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► **To cite this version:**

Fen Hu, Chuan Wang, Jun Du, Wei Sun, Jidong Yan, et al..  $\delta$ EF1 Promotes Breast Cancer Cell Proliferation through Down-regulating p21 Expression. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2010, 10.1016/j.bbadis.2009.12.002 . hal-00562950

**HAL Id: hal-00562950**

**<https://hal.science/hal-00562950>**

Submitted on 4 Feb 2011

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Accepted Manuscript

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PII: S0925-4439(09)00295-6  
DOI: doi:[10.1016/j.bbadis.2009.12.002](https://doi.org/10.1016/j.bbadis.2009.12.002)  
Reference: BBADIS 63041

To appear in: *BBA - Molecular Basis of Disease*

Received date: 14 August 2009  
Revised date: 20 November 2009  
Accepted date: 3 December 2009



Please cite this article as: Fen Hu, Chuan Wang, Jun Du, Wei Sun, Jidong Yan, Dong Mi, Jie Zhang, Yuhuan Qiao, Tianhui Zhu, Shuang Yang,  $\delta$ EF1 Promotes Breast Cancer Cell Proliferation through Down-regulating p21 Expression, *BBA - Molecular Basis of Disease* (2009), doi:[10.1016/j.bbadis.2009.12.002](https://doi.org/10.1016/j.bbadis.2009.12.002)

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**$\delta$ EF1 Promotes Breast Cancer Cell Proliferation through Down-regulating p21  
Expression**

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Running Title: Mechanism of Cell Cycle Regulation by  $\delta$ EF1 in Breast Cancer

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**Keywords:**  $\delta$ EF1; p21; CDK; cell cycle; breast cancer proliferation

**Abstract**

Although the zinc finger-homeodomain transcription factor  $\delta$ EF1 is implied as a regulatory factor at the crossroad between proliferation and differentiation in carcinogenesis, its potential effect in the regulation of cell cycle progression has not been well elucidated. In our present study, we provide novel finding that, in breast cancer, the ectopic expression of  $\delta$ EF1 in MDA-MB-231 cells significantly promoted cell proliferation by increasing the cell number in S phase of the cell cycle. In contrast,  $\delta$ EF1 knockdown by RNA interference exhibited an opposite effect, highlighting a potent role of  $\delta$ EF1 to promote G1-S transition of breast cancer cells. Moreover, we demonstrated that  $\delta$ EF1 down-regulated p21 and concurrently up-regulated the expressions of CDK2 and CDK4 during this process. Further,  $\delta$ EF1 inhibited p21 transcription by recruiting to the E<sub>2</sub>-box element on the p21 promoter. Depletion of endogenous  $\delta$ EF1 in MDA-MB-231 cells was sufficient to allow an inherent release of p21 expression, thus resulting in the cell cycle arrest. In addition, the stimulatory effect of  $\delta$ EF1 on cell proliferation through p21 regulation was supported by an inverse correlation of  $\delta$ EF1 and p21 expressions observed in both breast cancer cell lines and clinical tumor specimens. Taken together, these observations suggest a dual effect of  $\delta$ EF1 in promoting breast cancer cell proliferation, by differentially regulating the cell cycle regulatory proteins.

## 1. Introduction

Breast cancer cell progression is a coordinated process that comprises cell cycle dysregulation and a specific gene expression program to determine tissue identity. Many studies have provided knowledge about the molecules involved in cell proliferation and key regulatory transcription factors that control tissue specific gene expression [1,2]. At different phases, progression through cell cycle is regulated by sequential activation and subsequent inactivation of a series of cyclin-dependent kinases (CDKs), whose activity depends on interactions with timely expressed cyclins and cyclin-dependent kinase inhibitors (CDKIs) [3-5]. For instance, the transition from G1 to S phase requires the activation of two classes of CDKs, CDK4/6 and CDK2. As cells emerge from quiescence in response to mitogenic stimuli, D type cyclins are synthesized and associate with CDK4/6. The cyclin D-CDK4/6 complex hyperphosphorylates retinoblastoma protein (Rb), leading to its release from transcription factor E2F. The freed transcription factor E2F would then activate the genes responsible for cellular proliferation including cyclin E. Cyclin E binds to and activates CDK2 kinase contributing to G1-to-S phase progression [6-8]. Among the CDKIs, p16<sup>INK4A</sup> (p16), a member of the INK4 protein family, is specifically induced at the end of the G1 phase in response to pRB phosphorylation as a retrocontrol mechanism to inhibit CDK4/6. In addition, p21<sup>Cip1/WAF1</sup> (p21) and p27<sup>Kip1</sup> (p27), members of the Cip/Kip family, inhibit a broad range of CDKs, including CDK4/6 and CDK2 [9-11]. Given that decreased expression of p21 is associated with the promotion of tumor formation and a poor prognosis in many types of cancer [11,12], it is of significance to characterize the mechanisms underlying the transcriptional regulation of p21 genes, for an understanding of the genesis of cancers and for a search of novel therapeutic target for cancer [13,14].

Recent reports provided evidence that the zinc finger-homeodomain transcription factor  $\delta$ EF1 regulates gene expression to modulate cell differentiation and tissue-specific functions [15-21]. Presence of  $\delta$ EF1 has been implicated in the inhibition of differentiation of multiple cell lineages including muscle [17], cartilage [18], bone

[19,20], and neural cells [21]. Further studies revealed that  $\delta$ EF1 is also involved in the epithelial-mesenchymal transition (EMT) that takes place concomitant with the acquisition of progressive properties in tumors [22-27].  $\delta$ EF1 is expressed in invasive cells of various tumors, including lung [22], squamous [23], uterine [24], and breast cancers [25-27].  $\delta$ EF1 appears to be an early marker of malignant phenotype as well as a prognostic factor [28]. In addition,  $\delta$ EF1 is implicated at the crossroad between proliferation and differentiation by its role in repressing p73 expression [29-31].  $\delta$ EF1 could indeed act as a primary survival factor and inducer of cell movement, apart from its role in EMT. Notwithstanding, a direct role for  $\delta$ EF1 in cell proliferation and its relation with EMT or other  $\delta$ EF1-associated functions has yet to be established.

