleophosmin/B23, immunoblotting analysis was done with proteins extracted from control vector and Ras overexpressed U4 cells. Our results showed that Ras, c-Myc, and nucleophosmin/B23 were up-regulated by transient overexpression of Ras (Fig. 4A, top). Measurement of transcription from the pGL3-B23I1 promoter showed higher promoter activity in the Ras transiently overexpressed than in vector-transfected U4 cells (Fig. 4A, bottom). To further examine whether transcription of nucleophosmin/B23 is a downstream signaling event of Ras, our approach was to constitutively express Ras dominant-negative protein in U1 cells and look for any changes in the c-Myc transcriptional activation of nucleophosmin/B23 promoter. As a result, the expressions of Ras, c-Myc, nucleophosmin/B23, and p-ERK decreased in Dn-Ras-U1 cells (Fig. 4B). The Erk kinase activity was also

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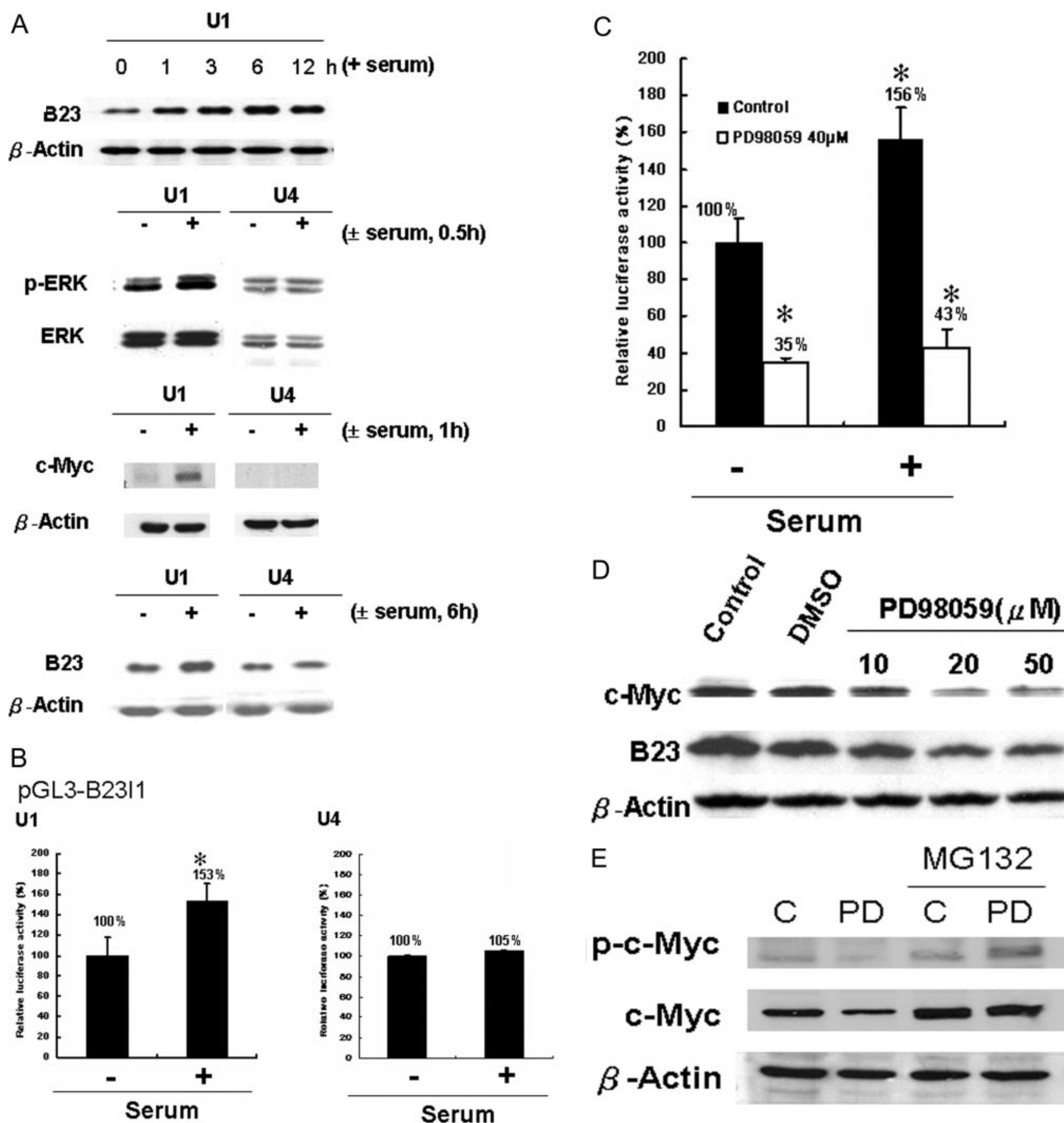

