Ectopic EBP2 expression enhances cyclin E1 expression and induces chromosome instability in HEK293 stable clones

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To explore the effects of deregulated expression of the EBNA1 binding protein 2 (EBP2) on cell growth, we generated human HEK293 stable clones constitutively expressing an EBP2-EGFP fusion protein. We found both RNA and protein levels of cyclin E1, a dominant oncoprotein, were elevated in the EBP2-EGFP stable clones. These findings were confirmed by flow cytometry bivariate analysis of cyclin expression versus DNA content. Moreover, the increase in p21 expression and the specific phosphorylation at Ser1981 of ATM and Ser15 of p53 were also observed in these stable clones, and these observations may explain the failure to observe an increase in Cdk2 kinase activity. In addition, after one year of passage culture, the EBP2-EGFP stable clones tended to lose 4 to 5 chromosomes per cell when compared to that of control cells. All of these findings provide a possible link between deregulated expression of EBP2 and tumor development. [BMB reports 2008; 41(10): 716-721]

INTRODUCTION

The EBNA1 binding protein 2 (p40/EBP2/NoBP) is a nucleolar protein that is expressed at high levels in human neoplasms (1). The human EBP2 protein exhibits the ability to interact with the viral EBNA1 protein (2) and human nucleolar FGF3 protein (3), as demonstrated by yeast two-hybrid analysis. Defects in the yeast EBP2 homologue, p40, inhibited the processing of 27S-A rRNA into 27S-B rRNA (4,5). Moreover, overexpression of human EBP2 protein increased mouse NIH3T3 cells growth and counteracted the inhibitory effect of nuclear FGF3 (3). In addition, suppression of human EBP2 by RNA silencing resulted in cell growth arrest (6). These findings suggest that the EBP2 protein plays a dual role in both rRNA processing and cell growth regulation.

Development and progression of tumors are often associated with chromosome abnormalities, including changes in structure and number (7,8). Centrosome hyperamplification has also been observed in various cancers and results in an unequal segregation of chromosomes (9). Deregression of the genes involved in DNA damage repair and cell cycle progression are implicated in the induction of chromosome instability. Recently, more attention has been focused on the correlation of cyclin E deregulation and chromosome instability (10,11).

The cyclin E-Cdk2 complex is an important regulator of entry into S phase during the mammalian cell cycle. Previous studies revealed that cdk2 knockout mice were viable and essentially developed normally (12). However, mice lacking both cyclins E1 and E2 died during midgestation due to placental abnormalities (13). These findings raise the possibility that some functions of cyclin E might be Cdk2-independent. In addition, ectopic cyclin E expression in established cell lines is associated with genetic instability and abnormal centrosome duplication (14,15), indicating that cyclin E also functions as a dominant oncoprotein.

To characterize the consequences of excess EBP2 during cell growth, we generated HEK293 stable clones that ectopically expressed an EBP2-EGFP fusion protein. Here, we demonstrated that ectopic EBP2-EGFP expression enhanced cyclin E1 expression. Moreover, it is noted that prolonged culture of the EBP2-EGFP stable clones induced chromosome instability. These findings provide a possible link between excess EBP2 expression and tumorigenesis.

RESULTS

Generation of EBP2-EGFP stable clones in HEK293 cells

HEK293 cells were transfected with the plasmid pEGFP-N1-EBP2-EGFP. The expression of the EBP2-EGFP fusion protein, which localized to nucleoli of interphase cells, was identified directly by fluorescent microscopy (data not shown). The EBP2-EGFP transfectants were treated with G418 for 2 weeks and the stable clones were selected by limiting dilution. The expression of 67-kD EBP2-EGFP fusion proteins in these stable clones was confirmed by Western blot analysis using anti-serum against EBP2 (Fig. 1A).
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Fig. 1. Increased cyclin E1 expression in EBP2-EGFP stable clones.
(A) The expression of the EBP2-EGFP fusion protein in parental cells (293), EGFP control cells (293G), and EBP2-EGFP stable clones (SC1 and SC3) was identified by Western blot analysis with antiserum against EBP2. The molecular weight of EBP2-EGFP and endogenous EBP2 were 67 kD and 40 kD, respectively. (B) Fifty micrograms of total protein isolated from the asynchronized cells were subjected to Western blot analysis for cyclin E1. The chemoluminescence intensity of cyclin E1 was normalized against that of β-actin and compared to the amount of wild-type HEK293. The average and the standard deviation of three independent experiments ± S.D. are shown (*indicates P < 0.01). (C) Stable clone 1 was transfected with the plasmid-based EGFP shRNA construct and selected using puromycin for 1 week. Fifty micrograms of total protein was used to investigate the expression levels of EBP2-EGFP, cyclin E1, and p21 using antibodies recognizing EGFP, cyclin E1, and p21, respectively. (D) Ten micrograms of total RNA was subjected to Northern blot analysis using DIG-labeled EBP2 RNA probes. The EtBr-stained image of 28S was used as the loading control.