To address this issue, we have performed studies on the gain or loss of function of  $\delta$ EF1 in the highly undifferentiated human breast carcinoma MDAMB-231 cell line. Flow cytometry assays after BrdU incorporation present evidence that  $\delta$ EF1 overexpression promoted cell cycle progression by increasing cell number in the S phase, whereas  $\delta$ EF1 knockdown exhibited an opposite effect. Importantly, to control breast cancer cell proliferation,  $\delta$ EF1 down-regulated p21 and concurrently up-regulated the expressions of CDK2 and CDK4. These data collectively suggest a significant role of  $\delta$ EF1 in breast cancer tumor growth and metastasis.

## 2. Materials and Methods

### 2.1. Tissue Samples

Fresh breast cancer tissues of invasive ductal carcinoma were obtained from the Tissue Banking Facility Jointly Supported by TMUCIH (Tianjin Medical University Cancer Institute and Hospital) & NFCR (National Foundation for Cancer Research). Tissue samples were stored at  $-70^{\circ}\text{C}$  until being used in the assay. All patients were histologically confirmed as having breast cancers and were recruited in the same department. The study was approved by the institutional ethics committee.

### 2.2. Cell Culture

MDA-MB-231, MCF-7, T47D, and ZR-75-1 cells were maintained in DMEM-high glucose medium (GIBCO) supplemented with 10% FBS (Hyclone), penicillin, and streptomycin. MDA-MB-231 cells were plated at a density of  $8 \times 10^4$  in 6-well plates for quantitative CHIP and western blotting assays. MDA-MB-231 cells were plated at a density of  $2 \times 10^4$  cells/well in 24-well plates for use in luciferase assays.

### 2.3. Plasmids Construction and Transfection

The generation of full-length  $\delta\text{EF1-myc}$  expression vector (pcDNA6B- $\delta$ -FL) has been previously described [19]. Human p21 promoter sequence (-557/+19) was obtained by PCR from human blood genomic DNA and cloned into the pGL3-basic vector (Promega) using the forward primer, 5'-GCTGAGCTCGTCTAGGTGCTCCAGGTGC-3', and reverse primer, 5'-GCAGATCTCGAGGAACTGAC-3'. Mutagenesis of the  $E_2$ -box in the human p21 promoter was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) with the forward primer: 5'-gtctaggtgctcCAGATGcttctgggagagg-3' and reverse primer: 5'-cctctcccagaagCATCTGgagcacctagac-3'.

MDA-MB-231 cells were plated in a 10-cm dish and were grown to confluent.  $2 \times 10^7$  cells were collected and washed twice with PBS, electroporated at 360 V, 950  $\mu\text{F}$  using a Gene Pulser II System (Bio-Rad). Transfected cells were resuspended in 2 ml of DMEM medium containing 10% FBS and seeded in a 6-well plate. Transient

transfection with pcDNA6B- $\delta$ -FL was performed with electroporation in MDA-MB-231 cells. Blasticidin-resistant clones were isolated over a period of 3-4 weeks. Overexpression of  $\delta$ EF1 was confirmed by western immunoblot analysis.

#### 2.4. Cell Proliferation Assay

MDA-MB-231 cells stably transfected with pcDNA6B- $\delta$ -FL or pcDNA6B vector control were plated in a 96-well plate at a concentration of 5,000 cells/well. At the indicated time point, the cell proliferation assay was performed by the addition of 10  $\mu$ l CCK-8 (Cell counting kit-8, Jindojin) solution to each well, followed by incubation at 37°C for 2 h. Absorbance was measured at a wavelength at 450 nm using a microplate reader.

#### 2.5. BrdU Incorporation and Flow cytometry

To evaluate proliferation, 5-bromo-2'-deoxyuridine (BrdU) incorporation was examined. MDA-MB-231 cells transiently transfected with pcDNA6B- $\delta$ -FL or pcDNA6B vector control were seeded onto 10-cm plates with a confluence level from 50% to 70%. Cells were incubated for 60 min with 10  $\mu$ M BrdU at 0, 24, 48, and 72 h after transfection. After the 60-min pulse, the cells were washed twice with PBS, removed from the plates, centrifuged at 500 $\times$ g for 5 min, and fixed with ice-cold 70% ethanol. Fixed cells proceeded for DNA fragment detection were washed with PBS, resuspended in 2 M HCl containing 0.5% (v/v) Triton X-100, and incubated at RT for 30 min. Cells were subsequently centrifuged and resuspended in 0.1 M Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> (pH 8.5) for 2 min. After a repeat centrifugation, cell pellets were resuspended in washing solution (PBS containing 0.5% (v/v) Tween 20 and 1% (w/v) BSA). The cells were spun down, and mouse monoclonal Ab against BrdU (sc-32323, Santa Cruz) diluted 1:100 in washing solution was added. The cells were incubated in the dark for 1 h at RT, washed and resuspended with washing solution. Alexa Fluor 488 goat anti-mouse IgG Ab (Invitrogen) diluted to a predetermined working concentration of 100-fold was added. After 1 h incubation at RT, cells were washed and resuspended in PBS.