Fig. 2. p-ERK, c-Myc, and nucleophosmin/B23 were increased upon serum-stimulated growth in U1 cells. A, U1 and U4 cells were made quiescent by serum starvation for 72 h. Cells were then grown in RPMI containing 10% fetal bovine serum for various times (0.5,1, 2, 3, 6, or 12 h). Cells were harvested, washed, and lysed. Equal amounts (20 μg) of proteins were separated by 12% SDS-PAGE and blotted onto PVDF membrane. ERK, p-ERK, c-Myc, nucleophosmin/B23 (B23), and β-actin were detected by Western blot using their specific antibodies and ECL reaction. B, nucleophosmin/B23 promoter activity increased upon serum stimulation in U1 cells. U1 and U4 cells (5×10^5 of U1 and 1×10^6 of U4) were transiently transfected with 1 μg of nucleophosmin/B23 promoter plasmid (pGL3-B23I1) and 1 μg of pSV-β-galactosidase (as an internal control). Cells were made quiescent by serum starvation for 72 h. Cells were then grown in RPMI containing 10% fetal bovine serum for 6 h (U1 and U4 cells). Cells were harvested and lysed by reporter lysis buffer. The cell extracts were analyzed for luciferase and β-galactosidase activities. Luciferase activities were normalized to the corresponding β-galactosidase values. Bars, means of triplicates ± S.D. *, $P < 0.05$, compared with nucleophosmin/B23 promoter activity upon serum stimulation and the control without serum stimulation. C, U1 cells (5×10^5) were transiently transfected with 1 μg of nucleophosmin/B23 promoter plasmid (pGL3-B23I1) and 1 μg of pSV-β-galactosidase (as an internal control). Cells were made quiescent by serum starvation for 72 h. The cells were grown in the serum-deprived medium in the presence of 40 μM MEK inhibitor, PD98059 (PD), for 1 h. Cells were then grown in RPMI containing 10% bovine calf serum for 6 h. Cells were harvested, and the promoter activity was determined. *, $P < 0.05$, compared with the control without serum stimulation and in the absence of inhibitor. D, U1 cells (2×10^5) were treated with 10 to 50 μM MEK inhibitor, PD98059 (PD), for 48 h. Cells were harvested, washed, and lysed. Equal amounts (20 μg) of proteins were separated by 10% SDS-PAGE and blotted onto PVDF membrane. c-Myc, nucleophosmin/B23 (B23), and β-actin were detected by Western blot using their specific antibodies and ECL reaction. E, U1 cells (2×10^5) grown in serum-free medium were not treated as control (C) or treated with 40 μM MEK inhibitor, PD98059 (PD), for 42h. Cells were then treated with 5 μM proteasome inhibitor, MG132, for 6 h before harvest. Equal amounts (20 μg) of proteins were separated by 10% SDS-PAGE and blotted onto PVDF membrane. c-Myc, c-Myc phosphorylated at Thr58 and Ser62 (p-c-Myc), and β-actin were detected by Western blot using their specific antibodies and ECL reaction.