Ectopic EBP2-EGFP expression increased cyclin E expression
To characterize the effect of EBP2-EGFP expression on cell cycle progression in HEK293 cells, cyclin D1, E1, A, and B were analyzed by Western blot assay. We found that cyclin E1 expression was elevated in the asynchronized EBP2-EGFP stable clones, but not cyclin D1, A, or B (Fig. 1B and data not shown). Moreover, the elevated cyclin E1 expression was reduced by suppressing the ectopic EBP2-EGFP expression via the expression of an EGFP short hairpin RNA (shRNA) (Fig. 1C).

To further investigate whether the increase in the cyclin E1 protein was due to the increase in transcription of cyclin E1 in the EBP2-EGFP stable clones, the mRNA levels of cyclin E1 were analyzed by Northern blot assay. As shown in Fig. 1D, we found that the mRNA levels of cyclin E1 were also augmented in the stable clones (SC1 and SC3). These data indicate that ectopic EBP2-EGFP expression in the HEK293 stable clones enhanced cyclin E1 expression at both the protein and mRNA levels.

Since the expression of cyclins fluctuate throughout the cell cycle, we performed flow cytometric bivariate analysis of cyclin expression versus DNA content to reveal changes in cyclin expression with respect to the cell cycle. We found that the amount of cyclin E1 was much higher in EBP2-EGFP stable clones during cell cycle progression relative to that of controls (Fig. 2). This result was consistent with the finding from Western blot analysis, as shown in Fig. 1B. The highest level of cyclin E1 was at the G1 phase, then gradually declined after entering S, and still further declined through G2/M. Expression of cyclins D1, A, and B were not apparently different between control cells (293 and 293G) and EBP2-EGFP stable clones (SC1 and SC3). These results indicate that cyclin E1 expression is indeed increased in the EBP2-EGFP stable clones and the accumulation of cyclin E1 protein is due to the increase in mRNA levels and not a defect in cyclin E1 degradation.

EBP2-EGFP-induced cyclin E expression does not increase Cdk2 activity
Previous studies showed that the effects of ectopic cyclin E1 expression on Cdk2 activity and cell cycle progression are different in murine and human cell lines (11,14,16). To assess the effect of ectopic EBP2-EGFP on Cdk2 activity and cell cycle progression, the stable clones were examined by flow cy-

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Fig. 3. Excess cyclin E1 expression did not influence Cdk2 kinase activity by initiating the ATM-p53-p21 system in HEK 293 stable clones. (A) Cdk2 expression in control cells and stable clones was determined by Western blot assay. (B) Cdk2 protein was immunoprecipitated from total protein and subjected to Cdk2 kinase activity assay. (C) The expression of p21 in the control cells and the stable clones was analyzed by Western blot analysis by using specific antibodies against p21. (D) The expression of p53 was determined by Western blot assay. (E) The expression and phosphorylation levels of ATM and ATR were analyzed by Western blot.