For nuclear staining with propidium iodide, the cells were treated with 50  $\mu$ l RNase A (1 mg/ml) (Sigma) followed by 100  $\mu$ l propidium iodide (100  $\mu$ g/ml). Dual parameter measurements of green-versus-red fluorescence signals were taken by a flow cytometry on FACcalibur (BD) equipped with argon laser excitation (power 200 mW) at 488 nm, a 510–540 nm interference filter for the detection of FITC green fluorescence, and a 610-nm long-pass filter for the detection of PI red fluorescence. At least 20,000 cells/sample were measured and subsequently analyzed by MODFIT software.

## 2.6. RNA Extraction and Quantitative RT-PCR

Total RNA was extracted from MDA-MB-231, MCF-7, T47D, and ZR-75-1 cells using the TRIzol Reagent (Invitrogen). 0.5  $\mu$ g of the total RNA from each sample was used for first strand cDNA synthesis (M-MLV Reverse Transcriptase, Promega). Specific transcripts of human CDK2, CDK4, CDK6, and human p21 were amplified by quantitative PCR using the following primers: CDK2, 5'-GTCCCCAGAGTCCGAAAGAT-3' (forward), and 5'-GCTTTCTGCCATTCTCATCG-3' (reverse); CDK4, 5'-ACGGGTGTAAGTGCCATCTG-3' (forward), and 5'-TGGTGTCGGTGCCTATGGGA-3' (reverse); CDK6, 5'-CCACTGAGGTTAGAGCCATC-3' (forward), and 5'-CGAATGCGTGGCGGAGATC-3' (reverse); p21, 5'-GCGGAACAAGGAGTCAGA-3' (forward), and 5'-GGAGAAACGGGAACCAG-3' (reverse). GAPDH was used as an internal control. Verification of the expression levels of genes was performed by quantitative RT-PCR using EvaGreen (Biotium). The expression level was expressed as the threshold cycle ( $C_T$ ) values of the target and reference gene-GAPDH, which is constitutively expressed. Comparison and calculation of  $C_T$  values was used to determine the relative mRNA levels expressed as the fold change of target genes relative to the reference gene.

### 2.7. Western Immunoblot Analysis and Antibodies

Preparation of total cell extracts and western blotting analysis with the appropriate antibodies was performed as described [19]. The following antibodies (Abs) were used: goat polyclonal Ab against the N-terminal epitope of  $\delta$ EF1 (ZEB-E20, Santa Cruz); rabbit polyclonal Ab against CDK2 (sc-163, Santa Cruz); rabbit polyclonal Ab against CDK4 (sc-260, Santa Cruz); mouse monoclonal Ab against p21 (sc-6246, Santa Cruz); mouse monoclonal Ab against p27 (sc-56454, Santa Cruz); mouse monoclonal Ab against Actin (A-4700, Sigma).

### 2.8. Preparation of Small Interfering RNAs

The target sequence of the siRNA for human  $\delta$ EF1 is 5'-TGATCAGCCTCAATCTGCA-3' as previously reported [25]. Sense and antisense oligonucleotides with the internal loop were synthesized (TaKaRa). These were annealed and ligated into the BamHI and HindIII sites of pSilencer 4.1-CMVneo (Ambion) to construct the  $\delta$ EF1-specific and the BMP-6-specific siRNA expression plasmids, respectively, according to the manufacturer's instructions. pSilencer 4.1-CMVneo expressing scrambled siRNAs (Ambion) were used as controls. Transient transfection with siRNAs was performed with electroporation in MDA-MB-231 cells. G418-resistant clones were isolated over a period of 3-4 weeks. Down-regulation of  $\delta$ EF1 was confirmed by western immunoblot analysis.

### 2.9. Luciferase Assay

MDA-MB-231 cells were co-transfected with wild-type or mutant human p21 promoter constructs in 24-well plates using Lipofectamine 2000 (Invitrogen). Lysates were prepared 24 h after transfection. The luciferase activity was then measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase activity was normalized using the Renilla luciferase activity.

### 2.10. Chromatin Immunoprecipitation (CHIP) and Quantitative CHIP Assays

ChIP assays were performed using reagents commercially obtained from Upstate, essentially according to the manufacturer's instructions. The antibodies (10  $\mu$ g) used in these experiments were rabbit polyclonal antibody against the N-terminal epitope of  $\delta$ EF1 (ZEB-E20, Santa Cruz), anti-FLAG M2 antibody (F3165, Sigma), and anti-mouse normal IgG (sc-2025, Santa Cruz). The amount of each specific DNA fragment in immunoprecipitates was determined by PCR or quantitative PCR reactions. The fragment of human p21 promoter, containing the E<sub>2</sub>-box, was PCR-amplified using the following primers: 5'-TAAACGGGACTGAAAA-3' (forward), and 5'-GAAAAGCCCCACAAT-3' (reverse). For quantitative CHIP, a standard curve was generated for p21 promoter using the following primers: 5'-TGTGAGGTAGATGGGAGC-3' (forward), and 5'-CAGATCATGCAGCAAAGA-3' (reverse). Copy numbers for the DNA fragments -577/-449 bp of p21 promoter in each anti-ZEB-immunoprecipitated sample were determined and compared to copy numbers of the DNA fragment without IP (input DNA). Anti-FLAG antibody was used as controls for IP reactions. The percentage of the input was then calculated. The final value was the percentage of the DNA input obtained from specific antibody-IP samples subtracting the percentage of DNA input obtained from control antibody-IP samples. The dissociation curve was determined for each quantitative PCR reaction to assure that a single band was produced. Each data point represents three independent samples.