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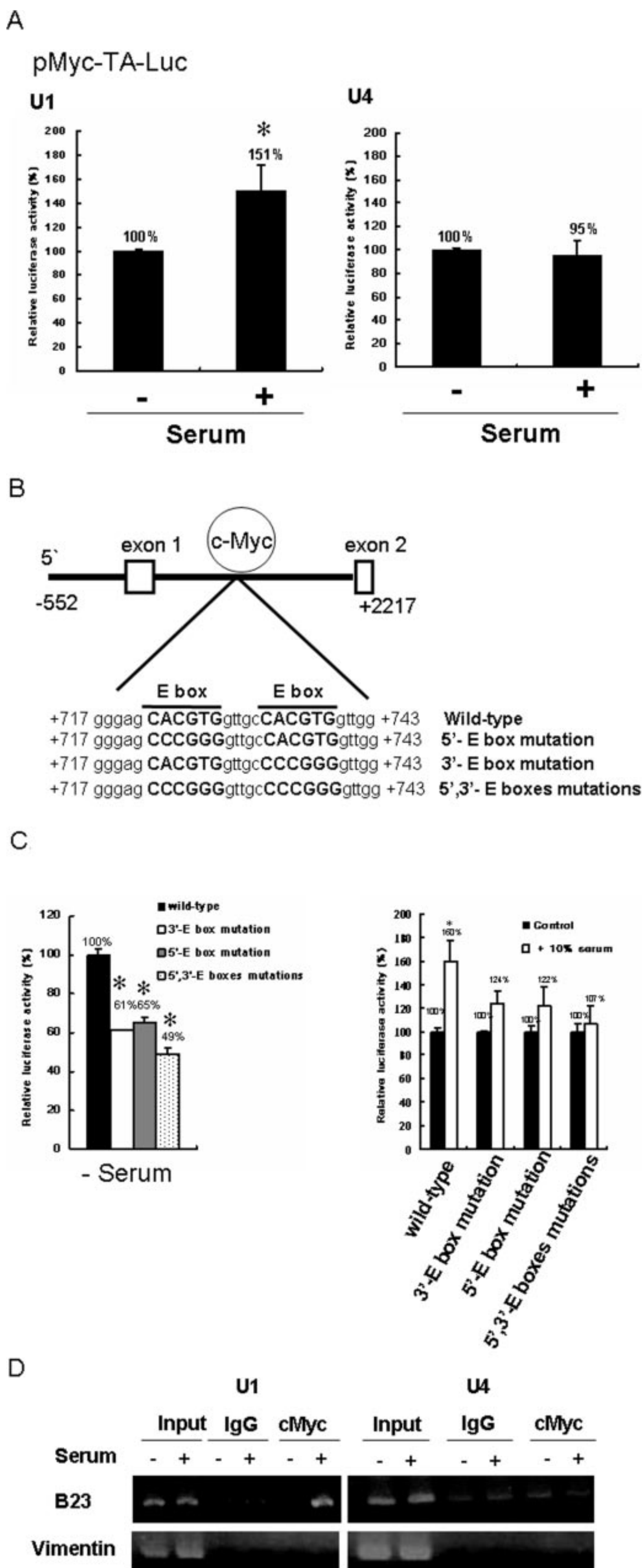

Fig. 3. c-Myc transcriptional activation of nucleophosmin/B23. A, c-Myc transcriptional activity increased upon serum stimulation in U1 cells. U1 and U4 cells (5×10^5 of U1 and 1×10^6 of U4) were transiently transfected with 1 μg of pMyc-TA-Luc and 1 μg of pSV-β-galactosidase (as an internal control). Cells were made quiescent by serum starvation for 72 h. Cells were then grown in RPMI containing 10% fetal bovine serum for 6 h (U1 and U4 cells). Cells were harvested and lysed by reporter lysis buffer. The cell extracts were analyzed for luciferase and β-galactosidase activities. Luciferase activities were normalized to the corresponding β-galactosidase values. Bars, means of triplicates ± S.D. *, $P < 0.05$, compared with c-Myc transcriptional activity upon serum stimulation and the control without serum stimulation. B, the schematic diagram of nucleophosmin/B23 promoter containing the c-Myc binding site and the point mutations in canonical E boxes. C, U1 cells were transiently transfected with 1 μg of wild-type nucleophosmin/B23 promoter plasmid or plasmids with point mutations in 5′-E box (5′-E box mutation), 3′-E box (3′-E box mutation) or in both boxes (5′-, 3′-E boxes mutations) of c-Myc-binding site and 1 μg of pSV-β-galactosidase (as an internal control). Cells were made quiescent by serum starvation for 72 h. Cells were then grown in RPMI containing 10% fetal bovine serum for 6 h. Cells were harvested, and the promoter activity was determined. Bars, means of triplicates ± S.D. *, $P < 0.05$, compared with nucleophosmin/B23 promoter activity upon serum stimulation and the control without serum stimulation. D, U1 or U4 cells (1×10^7 of U1 and 2×10^7 of U4) were made quiescent by serum starvation for 72 h. Cells were then grown in RPMI containing 10% fetal bovine serum for 6 h. Cells were harvested. Chromatin was collected, and ChIP analysis was performed for nucleophosmin/B23 (B23) and negative control vimentin using antibody against c-Myc.

found to be lower in DN-Ras-U1 cells compared with wild-type or vector-transfected U1 cells (Fig. 4C). Furthermore, nucleophosmin/B23 promoter activity, c-Myc transcriptional activity (Fig. 4D), and c-Myc binding to the nucleophosmin/B23 promoter (Fig. 4E) remained relatively unchanged in DN-Ras-U1 cells upon serum stimulation. Sequence corresponding to the vimentin gene, which is not a target of c-Myc, was not detected in the immunoprecipitate (Fig. 4E). Taken together, our results demonstrate the important role of Ras in the recruitment of c-Myc for transcriptional activation of nucleophosmin/B23 in tumor growth stimulation.

