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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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Running Title: Ras and c-Myc regulate nucleophosmin/B23

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Abbreviations: Dn-Ras, Ras dominant negative; MEK, MAPK/ERK activating kinase; ERK, extracellular-signal regulated kinase; MAPK, mitogen activated protein kinase; IRF-1, interferon regulatory factor-1; PI3K, phosphatidylinositol 3’-kinase.
Abstract

U1 bladder cancer cells of high malignancy exhibited higher proliferation capacity than U4 pre-malignant cells. Higher expression of Ras, c-Myc, and nucleophosmin/B23 as well as greater c-Myc transactivation and nucleophosmin/B23 promoter activities were detected in U1 cells as compared to U4 cells. Moreover, c-Myc and nucleophosmin/B23 were increased in U1 but not in U4 cells upon serum stimulation from quiescence. Similarly, 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 MEK inhibitor and was associated with recruitment of c-Myc to the promoter. Constitutive expression of Ras dominant negative (Dn-Ras-U1) 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 have indicated that Ras and c-Myc play important role in the up-regulation of nucleophosmin/B23 during proliferation of cells associated with high degree of malignancy, thus outlining a signaling cascade involving these factors in the cancer cells.
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Introduction

Transition cell carcinoma of the bladder is the second most malignancy of the genitourinary tract and the second most common cause of death from genitourinary tumors (Raghaven et al., 1990; Konetry & 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 (Foresman & Messing, 1997; Newling, 1996).

The nucleophosmin/B23 gene (also known as B23) appears to be involved in control of cell growth, cell differentiation, and programmed cell death (Hsu & Yung, 1998; Liu & Yung, 1998; You et al., 1999). Nucleophosmin/B23 has been known to have 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 HIV Type 1 Rev protein.
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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 (IRF-1), which is a tumor suppressor (Feuerstein et al., 1988; Borer et al., 1989; Inouye & Seto, 1994; Fankhauser et al., 1991; Adachi et al., 1993; Valdez et al., 1994; Tanaka et al., 1994; Kondo et al., 1997). Nucleophosmin/B23 is significantly more abundant in tumor and proliferating cells than normal resting cells. Nucleophosmin/B23 over-expression at the RNA and protein levels may contribute to onset of cancer (Feuerstein et al., 1988; Kondo et al., 1997; Chan et al., 1989). Blockage of nucleophosmin/B23 expression with its antisense oligonucleotides has shown that nucleophosmin/B23 is crucial for rendering cancer cells resistant to induction of differentiation and apoptosis (Hsu & Yung, 1998; Liu & Yung, 1998; You et al., 1999). It thus appears 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 & 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 interact with effector proteins such as Raf kinase, and phosphatidylinositol 3'-kinase (PI3K) proteins (Rodriguez-Viciana et al., 1997). Whereas these effector pathways exert essential roles in the signaling involved in Ras transformation, downstream factors play critical mediating role in 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 man 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 activation of genes involved in cell metabolism, proliferation, and apoptosis as well as through repression of genes that may promote cellular differentiation and cell cycle arrest.

Malignant progression is a complex and poorly understood process that appears 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 the 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 to those in the U4 pre-malignant 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 Ras dominant negative
(Dn-Ras-U1), no increase of nucleophosmin/B23 transcription or c-Myc binding to the promoter were 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.
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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 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% CO2 humidified incubator at 37°C. 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 mAb was kindly provided by Dr. P. K. Chan (Dept. 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, horse-radish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG were
from Chemicon International Inc. (Temecula, CA). Cell viability was assessed by 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 (Amersham Pharmacia Biotech, 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 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
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5'-GGACAAGAAUCCUUCAAGA-3'. It was synthesized and cloned into psilencer 2.0-U6 plasmid as described in 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 Corporation, Carlsbad, CA) method. Before transfection, cells (5x10^5-1x10^6) were seeded in 6-cm dishes overnight. Plasmid DNA (2 µg) and Lipofectamine™ Reagent (20 µg), 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-lipofetamin complexes formation. Cells were rinsed twice with PBS, and replaced to serum-free medium (1.5 ml), and then overlaid with DNA-liposome complexes. After 6 h of incubation at 37°C in CO₂ 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 5X10^5 and 0.5mg/ml of G418 (Calbiochem, San Diego, CA) was added to the cell culture at 48 hour after transfection. After selection with G418 for 3 weeks, individual clones were
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expanded to mass cultures and subsequently assayed for c-H-Ras expression. The transfectants were maintained in culture medium supplemented with 0.5mg/ml of G418.