### 3. Results

#### 3.1. *ΔEF1* overexpression promotes the proliferation of MDA-MB-231 breast cancer cells

We previously reported that  $\delta$ EF1 overexpression stimulates the proliferation of murine pre-myoblast C2C12 cells, which effect is independent of BMP-2 activity [19]. In the present study, we assess the effect of  $\delta$ EF1 in regulating cell cycle progression of breast cancer using cell proliferation assay. MDA-MB-231 breast cancer cells were stably transfected with full-length  $\delta$ EF1 expression plasmid or a siRNA plasmid targeting  $\delta$ EF1. Overexpression or knockdown of  $\delta$ EF1 was confirmed by western blotting (Fig. 1a and b). As seen in Fig. 1c, in  $\delta$ EF1-overexpressing cells, cell number increased significantly after 24 h, in comparison to the empty vector transfected cells. In contrast, depletion of  $\delta$ EF1 in MDA-MB-231 cells exhibited a decreased proliferative rate, compared to the control cells (Fig. 1c), indicating a promoting effect of  $\delta$ EF1 on proliferation of MDA-MB-231 cells. Furthermore, flow cytometric measurements after BrdU incorporation confirmed an increase in the number of cells in the S phase after  $\delta$ EF1 overexpression in MDA-MB-231 cells. Full-length  $\delta$ EF1 expression plasmid or the empty vector control was respectively introduced into MDA-MB-231 cells. At 24 h after transfection, the percentage of cells in the S phase of  $\delta$ EF1-overexpressing sample was  $70.8 \pm 1.2\%$ , while that of the control was  $61.4 \pm 0.9\%$  (Fig. 1d). At 48 h, the cell percentage in the S phase of  $\delta$ EF1-overexpressing sample was  $58.5 \pm 0.7\%$ , compared to that of the control which was  $50.7 \pm 0.4\%$  (Fig. 1d). Based on this, we hypothesized that knockdown of  $\delta$ EF1 using RNA interference would result in a decrease of cell number in the S phase. To do so, a siRNA expression plasmid targeting  $\delta$ EF1 or a scrambled control siRNA plasmid was transiently transfected into MDA-MB-231 cells. The knockdown of  $\delta$ EF1 expression was shown by western blotting (Fig. 1e). As shown in Fig. 1e, at 24 h after transfection, the cell number in the S phase of  $\delta$ EF1-interfered sample was  $33.0 \pm 1.0\%$ , while that of the control was  $46.6 \pm 0.8\%$ . At 48 h, the percentage of cells in the S phase of  $\delta$ EF1-interfered sample was  $42.3 \pm 1.3\%$ ,

compared to that of the control which was  $48.0 \pm 1.4\%$  (Fig. 1e). Taken together, these data suggest that  $\delta$ EF1 significantly promotes cell cycle progression of S phase in MDA-MB-231 cells.

### *3.2. $\delta$ EF1 controls MDA-MB-231 cell proliferation by concurrently down-regulating p21 and up-regulating CDK2 and CDK4*

To understand the molecular changes, we performed the quantitative RT-PCR to examine the effect of  $\delta$ EF1 on the CDK inhibitors p21 and p27 involved in the G1-S transition. As shown in Fig. 2a, p21 mRNA level was significantly down-regulated after  $\delta$ EF1 overexpression.  $\delta$ EF1 transfection for 3 h resulted in an up to 40% decrease in the expression of p21 mRNA, compared to the basal level (Fig. 2a). The inhibition was further down to about 70% between 6 to 24 h (Fig. 2a). At the protein level,  $\delta$ EF1 transfection resulted in an up to 60% decrease in the expression of p21 protein as early as 12 h, compared to the empty vector control. In 24 h, repression of p21 expression by  $\delta$ EF1 became milder and was maintained at about 20% (Fig. 2b). In contrast, depletion of  $\delta$ EF1 exhibited a marked up-regulation (up to 3.8-fold relative to that of the control at 24 h) in the mRNA level of p21 in MDA-MB-231 cells (Fig. 2c). However, the expression of p27 was not significantly affected by  $\delta$ EF1 overexpression (Fig. 2b). These observations indicated that down-regulation of p21 occurred at the transcriptional level and was specifically mediated by  $\delta$ EF1, contributing to the promotion of breast cancer cell proliferation.

We further moved on to assess the potential effect of  $\delta$ EF1 on cell cycle regulatory protein CDKs, including CDK2, CDK4 and CDK6 involved in the G1-S transition. Full-length  $\delta$ EF1 expression plasmid was transiently transfected into MDA-MB-231 cells. Total RNAs were collected at 0, 24, 48, and 72 h following each transfection. The results of quantitative RT-PCR showed that  $\delta$ EF1 overexpression up-regulated the mRNA levels of CDK2 and CDK4 in a time-dependent manner.  $\delta$ EF1 overexpression resulted in an up to 1.8-fold increase in the expression of CDK2 mRNA at 3 h, compared to the basal level. In addition,  $\delta$ EF1 transfection for 6-12 h

also showed an induction to 3~3.5-fold in the expression of CDK4 mRNA (Fig. 3a). On the other hand, depletion of  $\delta$ EF1 expression in MDA-MB-231 cells resulted in a decreased mRNA level of CDK2 and CDK4, compared to the control cells (Fig. 3b). CDK6 mRNA level was not as affected (data not shown). Moreover, western blotting demonstrated that both of CDK2 and CDK4 protein levels were markedly up-regulated by  $\delta$ EF1 transfection at 12-24 h. The above observations suggested a dual effect of  $\delta$ EF1 in promoting MDA-MB-231 cell proliferation, through concurrently down-regulating p21 and up-regulating CDK2 and CDK4.