To further define that nucleophosmin/B23 overexpression is a prerequisite for cell proliferation, we compared the growth capacity of U1-vector cells and U1-siB23 cells. Lowered expression of nucleophosmin/B23 was observed in U1-siB23 cells (Fig. 5A). Furthermore, U1-siB23 cells exhibited lower proliferation capacity than U1-vector cells (Fig. 5B). The efficiency of colony formation in soft agar of U1-siB23 cells was also found to be significantly lower than U1-vector cells (Fig. 5C).

Discussion

One important difference between cancer and normal cells is hyperactivity and pleomorphism of the nucleoli (Busch et al., 1963). The nucleolus in cancer cells undergoes extreme variations in size, shape, fine structure, and cytochemical composition (Bernhard and Granboulan, 1968). Uncontrolled cell proliferation is the hallmark of cancer, and tumor cells have typically acquired damage to genes that directly regulate their cell cycles and cell growth. Although rRNA transcription, processing, and ribosome assembly have been established as major functions of the nucleolus, previous studies suggest that nucleolus participates in many other aspects of gene expression as well (Pederson, 1998). New results indicate that biosyntheses of signal-recognition particle RNA and telomerase RNA involve a nucleolar stage, and nucleolus is a site critical to cellular aging (Johnson et al., 1998).

U4 cell line was derived from a bladder biopsy specimen diagnosed as severe atypia of the urothelium. U4 cells can grow in culture but fail to produce tumor in nude mice and could not form colonies in soft agar (Lin et al., 1985). Based

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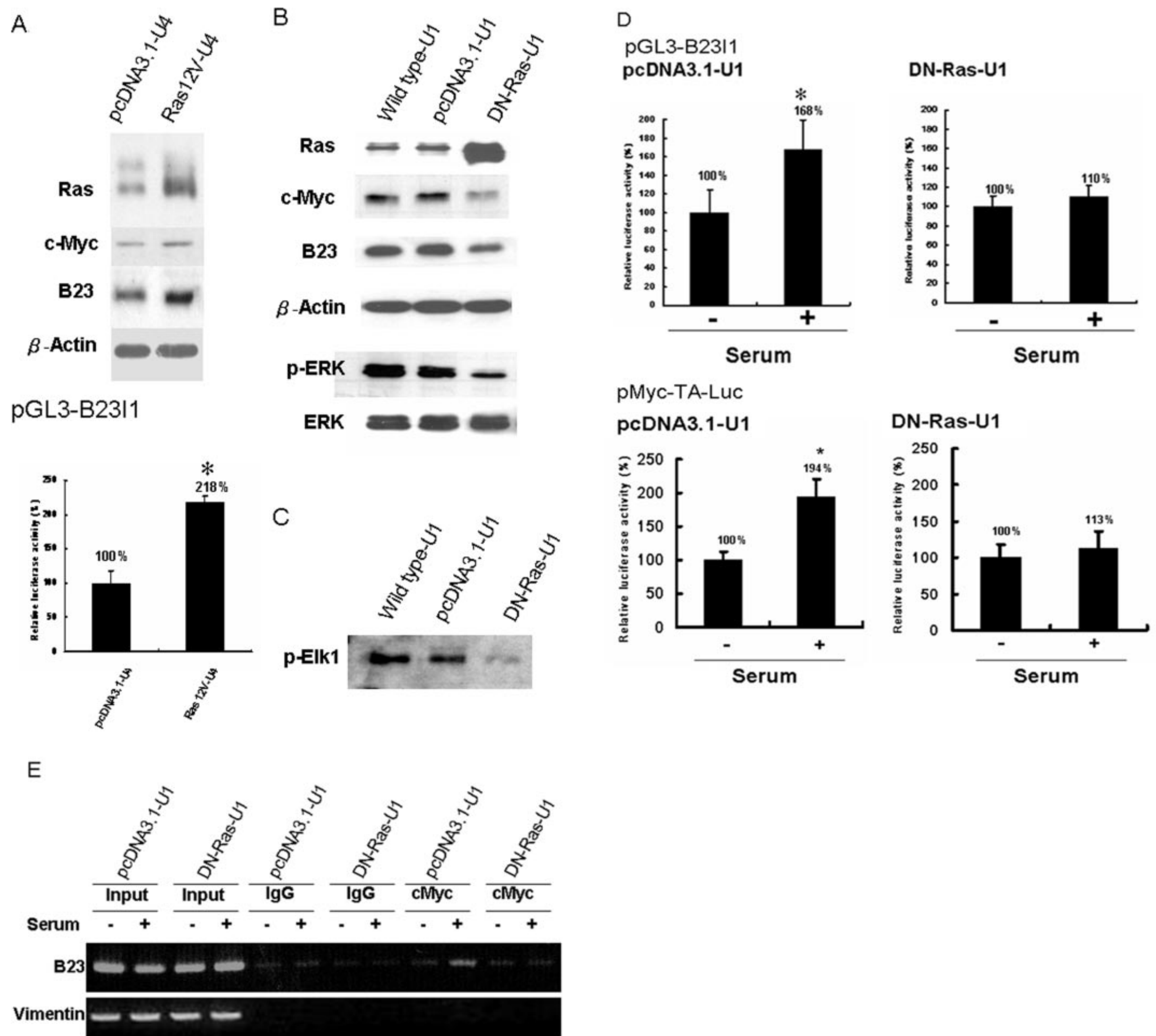