Disclosures
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In this study, we generated HEK293 stable clones that constitutively expressed the EBP2-EGFP fusion protein. We found that ectopic expression of EBP2-EGFP enhanced cyclin E1 expression at both the mRNA and protein levels in the HEK293 stable clones, but did not affect the expression of other cyclins. These findings were confirmed by flow cytometry bivariate analysis of cyclins versus DNA content. In addition, the augmented cyclin E1 expression was restored by suppression of the ectopic EBP2-EGFP expression via EGFP shRNA. The transcription of cyclin E is mainly regulated by the mitogen-cyclin D-Rb-E2F pathway (18,19) and the neoplasms produced with aberrant cyclin E1 expression are most frequently a result of mutations in this pathway (20,21). In this study, we did not detect a marked difference in cell proliferation or cell cycle progression (data not shown). However, cyclin E1 expression, a dominant oncprotein, was indeed augmented in the EBP2-EGFP stable clones. This result indicates that excess cyclin E1 expression caused by excess EBP2 expression may play a role in tumorigenesis.
Overexpression of cyclin E1 can increase Cdk2 kinase activity in primary REF and MEF cells, but not in primary human fibroblasts (11,14,16). This discrepancy might be a result of cell differences. In cultured normal human cells, cyclin E expression is tightly controlled, limited to a short period in late G1 and early S phases. In contrast, cyclin E expression is controlled less strictly, and increased levels of cyclin E often can be detected during early-mid G1 phase in cultured mouse cells (10). Minella et al. has reported that cyclin E overexpression can initiate a p53-dependent response to block excess Cdk2 activity by inducing p21. In addition, phosphorylation at Ser15 of p53 is one of the cell's responses to stresses. DNA damage could activate the ATM and ATR proteins to induce p53 phosphorylation at serine 15 and thus increase p21 expression (16). These findings indicate that in response to cyclin E deregulation, p53 and p21 form an inducible barrier to prevent cell cycle anomalies. In this study, we indeed found that excess cyclin E1 expression resulted from ectopic EBP2-EGFP expression could induce induced p21 expression, and this event may protect Cdk2 function from deregulation in the presence of excess cyclin E. This may be the reason that cell proliferation and the cell cycle were not significantly altered in the EBP2-EGFP stable clones. On the other hand, we also found that the induction of p21 expression was not due to increased p53 expression, as the total p53 protein levels were not significantly different between control and EBP2-EGFP stable clones. This may be the result of increased phosphorylation at Ser15 of p53 via ATM activation (but not ATR) in the EBP2-EGFP stable clones.

Chromosome instability is believed to play an important role in carcinogenesis through promoting accumulation of mutations responsible for the malignant phenotypes (9,22). The association of cyclin E1 with centrosome amplification has been reported. In mouse embryonic fibroblasts, ectopic cyclin E1 overexpression can induce centrosome overproduction (23) and cause chromosome instability (14). In addition, it was found that centrosome duplication is normal in cdk2 null MEF cells. This indicates that Cdk2 activity is not required for normal centrosome duplication. Moreover, a kinase-deficient cyclin E1 mutant can still localize to centrosomes and chromatin during G0 to S phase progression, followed by stimulation of DNA synthesis (24,25). These finding indicated that the Cdk-independent functions of cyclin E play a vital role in cell cycle progression and cell transformation. Whether the chromosome instability in EBP2-EGFP clones is due to cyclin E1-induced centrosome overproduction will be investigated in a future study.

In conclusion, our study demonstrated that ectopic EBP2-EGFP expression elevated the expression of multifunctional cyclin E1, a dominant oncprotein, in stable clones. In addition, we also noted that prolonged culture of HEK293 cells under such an intracellular situation leads to chromosome instability. These findings suggest a possible role for EBP2 deregulation in tumorigenesis.

### MATERIALS AND METHODS

**EBP2-EGFP stable clone selection**

The human embryonic kidney cell line (HEK293) was cultured in DMEM (Gibco, Invitrogen Corporation, USA) supplemented with 10% FBS as well as 100 U/ml of both penicillin and streptomycin (Gibco, Invitrogen Corporation, USA). The human EBP2 cDNA was cloned into the pEGFP-N1 plasmid (Clontech Laboratories, USA) to generate the recombinant EBP2-EGFP. HEK293 cells were transfected using Lipofectamine (Invitrogen Corporation, USA). For stable clone selection, the transfected cells were cultured in the presence of 800 μg/ml G418 sulfate (Calbiochem, EMD Chemicals, Inc., USA). Stably transfected single cell-derived clones were obtained by limiting dilution.