### 3.3. $\delta$ EF1 represses the transcriptional activity of p21 promoter by binding to the E<sub>2</sub> box (CAGGTG) element

Having found that  $\delta$ EF1 inhibited p21 expression in MDA-MB-231 cells, we were curious about whether  $\delta$ EF1 is a *bona fide* repressor of p21 transcription. As shown in Fig. 4b, a reporter gene assay showed that  $\delta$ EF1 overexpression significantly repressed human p21 promoter activity of the wild-type -557/+19 reporter gene. The repression was about 50% relative to the control without  $\delta$ EF1 transfection. Meanwhile, we found that  $\delta$ EF1 regulated the promoter activity of p21 in a dose-dependent manner (Fig. 4c), confirming a role of  $\delta$ EF1 in the negative regulation of p21 transcription.

$\delta$ EF1 has been reported to function as a transcriptional repressor by directly binding to the E<sub>2</sub> box [CA(C/G)(C/G)TG] in the promoter region of target genes with its zinc finger clusters which are located close to the N and C termini of the molecule [32,33]. In this present report, a search using the transcription factor database TESS identified an E<sub>2</sub> box (CAGGTG) that is located at position -545/-540 of human p21 promoter. In order to investigate whether  $\delta$ EF1 regulates the transcriptional activity of human p21 promoter through this putative response element, a truncated p21 promoter reporter p21-tE<sub>2</sub> was constructed (Fig. 4a). The results showed that, by E<sub>2</sub> box depletion, p21-tE<sub>2</sub> exhibited an increased luciferase activity relative to the -557/+19 promoter p21-wt (Fig. 4b). Further,  $\delta$ EF1-induced transrepression of p21-

tE<sub>2</sub> was blunted (by ~20%) compared to the control without  $\delta$ EF1 transfection (Fig. 4b), indicating that deletion of the E<sub>2</sub> box on human p21 promoter significantly attenuated the repressive effect of p21 transcription by  $\delta$ EF1. In addition, the E<sub>2</sub> box on human p21 promoter was mutated to generate the p21-mE<sub>2</sub> construct as shown in Fig. 4a. Luciferase assay showed that E<sub>2</sub> box mutation also resulted in an increase of the luciferase activity of p21-mE<sub>2</sub>, in comparison to the p21-wt promoter (Fig. 4b). Similarly, the repression of p21-mE<sub>2</sub> promoter activity by  $\delta$ EF1 was markedly weakened (Fig. 4b). These data confirmed that  $\delta$ EF1 inhibited the transcription of p21 through engaging the E<sub>2</sub> box on the p21 promoter.

We further hypothesized that knockdown of  $\delta$ EF1 using RNA interference would result in an activation of the p21 promoter. To do so, a siRNA expression plasmid targeting  $\delta$ EF1 or a scrambled control siRNA plasmid was co-transfected with the p21 promoter reporter into the MDA-MB-231 cells. The knockdown of  $\delta$ EF1 mRNA expression was confirmed by RT-PCR (Fig. 4d). The results showed that  $\delta$ EF1 depletion resulted in a significant increase in the promoter activity of p21, compared to the cells transfected with the control (Fig. 4d). Therefore, the down-regulation of endogenous  $\delta$ EF1 in breast cancer cells is sufficient to allow an inherent release of p21 expression.

In order to determine whether endogenous  $\delta$ EF1 binding to the human p21 promoter is mediated through the E<sub>2</sub>-box element, we performed quantitative CHIP assays. Specifically, total protein lysates from MDA-MB-231 cells were incubated with an anti-ZEB antibody in order to precipitate the endogenous  $\delta$ EF1. As shown in Fig. 5a,  $\delta$ EF1 bound to the p21 promoter at the basal state in an E<sub>2</sub>-box-dependent manner.  $\delta$ EF1 transfection increased its binding to the endogenous p21 promoter by 2.2 folds, compared to the control (Fig. 5b). The data suggested that  $\delta$ EF1 overexpression inhibited p21 transcription by its recruitment to the p21 promoter.

### *3.4. Expression levels of $\delta$ EF1 and p21 are inversely correlated in breast cancer*

Loss of p21 expression has been reported to predict aggressive behavior in several

cancer malignancies, including breast cancer [12,34]. To test whether  $\delta$ EF1 mediates the transcriptional repression of p21 in human tumor cells, we first performed quantitative RT-PCR to examine the expression of  $\delta$ EF1 and p21 in a panel of 4 human breast cancer cell lines. Interestingly, MDA-MB-231 cells that expressed high levels of  $\delta$ EF1 exhibited very low of p21 transcripts (Fig. 6a). Conversely, the p21-positive cells had strongly reduced  $\delta$ EF1 mRNA levels, as shown in MCF-7, T47D, and ZR-75-1 cells (Fig. 6a). Similarly, the inverse correlation of  $\delta$ EF1 and p21 expressions in these cell lines was also shown by western blotting (Fig. 6a).

We are further interested in the relationship between  $\delta$ EF1 and p21 in human breast cancer tissue samples. We collected 22 pairs (cancer and paracancer) of matched advanced breast tumor specimens of invasive ductal carcinoma, among which two cancers (9%) had low (I) histological grade (T1 and T2), 16 cancers (73%) had intermediate (II) grade (T3 to T18), and four cancers (18%) had high (III) histological grade (T19 to T22). As shown in Fig. 6b, an inverse correlation between  $\delta$ EF1 and p21 was clearly evident in T1, T3, T4, T7, T8, T10-14, T17, T18, T20, T22 (14 out of 22 tissue samples). Moreover, we found that  $\delta$ EF1 expression was much higher in tumor tissue than that in the corresponding normal tissues, whereas p21 expression level was higher in normal tissues (Fig. 6c). Together, these data suggest a strong inverse relationship between the expressions of  $\delta$ EF1 and p21, which may contribute to the invasive and metastatic capacities of breast cancer cells.