Fig. 4. Ras-dependent regulation of nucleophosmin/B23. A, oncogenic Ras transiently overexpressed (Ras12V-U4) or vector-transfected U4 cells (pcDNA3.1-U4) were grown in RPMI containing 10% fetal bovine serum for 72 h. Cells were harvested, washed, and lysed. Equal amounts (20 μg) of proteins were separated by 12% SDS-PAGE and blotted onto PVDF membrane. Ras, c-myc, nucleophosmin/B23 (B23), and β-actin were detected by Western blot using their specific antibodies and ECL reaction (top). Oncogenic Ras transiently overexpressed (Ras12V-U4) or vector-transfected U4 cells (pcDNA3.1-U4) were also transiently transfected with 1 μg of nucleophosmin/B23 promoter plasmid (pGL3-B23I1) and 1 μg of pSV-β-galactosidase (as an internal control) in RPMI containing 10% fetal bovine serum for 72 h. Cells were harvested and lysed by reporter lysis buffer. The cell extracts were analyzed for luciferase and β-galactosidase activities. Luciferase activities were normalized to the corresponding β-galactosidase values. Bars, means of triplicates ± S.D. $*$, $P < 0.05$, compared with nucleophosmin/B23 promoter in vector-transfected control cells (bottom). B, 5×10^5 of U1 cells (wild-type U1), vector stably expressed U1 cells (pcDNA3.1-U1), and dominant-negative Ras stably expressed U1 cells (DN-Ras-U1) were grown in RPMI containing 10% fetal bovine serum for 24 h. Cells were harvested, washed, and lysed. Equal amounts (20 μg) of proteins were separated by 12% SDS-PAGE and blotted onto PVDF membrane. Ras, c-myc, nucleophosmin/B23 (B23), p-ERK, ERK, and β-actin were detected by Western blot using their specific antibodies and ECL reaction. C, lysates from U1 cells (wild-type U1), vector stably expressed U1 cells (pcDNA3.1-U1), and dominant-negative Ras stably expressed U1 cells (DN-Ras-U1) were subjected to immunoprecipitation with antibody to active MAPK. The immunoprecipitates were incubated with ATP and ELK1 fusion protein in the kinase buffer for 30 min at 30°C. The labeled Elk-1 were analyzed by SDS-PAGE and immunoblotting with phosphoserine monoclonal antibody. D, 5×10^5 of vector stably expressed (pcDNA3.1-U1) and dominant-negative Ras stably expressed U1 cells (DN-Ras-U1) were transiently transfected with 1 μg of nucleophosmin/B23 promoter plasmid (pGL3-B23I1) or 1 μg of pMyc-TA-Luc and 1 μg of pSV-β-galactosidase (as an internal control). Cells were made quiescent by serum starvation for 72 h. Cells were then grown in RPMI containing 10% fetal bovine serum for 6 h. Cells were harvested and the promoter activity was determined. Bars, means of triplicates ± S.D. $*$, $P < 0.05$, compared with nucleophosmin/B23 promoter activity upon serum stimulation and the control without serum stimulation. E, 1×10^7 of vector stably expressed (pcDNA3.1-U1) and dominant-negative Ras stably expressed U1 cells (DN-Ras-U1) were made quiescent by serum starvation for 72 h. Cells were then grown in RPMI containing 10% fetal bovine serum for 6 h. Cells were harvested. Chromatin was collected, and ChIP analysis was performed for nucleophosmin/B23 (B23) and negative control vimentin using antibody against c-Myc.