**Soft Agar Assay.** Cells were cultured in 6-well plates (Nagle 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 2x10³ cells per 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 total RNA was reverse transcribed into cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) and random hexamer by incubating the reaction mixture (20 µl) at 37°C for 50 min. The polymerase chain reaction (PCR) was performed in a final volume of 25 µl solution containing 1 µl of reverse
transcribed cDNA, 75mM Tris-HCl (pH 8.8), 0.01% Tween 20, 2 mM MgCl₂, 200 µM of each dNTP, 0.5 µM of each primer (B23, forward: 5’-GAATTCTTGTTGAAGCAGAGGCAATG-3’, reverse: 5’-AAGCTTACTTCCTCCACTGCCAGAGA-3’, β-Actin, forward: 5’-GAATTCAGAAAATCTGGCACCAC-3’, reverse: 5’-AAGCTTCCATCTCTTGCTCGAAGTCC-3’) and 1U Taq DNA polymerase (GENEMARK Technology, Taipei, Taiwan). After an initial denaturation for 7 min at 95°, 22 cycles of amplification (95° for 1 min, 60° for 1 min and 72° for 1 min) were performed followed by a 7-min extension at 72°. 5µl 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 Na₂HPO₄, 100 mM NaCl, 0.2 mM PMSF). Lysates were boiled in SDS sample buffer [62.5 mM Tris (pH 6.8), 5% β-mercaptoethanol (Merck & Co., Inc. Whitehouse station, NJ), 10% glycerol, 2% SDS, 0.001% bromophenol blue], and then was fractionated by 12% SDS-PAGE. Separated proteins in SDS-PAGE were electrotransferred to Hybond-PVDF membrane (Amersham...
Pharmacia Biotech, Uppsala, Sweden). The PVDF membrane was then soaked in a blocking solution containing 5% (w/v) non-fat milk in TBST [20 mM Tris, pH 7.5, 0.5 M NaCl, 0.1% (v/v) Tween-20] for 1 h at room temperature. Antibody against indicated protein was diluted in TBST. The soaked PVDF membrane was then incubated with antibody for overnight at 4°C, and then washed with TBST three times for 15 min each and incubated in horse-radish 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, Amersham Pharmacia Biotech) and exposed to the X-ray film (Fuji Photo Film Corporation, Tokyo, Japan).

**Luciferase and β–Galactosidase Activity Assays.** Cells (5x10^5 -1x10^6) 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 hours. Cells were washed with PBS twice and lysed in Reporter Lysis Buffer (Promega). 20 µl of cell lysate was mixed with 50 µl of the luciferase assay substrate (Promega). Luciferase activity was quantified in a LMax II microplate
reader (Molecular Devices). For β–galactosidase activity assay, 100 µl of cell lysate was mixed with 100 µl of the 2X reaction buffer [200 mM sodium phosphate buffer (pH7.3), 2 mM MgCl₂, 100 mM β-mercaptoethanol, 1.33 mg/ml ONPG (o-nitrophenyl-β-D-galactopyranoside)] and was incubated at 37°C for 30 min. 500 µl of Na₂CO₃ 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, 125mM) 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 [5mM Pipes (pH8.0), 85mMKCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1µg/ml leupeptin and 1µg/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 [50mM Tris (ph 8.1), 10mM EDTA, 1% SDS 1 mM phenylmethylsulfonyl fluoride, 1µg/ml leupeptin and 1µg/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
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for 1 hour at 4°C with agitation. Protein concentration of precleared supernatant was
determined and 300µg 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, 0.1M
NaHCO3 were carried out. Crosslinks were reversed by addition of NaCl to a final
concentration of 200 mM, and RNA was removed by addition of 10 µg of RNase A per
sample followed by incubation at 65°C for 5 hr. The samples were then mixed with 10 µl
of 0.5 mM EDTA, 20 µl of 1M Tris-HCl, pH 6.5 and 2 µl of 10 mg/ml Proteinase K and
incubated for 1 hr at 45°C. DNA was extracted with phenol:chloroform:isoamyl alcohol
(25:24:1) and resuspended in 20 µl of H2O. Total input samples were resuspended in
100 µl of H2O before PCR. PCR reactions contained 1 µl of immunoprecipitate, 75mM
Tris-HCl (pH8.8), 0.01% Tween 20, 2 mM MgCl2, 200 µM of each dNTP, 0.5 µM of each
primer and 1U 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 AC091980). The
control primers for human vimentin promoter designed from human genomic sequence
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(GenBank™ accession 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). Briefly, Cells were washed twice by ice-cold PBS and harvested in the ice-cold cell lysis buffer [20mM Tris (ph7.5), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 1µg/ml leupeptin and 1µg/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 Elk-1 fusion protein and 200 µM ATP in the kinase buffer [25mM Tris (pH 7.5), 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na₃VO₄, 10 mM MgCl₂] 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-phospho-serine monoclonal antibody overnight at 4°C followed by HRP-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).
Results