#### 4. Discussion

Breast carcinogenesis is the result of cell cycle disorganization, leading to an uncontrolled cellular proliferation. Specific cellular processes-mechanisms that control cell cycle progression and checkpoint traversation through the intermitotic phases are deregulated. We previously uncovered that one of the zinc finger-homeodomain transcription factors,  $\delta$ EF1, might contribute to the regulation of cell cycle progression in C2C12 cells. The current study was designed to investigate the mechanism by which  $\delta$ EF1 promotes cell proliferation in breast cancer. Our results show that  $\delta$ EF1 significantly promotes cell cycle progression by increasing the cell number in the S phase in MDA-MB-231 cells. During this process,  $\delta$ EF1 specifically down-regulates p21 transcription by binding to the E<sub>2</sub> box (CAGGTG) on its promoter. Moreover, the inverse correlation of  $\delta$ EF1 and p21 expressions is present in both breast cancer cell lines and in the clinical tumor specimens. Our observations provide the novel evidence, at the cellular and molecular level, that  $\delta$ EF1 controls proliferation by regulating cell cycle regulatory proteins, such as p21, CDK2, and CDK4, in breast cancer progression.

Recent evidences support  $\delta$ EF1 as a potential regulatory factor at the crossroad between proliferation and differentiation in various cells. p73, a new p53 homolog, has been well defined to be closely related to growth arrest, apoptosis, and differentiation [29,35,36]. Fontemaggi et al. reported the binding of  $\delta$ EF1 to specific E boxes within the first intron of the p73 gene, and that implies that p73 is a specific target for transcriptional repression by  $\delta$ EF1 during muscle and neuronal differentiation [29]. They further demonstrated a distinct pattern of *in vivo* recruitment of  $\delta$ EF1 to p73 regulatory regions is found between proliferating and differentiating muscle cells, suggesting a role of  $\delta$ EF1 as a survival factor and inducer of cell movement [30]. These previous observations are consistent with our results that  $\delta$ EF1 stimulates proliferation of pre-myoblast C2C12 cells and concurrently inhibits BMP-2-induced cell differentiation, an effect that is not mediated via the canonical BMP/Smad signaling pathway, but instead by differential regulation of the

AP-1 pathway [19]. These raised a critical and intriguing issue with regard to whether  $\delta$ EF1 may trigger mutually exclusive cellular effects on tumor cells, i.e. regulated cell cycle versus deregulated cell proliferation leading to tumor progression. It has been known that EMT describes the differentiation switch between polarized epithelial cells and contractile and motile mesenchymal cells, and facilitates cell movements during carcinogenesis [37,38]. A growing body of evidence have strongly indicated a significant role of  $\delta$ EF1 in the regulation of EMT progression in a variety of carcinomas, including breast cancer [22-27,39]. Previous research from our group demonstrated that  $\delta$ EF1 overexpression represses BMP-6-induced up-regulation of E-cadherin in MDA-MB-231 cells, thus inducing breast cancer EMT [26]. Importantly, a dual mechanism for BMP-6-regulated  $\delta$ EF1 expression is elucidated, involving cross-talks between AP-1-mediated transcriptional repression and miRNA-192-mediated translational inhibition [39]. However, the proliferative nature of breast cancer regulated by  $\delta$ EF1 has not been recognized yet. In the present study, we provided novel evidence that  $\delta$ EF1 overexpression significantly promotes cell cycle progression by increasing the cell number in the S phase in MDA-MB-231 cells. On the other hand, RNA interference targeting  $\delta$ EF1 exhibited an opposite effect to inhibit G1-S transition of the cell cycle, confirming a potential role of  $\delta$ EF1 in the regulation of cell proliferation. Taken together, these findings strongly suggest a possible mechanism that simultaneous growth promotion and migration of tumor cell may occur during metastasis.

Several studies described p21 as a potent regulator of cell proliferation in various cell culture models [40]. For instance, in NIH 3T3 cells, hepatitis C virus (HCV) core protein represses the p21 promoter through the TGF- $\beta$ -responsive element (T $\beta$ RE), suggesting that HCV core protein stimulates cell cycle progression by repressing p21 transcription through inhibition of a TGF- $\beta$  pathway [41]. Similarly, the AP-1 transcription factor c-Jun, a key inducer of hepatocyte proliferation, controls liver regeneration by repressing p53/p21 activity [42]. These results coincide with our findings presented in this report that  $\delta$ EF1 is a native repressor of p21 gene in MDA-

MB-231 breast cancer cells. Significant down-regulation of p21 transcription induced by  $\delta$ EF1 was observed within 3 h after  $\delta$ EF1 transfection, suggesting a direct mechanism is involved in this regulation. In our study, the transfection of  $\delta$ EF1 did not appear to completely shut down the p21 gene, but the level of p21 mRNA dropped swiftly to 60% in 3 h and maintained later on around 30% of the control for up to 24 h. We thus speculate that the normal half life of p21 mRNA is relatively short, perhaps less than 3 h. Regulation of such a short-lived mRNA by  $\delta$ EF1 to cause significant changes in cellular functions indicated the p21/ $\delta$ EF1 balance plays a critical role in the complex network that governs breast cancer cell function, and likely tumor progression. We further found that  $\delta$ EF1 inhibited the promoter activity of human p21 in a dose-dependent manner. Depletion of  $\delta$ EF1 by RNA interference was sufficient to allow a native release of p21 expression. Importantly, we discovered that deletion or mutation of the E<sub>2</sub> box on p21 promoter remarkably abolished the repressive effect of p21 transcription induced by  $\delta$ EF1.  $\delta$ EF1 transfection in MDA-MB-231 cells resulted in a marked increase of the binding affinity of  $\delta$ EF1 to the endogenous p21 promoter, indicating a significant role of the E<sub>2</sub> box element in mediating  $\delta$ EF1-inhibited expression of p21. These data collectively supports the role of  $\delta$ EF1 as a potential activator in the regulation of breast cancer proliferation, which effect is mediated by modulating p21 expression.