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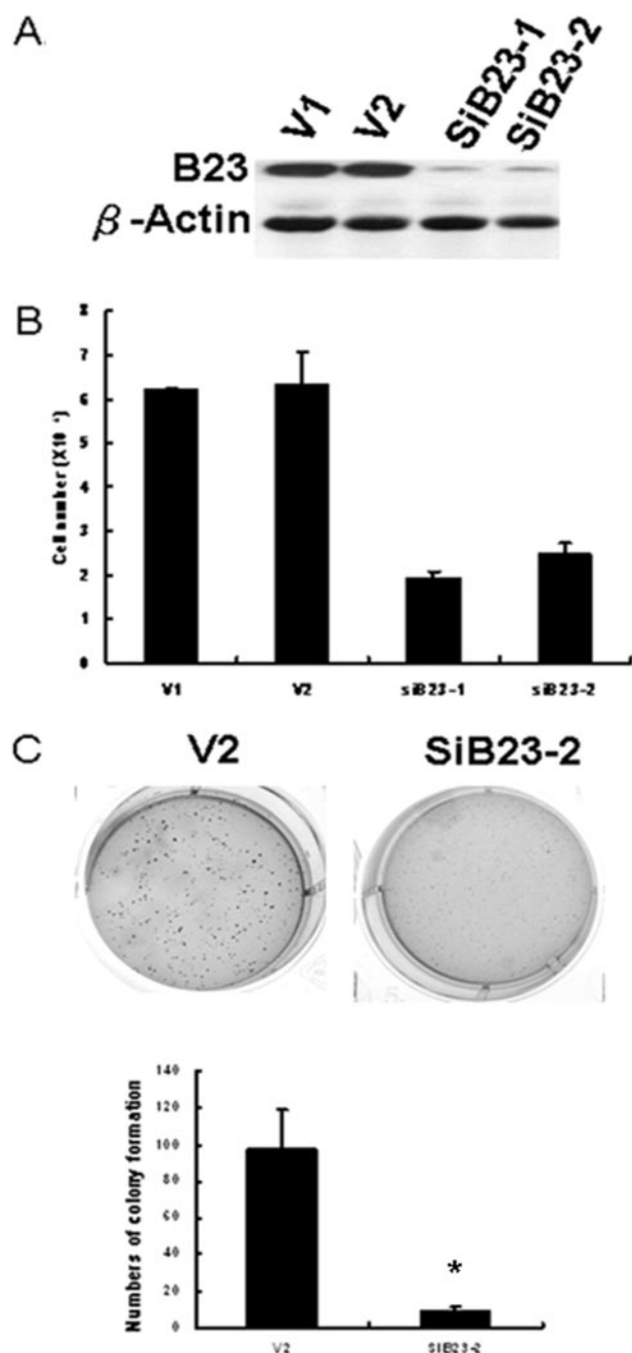

Fig. 5. Cell growth inhibition in U1-siB23 cells. A, U1-vector (V1, V2) or U1-siB23 (SiB23-1, SiB23-2) cells (1×10^5 cells) were grown in RPMI containing 10% fetal bovine serum for 48 h. Cells were harvested and washed, and the lysates were centrifuged at 12,000*g* for 30 min at 4°C. Equal amounts (30 μg) of proteins were separated by 10% SDS-PAGE and blotted onto PVDF membrane. Nucleophosmin/B23 (B23) and β-actin were detected by Western blot using their specific antibodies and ECL reaction. B, U1-vector (V1, V2) or U1-siB23 (SiB23-1, SiB23-2) cells (1×10^4 cells/dish) were seeded onto 6-cm dishes. The cells were grown in RPMI containing 10% fetal bovine serum for 72 h. Cells were harvested, and viable cells were determined by tryphan blue exclusion method; cell numbers were obtained by counting with a hemocytometer. Points, means of triplicates ± S.D. C, U1-vector (V2) or U1-siB23 (SiB23-2) cells were cultured in six-well plates. Cell suspensions in RPMI containing 0.35% agar and 10% fetal calf serum were layered over the 0.5% base layer to a final cell density of 8×10^3 cells/well. The colonies were stained (top) 28 days after seeding. Colonies with more than 30 cells were scored (bottom). Values were expressed as the means ± S.D. for three determinations. Bars, means of triplicates ± S.D. *, $P < 0.05$, compared with U1-vector (V2) cells.