Ras, c-Myc and Nucleophosmin/B23 Were Highly Expressed in U1 Cells. U1 cells exhibited higher proliferation capacity than U4 cells (Fig. 1A). As compared to 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 was found to be significantly higher than U4 cells, consistent with a known important feature of such transformed cells (Fig. 1B). Interestingly, 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 as compared to 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, 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).
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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 12h) after serum addition and lysates were subjected to immunoblotting analysis. Level of nucleophosmin/B23 was found to be increased at 1h and peaked at 6h 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 when compared to 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%).
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in the promoter activity at 6h after re-supplementation 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).

Since 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 a MEK inhibitor, PD98059, depressed the nucleophosmin/B23 promoter activity in minus serum condition as well as 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 Thr 58 and Ser 62 is associated with degradation of Myc. Using antibody that recognizes c-Myc that is doubly phosphorylated at Thr 58 and Ser 62, 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 as compared to the control. Our results have demonstrated that MAPK/ERK and phosphorylation of c-Myc are
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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 6h post serum re-supplementation 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). Fig.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 at 6h after re-supplementation 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 over-expressed U4 cells. Our results showed that Ras, c-Myc and nucleophosmin/B23 were up-regulated by transient over-expression of
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Ras (Fig. 4A, upper panel). Measurement of transcription from the pGL3-B23I1 promoter showed higher promoter activity in the Ras transiently over-expressed than in vector-transfected U4 cells (Fig. 4A, lower panel). 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 pERK decreased in U1 cells constitutively expressing Ras dominant negative (Dn-Ras-U1) (Fig. 4B). The Erk kinase activity was also found to be lower in DN-Ras-U1 cells as compared to 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 have demonstrated 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 over-expression 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 & 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 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 on the same characterizations including growth, morphology, colony formation and tumorigenicity in nude mice, U1 cells represent cells with high degree
of malignancy, while U4 cells are considered to be pre-malignant (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 as compared to 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 role 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 & Seto, 1994; Mai et al., 2005; Li et al., 2004) 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 expression of c-Myc correlates with nucleophosmin/B23 expression (Guo et al., 2000; Gnomom et al., 2001; Kim et al., 2000; 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 dramatic increase in liver nucleophosmin/B23 mRNA, which correlates with increasing Myc protein levels 3-5 days post-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 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 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 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 Ras
dominant negative abrogates the serum-induced increase of nucleophosmin/B23 promoter activity as well as 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 (Kondo et al., 1997; Chan et al., 1989; Mulcahy et al., 1985; Cole, 1986), 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 through 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 over-expressed nucleophosmin/B23 could reach a higher cell density and exhibit anchorage-independent growth and tumorigeneicity in nude mice (Kondo et al., 1997; Chan et al., 1989). 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 recently have reported that nucleophosmin/B23 over-expression is associated with bladder cancer recurrence and progression. Patients with nucleophosmin/B23 mRNA over-expression 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 importantly 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 while in cancer cells over-expression of nucleophosmin/B23 could contribute to p53 inactivation and tumor progression. Additionally, we have recently shown 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 recently shown 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. While there are no significant differences in p53 expressions in U1 and U4 cells (our 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
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promoter. These results demonstrate a signaling cascade involving Ras, c-Myc and nucleophosmin/B23 in cancer growth.
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References


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Nucleophosmin/B23 regulates the stability and transcriptional activity of p53.

*Nature Cell Bio* **4**:529-533


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Tsui KH, Cheng AJ, Chang PL, Pan TL and Yung BYM. (2004) Association of


Footnotes

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Figure legends

Figure 1 ----- Ras, c-Myc and nucleophosmin/B23 were highly expressed in U1 cells. (A) U1 or U4 cells (1x10^5 cells per 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 indicated times. Viable cells were determined by trypan blue exclusion method, and cell numbers were obtained by counting with a hemocytometer. Points, means of triplicates ± standard deviations. (B) U1 or U4 cells were cultured in 6-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 2x10^3 cells per well. The colonies were stained (Upper Panel) and scored (Lower Panel) 28 days after seeding. Values were expressed as the means ± standard deviations for three determinations. (C) U1 and U4 cells were grown in RPMI containing 10% fetal bovine serum for 24h. 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 2µg 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 (5x10^5 of U1 and 1x10^6 of U4) were transiently transfected with 1µg pMyc-TA-Luc, pTA-Luc, 1µg nucleophosmin/B23 promoter plasmid (pGL3-B23I1) and 1µg
pSV-β-galactosidase (as an internal control) for 48h. 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 ± SD. *P < 0.05, as compared with U1 cells.