We report here that there is an inverse correlation of  $\delta$ EF1 and p21 expressions in both breast cancer cell lines and in clinical tumor specimens. We previously found that  $\delta$ EF1 is expressed at a higher level in ER negative breast cancer cells, whereas BMP-6 expression is relatively higher in estrogen receptor (ER) positive cells [26]. These are consistent with the recent reports that in ER negative breast cancer cell lines, ER $\alpha$  status is associated with low basal levels of p21 gene expression [43,44]. ER $\alpha$  itself is able to transactivate the p21 promoter and to exhibit a growth inhibitory activity in ER negative cells [44]. Furthermore, the above results of concurrent increases in BMP-6 and p21 in breast cancer cells suggest that BMP-6-induced  $\delta$ EF1 inhibition is responsible, at least in part, for restoring p21 expression at both *in vitro*

in breast carcinoma but also *in vivo* situations. On the other hand, repression of  $\delta$ EF1 by BMP-6 has been implied as a key event in mediating BMP-6-induced EMT inhibition in breast cancer cells [26,39]. Meanwhile, BMP-6 addition to MDA-MB-231 cells resulted in a significant decreased cell numbers from G0/G1 into S phase, supporting a potential effect of BMP-6 in inducing cell cycle arrest of breast cancer (un-published data). In the present research, we found that artificial removal of  $\delta$ EF1 by RNA interference resulted in an inhibition of G1-S transition of the cell cycle in MDA-MB-231 cells, which effect is mediated via regulation of the p21 gene. Taken together, our result supports a plausible mechanism that  $\delta$ EF1 and p21 are involved in BMP-6-regulated cell proliferation of breast cancer. Importantly, factors that remove/decrease  $\delta$ EF1 level would subsequently increase the expression of p21, thus inhibiting breast cancer progression.

Our results present in this study also showed that, besides down-regulating p21 expression,  $\delta$ EF1 concurrently up-regulated expressions of CDK2 and CDK4 at both mRNA and protein levels, indicating that  $\delta$ EF1 functions simultaneously as inhibitor and activator during promoting breast cancer cell proliferation. Traditionally,  $\delta$ EF1 has been identified as a widely expressed transcriptional repressor in various cellular processes, via interactions with co-repressors or competition with activators for DNA binding sites [15,18,19,23-26,29,30]. However, several recent reports indicated that  $\delta$ EF1 can also function as a transcriptional activator in regulating the specific genes, including the vitamin D(3) receptor and ovalbumin [45,46]. These are coincided with the finding from our group that  $\delta$ EF1 exhibits activation on microRNA-21 transcription during mediating breast cancer EMT [47]. Collectively, we speculate a dual effect of  $\delta$ EF1 in control of breast cancer proliferation, by differentially regulating p21, CDK2, and CDK4. However, the mechanism through which  $\delta$ EF1 activates CDK2 and/or CDK4 expression requires to be further elucidated.

In conclusion, we have provided novel findings of a potential mechanism for  $\delta$ EF1-regulated cell proliferation in breast cancer, which effect is mediated through repressing p21 transcription by binding to the E2 box on its promoter. Moreover,

$\delta$ EF1 appeared to be a key regulatory factor at the crossroad between proliferation and differentiation, thus underscoring tumor progression and metastasis. Together,  $\delta$ EF1 may represent a novel target molecule or therapeutic approach for repression of systemic breast cancer progression.

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**Acknowledgements**

This work is supported by grants from the National Nature Science Foundation of China (No. 30700471) and from Tianjin Natural Science Foundation (No. 09JCYBJC09100) to S. Yang. We thank Dr. Darren Ji at PharmaLegacy Laboratories (Shanghai) Co, Ltd, Shanghai, China, for his critical reading and comments on the manuscript.