on the same characterizations, including growth, morphology, colony formation, and tumorigenicity in nude mice, U1 cells represent cells with high degree of malignancy, whereas U4 cells are considered to be premalignant (Lin et al., 1985). Over the past decade, many studies have been directed on the analysis of the genes in the control of cell growth and their participation in the development of cancer. Nucleolar protein nucleophosmin/B23, oncogenes Ras and c-Myc have been shown to be associated with cancers. The steady-state level of nucleophosmin/B23 mRNA is found to be significantly higher in abnormal growth than in normal growth (Chan et al., 1989). There is also a close correlation between DNA synthesis and induction of nucleophosmin/B23 in cell growth stimulated by serum (Feuerstein et al., 1988). In the analysis of clinical gastric cancer tissues, cancers of later stages seem to have higher nucleophosmin/B23 mRNA levels in relative to the matched adjacent "normal" gastric mucosa (You et al., 1999). In the present study, we showed that Ras, c-Myc, and nucleophosmin/B23 are highly expressed in U1 cells compared with U4 cells. c-Myc and nucleophosmin/B23 are increased in U1 cells but not in U4 cells upon serum stimulation from quiescence. Our results have indicated that U4 cells with relatively lower expression of Ras are less prone to serum-mediated induction of c-Myc and nucleophosmin/B23 expression. Expression and up-regulation of c-Myc and nucleophosmin/B23 may be importantly linked to oncogenic growth in U1 cells.

c-Myc and nucleophosmin/B23 may thus play important roles in cellular proliferation associated with high degree of malignancy. Furthermore, there is very low c-Myc expression in U1 cells in the absence of serum. Other transcriptional factors such as YY1 and HIF1 that have been reported to play a role in regulating nucleophosmin/B23 promoter (Inouye and Seto, 1994; Li et al., 2004; Mai et al., 2005) could be involved in control of nucleophosmin/B23 promoter activity in U1 cells in the absence of serum. This may explain why U1 cells with such low c-Myc expression and basal level of nucleophosmin/B23 promoter activity could proliferate in the absence of serum.

Previous studies have shown that the expression of c-Myc correlates with nucleophosmin/B23 expression (Guo et al., 2000; Kim et al., 2000; Gnomom et al., 2001; Neiman et al., 2001). Wild-type Rat1 fibroblasts maintain a nucleophosmin/B23 transcript level 3.5-fold higher than fibroblasts bearing deletion of myc (Guo et al., 2000). In myc-overexpressing avian bursal neoplasia, nucleophosmin/B23 transcript level is 3.5-fold higher than in normal bursa (Gnomom et al., 2001). Adenoviral transfection of c-Myc into mice leads to a dramatic increase in liver nucleophosmin/B23 mRNA, which correlates with increasing Myc protein levels 3 to 5 days after infection (Kim et al., 2000). These studies indicate that increased Myc expression results in elevated nucleophosmin/B23 transcript level. Not only Myc binding site has been identified, but also c-Myc has been shown to be able to bind nucleophosmin/B23 promoter in vivo and in vitro. C-myc may play an active role in the transcription of nucleophosmin/B23 promoter (Zeller et al., 2001).

Elevated c-Myc expression is observed in a wide variety of human tumors (Nesbit et al., 1999). In many cases this is caused, at least in part, by gene amplification. In addition, point mutations are frequently found in the coding regions of c-Myc in some tumors. Hot spots for these mutations are

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found clustered around MAPK/ERK phosphorylation site (Sears et al., 1999, 2000). Activation of the Ras pathway extends the half-life of c-Myc and thus the accumulation of c-Myc activity. Phosphorylations through the action of MAPK/ERK pathway are critical for controlling c-Myc protein accumulation (Sears et al., 1999, 2000). Our present results showed that a MEK inhibitor, PD98059, could depress the nucleophosmin/B23 transcription in minus serum condition and during serum stimulation. Taken together, nucleophosmin/B23 is regulated in the signaling pathway involving MAPK/ERK and c-Myc.