*Figure 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 72h. Cells were then grown in RPMI containing 10% fetal bovine serum for various times (0.5, 1, 2, 3, 6 or 12h). 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 (5x10^5 of U1 and 1x10^6 of U4) were transiently transfected with 1µg nucleophosmin/B23 promoter plasmid (pGL3-B23I1) and 1µg pSV-β-galactosidase (as an internal control). Cells were made quiescent by serum starvation for 72h. Cells were then grown in RPMI containing 10% fetal bovine serum for 6h (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 ± SD. *P < 0.05, as compared with nucleophosmin/B23 promoter activity upon serum stimulation and the control without serum stimulation. (C) U1 cells (5x10^5) were transiently transfected with 1µg nucleophosmin/B23 promoter
plasmid (pGL3-B23I1) and 1µg pSV-β-galactosidase (as an internal control). Cells were made quiescent by serum starvation for 72h. The cells were grown in the serum-deprived medium in the presence of 40µM MEK inhibitor, PD98059 (PD) for 1 h. Cells were then grown in RPMI containing 10% bovine calf serum for 6h. Cells were harvested and the promoter activity was determined. *P < 0.05, as compared with the control without serum stimulation and in the absence of inhibitor. (D) U1 cells (2x10^5) were treated with 10-50µM 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 (2x10^5) grown in serum-free medium were not treated as control (C) or treated with 40µM MEK inhibitor, PD98059 (PD) for 42h. Cells were then treated with 5 µM proteasome inhibitor MG132 for 6h 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 Thr 58 and Ser 62 (p-c-Myc), and β-actin were detected by Western blot using their specific antibodies and ECL reaction.

Figure 3 ------- c-Myc transcriptional activation of nucleophosmin/B23. (A) C-Myc transcriptional activity increased upon serum stimulation in U1 cells. U1 and U4 cells (5x10^5 of U1 and 1x10^6 of U4) were transiently transfected with 1µg pMyc-TA-Luc and 1µg pSV-β-galactosidase (as an internal control). Cells were made quiescent by serum starvation for 72h. Cells were then grown in RPMI containing 10% fetal bovine serum for 6h (U1 and U4 cells). Cells were harvested and lysed by reporter
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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 ± SD. *P < 0.05, as 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 1µg 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 1µg pSV-β-galactosidase (as an internal control). Cells were made quiescent by serum starvation for 72h. Cells were then grown in RPMI containing 10% fetal bovine serum for 6h. Cells were harvested and the promoter activity was determined. Bars, means of triplicates ± SD. *P < 0.05, as compared with nucleophosmin/B23 promoter activity upon serum stimulation and the control without serum stimulation. (D) U1 or U4 cells (1x10^7 of U1 and 2x10^7 of U4) were made quiescent by serum starvation for 72h. Cells were then grown in RPMI containing 10% fetal bovine serum for 6h. Cells were harvested. Chromatin was collected and ChIP analysis was performed for nucleophosmin/B23 (B23) and negative control vimentin using antibody against c-Myc.