**Knockdown of ectopic EBP2-EGFP by EGFP shRNA**

The plasmid-based EGFP shRNA construct was purchased from Open-Biosystems (Birmingham, AL). The EBP2-EGFP stable clone (SC1) was transfected with the EGFP shRNA construct using Lipofectamine (Invitrogen Corporation, USA) under the conditions suggested by the manufacturer. A stably expressing EGFP shRNA clone was generated by adding 2 μg/ml puromycin (Sigma-Aldrich Corporation, USA) 24 hours post-transfection. Populations of resistant clones were detected 7 days post-transfection.

**Northern blot analysis**

Ten micrograms of RNA was fractionated by electrophoresis and electro-transferred onto a nylon membrane (Roche...
Corporation, Switzerland). The DIG-labeled EBP2 RNA probes were used for hybridization and detected by anti-DIG antibodies conjugated with alkaline phosphatase (Roche Corporation, Switzerland). CSPD substrate (Roche Corporation, Switzerland) was added and the chemiluminescence signal was recorded on x-ray films.

Western blot analysis
Cells were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% Triton X-100, 0.1 mM sodium orthovanadate, 50 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride). Fifty micrograms of protein was fractionated via 6 or 10% SDS-PAGE and transferred to a PVDF membrane (Pall Corporation, USA). Primary antibodies used in this study included those against β-actin (Sigma-Aldrich Corporation, USA); p21, Cdk2, cyclin E (Santa Cruz Biotechnology, Inc., USA); ATM, ATR, phospho-ATR (Ser428) (Cell Signaling Technology, Inc. USA), and phospho-ATM (Ser1981) (Rockland, Gilbertsville, PA). EBP2 antiserum was generated by immunizing rabbits with synthetic peptide (amino acid residues 253-271: KKKGSKWNT RESYDDVSSF). An anti-phospho-p53 Sampler Kit (Cell Signaling Technology, Inc. USA) was used to identify the specific phosphorylation sites of p53. Bound primary antibodies were detected with goat anti-mouse or rabbit IgG conjugated with HRP. The chemiluminescence signal was recorded on x-ray films.

Flow cytometric bivariate analysis
Flow cytometric bivariate analysis was performed according to Gong et al. (26) with some modifications. Briefly, the cells were trypsinized and fixed with 80% ethanol. The fixed cells were blocked in buffer (0.1% Triton X-100 and 1% FBS in PBS) and hybridized with 1:200 diluted primary antibodies in washing buffer (0.1% Triton X-100 and 1% FBS in PBS). Primary antibodies included anti-cyclin D1 (H295), cyclin E1 (HE-12), cyclin A (H432), and cyclin B (GNS1) (Santa Cruz). Primary antibodies were detected by 1:500 diluted FITC-conjugated goat anti-mouse or rabbit IgG (Santa Cruz). Finally, cells were stained with propidium iodide and subjected to flow cytometric analysis.

Cdk2 kinase activity assay
Cdk2 proteins were immunoprecipitated using an anti-Cdk2 polyclonal antibody (M2, Santa Cruz) and protein A/G conjugated agarose beads (Calbiochem, EMD Chemicals, Inc., USA). Beads were washed with lysis buffer, resuspended in SDS-PAGE loading buffer and subjected to electrophoresis. The immunoprecipitated proteins were examined by Western blot. The kinase activity of Cdk2 was assayed according to Mazumder et al. (27) with some modifications. Briefly, the immunoprecipitated beads were washed with kinase buffer (50 mM HEPES, pH 7.3, 10 mM MgCl2, 1 mM DTT, and 5 mM MnCl2) and suspended in 40 μl of kinase buffer containing 2 μg of histone H1 as the substrate (Sigma), plus 50 μM ATP and 10 μCi of [32P] ATP. After incubation at 37°C for 30 min, the reactions were stopped with SDS-PAGE loading buffer, boiled for 5 min, and resolved via 10% SDS-PAGE. Phosphorylated histone H1 proteins were visualized by autoradiography.

Karyotype analysis
Sterilized 22 × 22 mm cover slips were placed into 6 well plates. 5 × 10^5 cells were seeded onto the cover slips for 24 hours. Cytological preparations were performed following the standard procedures (28).

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