In our analysis of the possible physiological role of Ras on c-Myc and nucleophosmin/B23, our data have revealed that constitutive expression of dominant-negative Ras abrogates the serum-induced increase of nucleophosmin/B23 promoter activity and the binding of c-Myc to the promoter in U1 cells. The present study has also linked Ras, c-Myc, and nucleophosmin/B23 to cancer growth. Although previous studies suggested the role of Ras, c-Myc, and nucleophosmin/B23 in transformation (Mulcahy et al., 1985; Cole, 1986; Chan et al., 1989; Kondo et al., 1997), it was unclear whether nucleophosmin/B23 acts as a mediator of Ras oncogenic transformation. Here, we have shown that oncogenic Ras activates nucleophosmin/B23 expression by inducing c-Myc binding to the promoter. Our study has revealed an important molecular mechanism for the Ras oncogenic function through which up-regulation of nucleolar protein nucleophosmin/B23 is achieved.

Previous results have demonstrated that cells with overexpressed nucleophosmin/B23 could reach a higher cell density and exhibit anchorage-independent growth and tumorigenicity in nude mice (Chan et al., 1989; Kondo et al., 1997). Nucleophosmin/B23 is notably correlated with growth capacity and malignancy of cancer cells. Our discovery thus strengthens the notion that nucleophosmin/B23, being a special target of c-Myc-Ras-activated pathway, plays a significant role in neoplastic growth of human cancers. In agreement with this finding, we have reported recently that nucleophosmin/B23 overexpression is associated with bladder cancer recurrence and progression. Patients with nucleophosmin/B23 mRNA overexpression are at significantly greater risk of disease recurrence and progression than those having low expressions of nucleophosmin/B23 (Tsui et al., 2004). Considering that Ras and c-Myc are significantly associated with tumor progression in many types of malignant cells, nucleophosmin/B23 together with Ras and c-Myc could then be potential molecular targets for therapeutic intervention of cancer development.

Nucleophosmin/B23 has been shown to be a natural repressor of p53 that may contribute to dampening p53 function during cellular growth or in the presence of low levels of DNA damage (Maiguel et al., 2004). Previous data suggest that nucleophosmin/B23 could contribute to suppressing p53 activation until its functions are absolutely required, whereas in cancer cells, overexpression of nucleophosmin/B23 could contribute to p53 inactivation and tumor progression. In addition, we have shown recently that p53 protein and its transcriptional activities are elevated in U1 cells treated with nucleophosmin/B23-siRNA (Chan et al., 2005). Together with the present study, these data illustrate the significance of the functional linkages among Ras, c-Myc, and nucleophosmin/B23 and further strengthen the role of nucleophosmin/B23 in cancer progression. On the other hand, nucleophosmin/B23 has been implicated in the stabilization of p53 and senescence in normal fibroblasts in two apparently contradictory reports (Colombo et al., 2002; Kurki et al., 2004). It has been shown recently that nucleophosmin/B23 is required for the stability of tumor suppressor Arf and cells null for p53 and nucleophosmin/B23 are more susceptible to transformation by activated oncogene (Colombo et al., 2005). Such discrepancy may be due to the levels of nucleophosmin/B23 activity in the systems under study as explained by Maiguel et al. (2004). Given that nucleophosmin/B23 is functionally associated with p53 and Arf, it is thus important in the future to determine how nucleophosmin/B23 differentially regulates p53 and Arf in systems of different levels of nucleophosmin/B23 activity. Although there are no significant differences in p53 expressions in U1 and U4 cells (Y.-C. Liang, unpublished data), the issues of whether there are different p53 genotypes or Arf-p53 status and whether nucleophosmin/B23 differentially regulates p53 and Arf in U1 and U4 cells warrant further investigation.

In conclusion, our results have provided strong evidence that Ras has a role in the up-regulation of nucleophosmin/B23 in cancer cells. The positive regulation of nucleophosmin/B23 is achieved by the binding of c-Myc to the nucleophosmin/B23 promoter. These results demonstrate a signaling cascade involving Ras, c-Myc, and nucleophosmin/B23 in cancer growth.

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