Figure 4 ------ Ras-dependent regulation of nucleophosmin/B23. (A) Oncogenic Ras transiently over-expressed (Ras12V-U4) or vector-transfected U4 cells (pcDNA3.1-U4) were grown in RPMI containing 10% fetal bovine serum for 72h. Cells were harvested, washed and lysed. Equal amounts (20 µg) of proteins were
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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 (Upper panel). Oncogenic Ras transiently over-expressed (Ras12V-U4) or vector-transfected U4 cells (pcDNA3.1-U4) were also transiently transfected with 1µg nucleophosmin/B23 promoter plasmid (pGL3-B23I1) and 1µg pSV-β-galactosidase (as an internal control) in RPMI containing 10% fetal bovine serum for 72h. 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 ± SD. *P < 0.05, as compared to nucleophosmin/B23 promoter in vector-transfected control cells (Lower panel). (B) 5x10^5 of U1 cells (Wild type-U1), vector stably-expressed U1 cells (pcDNA3.1-U1) and Ras dominant negative stably-expressed U1 cells (DN-Ras-U1) were grown in RPMI containing 10% fetal bovine serum for 24h. 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 phospho-serine monoclonal antibody. (D) 5x10^5 of Vector stably-expressed (pcDNA3.1-U1) and Ras dominant negative stably-expressed U1
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cells (DN-Ras-U1) were transiently transfected with 1µg nucleophosmin/B23 promoter plasmid (pGL3-B23I1) or 1µg pMyc-TA-Luc and 1µg pSV-β-galactosidase (as an internal control). Cells were made quiescent by serum starvation for 72h. Cells were then grown in RPMI containing 10% fetal bovine serum for 6h. Cells were harvested and the promoter activity was determined. Bars, means of triplicates ± SD. *P < 0.05, as compared with nucleophosmin/B23 promoter activity upon serum stimulation and the control without serum stimulation. (E) 1x10^7 of Vector stably-expressed (pcDNA3.1-U1) and Ras dominant negative stably-expressed U1 cells (DN-Ras-U1) were made quiescent by serum starvation for 72h. Cells were then grown in RPMI containing 10% fetal bovine serum for 6h. Cells were harvested. Chromatin was collected and ChIP analysis was performed for nucleophosmin/B23 (B23) and negative control vimentin using antibody against c-Myc.

**Figure 5**---- Cell growth inhibition in U1-siB23 cells. (A) U1-vector (V1, V2) or U1-siB23 (SiB23-1, SiB23-2) cells (1x10^5 cells) were grown in RPMI containing 10% fetal bovine serum for 48h. Cells were harvested, washed and the lysates were centrifuged at 12,000 X 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 (1x10^4 cells per dish) were seeded onto 6-cm dishes. The cells were grown in RPMI containing 10% fetal bovine serum for 72h. Cells were harvested and viable cells were determined by tryphan blue exclusion method, and cell numbers were obtained by counting with a
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hemocytometer. Points, means of triplicates ± standard deviations. (C) U1-vector (V2) or U1-siB23 (SiB23-2) cells were cultured in 6-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 8x10^3 cells per well. The colonies were stained (Upper Panel) 28 days after seeding. Colonies with more than 30 cells were scored (Lower Panel). Values were expressed as the means ± standard deviations for three determinations. Bars, means of triplicates ± SD. *P < 0.05, as compared with U1-vector (V2) cells.
Figure 1

A. 

![Graph showing cell numbers over days in culture for different conditions: U1 + serum, U4 + serum, U1 - serum, U4 - serum.](image)

B. 

![Images of U1 and U4 cells with captions: Microscope observation 100X.](image)

![Bar chart showing numbers of colony formation.](image)
Figure 1

C.

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<td>P-ERK</td>
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<td>ERK</td>
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Protein expression and mRNA levels are shown for different conditions U1 and U4.
Figure 2

A.

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(+ serum)

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(+ serum, 0.5h)

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(± serum, 1h)

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(± serum, 6h)

<table>
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Figure 2

B.

pGL3-B2311

U1

U4

Serum

Serum

Relative luciferase activity [%]

0 20 40 60 80 100 120 140 160 180 200

- +

100% 153% 100% 105%

*
Figure 2 E.

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Figure 3

A.

pMyc-TA-Luc

U1

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U4

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B.

5' -552 exon 1 c-Myc exon 2 +2217

E box

+717 gggag CACGTGgttgC CACGTGgttgg +743
+717 gggag CCCGGGgttgCCACGTGgttgg +743
+717 gggag CACGTGgttgCCC GG Ggttgg +743
+717 gggag CCCGGGgttgC CCC GG Ggttgg +743

Wild-type
5'-E box mutation
3'-E box mutation
5',3'-E boxes mutations
Figure 3

C.

- Serum

D.

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Figure 4

A.

pcDNA3.1-U4
Ras12V-U4

Ras

B.

Wild type-U1
pcDNA3.1-U1
DN-Ras-U1

Ras

c-Myc

B23

β-Actin

pGL3-B23I1

218% ±

p-ERK

ERK

*C

p-Elk1

Relative luciferase activity (%)
Figure 4

D.

pGL3-B23I1
pcDNA3.1-U1

DN-Ras-U1

Relative luciferase activity (%)

-  100%
  +  168%

Serum

pMyc-TA-Luc
pcDNA3.1-U1

DN-Ras-U1

Relative luciferase activity (%)

-  100%
  +  194%

Serum

Downloaded from molpharm.aspetjournals.org at ASPET Journals on May 20, 2022
E.

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B23

Vimentin