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## Reference

- [1] M.M. Lipinski, T. Jacks, The retinoblastoma gene family in differentiation and development. *Oncogene* 18 (1999) 7873-7882.
- [2] D. Lyden, A.Z. Young, D. Zagzag, W. Yan, W. Gerald, R. O'Reilly, B.L. Bader, R.O. Hynes, Y. Zhuang, K. Manova, R. Benezra, Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature* 401 (1999) 670-677.
- [3] G. Draetta, Cell cycle control in eukaryotes: molecular mechanisms of cdc2 activation. *Trends Biochem. Sci.* 15 (1990) 378-383.
- [4] C.J. Sherr, G1 phase progression: cycling on cue. *Cell* 79 (1994) 551-555.
- [5] C.E. Caldon, R.J. Daly, R.L. Sutherland, E.A. Musgrove, Cell cycle control in breast cancer cells. *J. Cell Biochem.* 97 (2006) 261-274.
- [6] T. Hunter, J. Pines, Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. *Cell* 79 (1994) 573-582.
- [7] J.S. Foster, D.C. Henley, A. Bukovsky, P. Seth, J. Wimalasena, Multifaceted regulation of cell cycle progression by estrogen: regulation of Cdk inhibitors and Cdc25A independent of cyclin D1-Cdk4 function. *Mol. Cell Biol.* 21 (2001) 794-810.
- [8] R.Y. Poon, T. Hunter, Dephosphorylation of Cdk2 Thr160 by the cyclin-dependent kinase-interacting phosphatase KAP in the absence of cyclin. *Science* 270 (1995) 90-93.
- [9] B.T. Zafonte, J. Hult, D.F. Amanatullah, C. Albanese, C. Wang, E. Rosen, A. Reutens, J.A. Sparano, M.P. Lisanti, R.G. Pestell, Cell-cycle dysregulation in breast cancer: breast cancer therapies targeting the cell cycle. *Front. Biosci.* 5 (2000) D938-961.
- [10] P. DeInnocentes, P. Agarwal, R.C. Bird, Phenotype-rescue of cyclin-dependent kinase inhibitor p16/INK4A defects in a spontaneous canine cell model of breast cancer. *J. Cell Biochem.* 106 (2009) 491-505.
- [11] A.M. Abukhdeir, B.H. Park, p21 and p27: roles in carcinogenesis and drug resistance. *Expert. Rev. Mol. Med.* 10 (2008) e19.
- [12] W. Domagala, M. Welcker, M. Chosia, M. Karbowiczek, B. Harezga, J. Bartkova, J. Bartek, M. Osborn, p21/WAF1/Cip1 expression in invasive ductal breast carcinoma: relationship to p53, proliferation rate, and survival at 5 years. *Virchows Arch* 439 (2001) 132-140.
- [13] C. Swanton, Cell-cycle targeted therapies. *Lancet. Oncol.* 5 (2004) 27-36.
- [14] D. Santamaria, S. Ortega, Cyclins and CDKS in development and cancer: lessons from genetically modified mice. *Front. Biosci.* 11 (2006) 1164-1188.
- [15] J. Funahashi, Y. Kamachi, K. Goto, H. Kondoh, Identification of nuclear factor delta EF1 and its binding site essential for lens-specific activity of the delta 1-crystallin enhancer. *Nucleic Acids Res.* 19 (1991) 3543-3547.
- [16] Z.C. Lai, E. Rushton, M. Bate, G.M. Rubin, Loss of function of the *Drosophila* zfh-1 gene results in abnormal development of mesodermally derived tissues. *Proc. Natl. Acad. Sci. USA.* 90 (1993) 4122-4126.
- [17] G. Nishimura, I. Manabe, K. Tsushima, K. Fujii, Y. Oishi, Y. Imai, K. Maemura, M. Miyagishi, Y. Higashi, H. Kondoh, R. Nagai, DeltaEF1 mediates TGF-beta signaling in vascular smooth muscle cell differentiation. *Dev. Cell* 11 (2006) 93-104.
- [18] D. Murray, P. Precht, R. Balakir, W.E. Horton, The transcription factor deltaEF1 is inversely expressed with type II collagen mRNA and can repress Col2a1 promoter activity in transfected chondrocytes. *J. Biol. Chem.* 275 (2000) 3610-3618.

- [19] S. Yang, L. Zhao, J. Yang, D. Chai, M. Zhang, J. Zhang, X. Ji, T. Zhu, deltaEF1 represses BMP-2-induced differentiation of C2C12 myoblasts into the osteoblast lineage. *J. Biomed. Sci.* 14 (2007) 663-679.
- [20] T. Takagi, H. Moribe, H. Kondoh, Y. Higashi, DeltaEF1, a zinc finger and homeodomain transcription factor, is required for skeleton patterning in multiple lineages. *Development* 125 (1998) 21-31.
- [21] D.S. Darling, R.P. Stearman, Y. Qi, M.S. Qiu, J.P. Feller, Expression of Zfh1/deltaEF1 protein in palate, neural progenitors, and differentiated neurons. *Gene Expr. Patterns* 3 (2003) 709-717.
- [22] T. Ohira, R.M. Gemmill, K. Ferguson, S. Kusy, J. Roche, E. Brambilla, C. Zeng, A. Baron, L. Bemis, P. Erickson, E. Wilder, A. Rustgi, J. Kitajewski, E. Gabrielson, R. Bremnes, W. Franklin, H.A. Drabkin, WNT7a induces E-cadherin in lung cancer cells. *Proc. Natl. Acad. Sci. USA.* 100 (2003) 10429-10434.
- [23] Y. Haddad, W. Choi, D.J. McConkey, Delta-crystallin enhancer binding factor 1 controls the epithelial to mesenchymal transition phenotype and resistance to the epidermal growth factor receptor inhibitor erlotinib in human head and neck squamous cell carcinoma lines. *Clin. Cancer Res.* 15 (2009) 532-542.
- [24] N.S. Spoelstra, N.G. Manning, Y. Higashi, D. Darling, M. Singh, K.R. Shroyer, R.R. Broaddus, K.B. Horwitz, J.K. Richer, The transcription factor ZEB1 is aberrantly expressed in aggressive uterine cancers. *Cancer Res.* 66 (2006) 3893-3902.
- [25] A. Eger, K. Aigner, S. Sonderegger, B. Dampier, S. Oehler, M. Schreiber, G. Berx, A. Cano, H. Beug, R. Foisner, DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. *Oncogene* 24 (2005) 2375-2385.
- [26] S. Yang, J. Du, Z. Wang, W. Yuan, Y. Qiao, M. Zhang, J. Zhang, S. Gao, J. Yin, B. Sun, T. Zhu, BMP-6 promotes E-cadherin expression through repressing deltaEF1 in breast cancer cells. *BMC Cancer* 7 (2007) 211.
- [27] T. Shirakihara, M. Saitoh, K. Miyazono, Differential regulation of epithelial and mesenchymal markers by deltaEF1 proteins in epithelial mesenchymal transition induced by TGF-beta. *Mol. Biol. Cell* 18 (2007) 3533-3544.
- [28] E.M. Hurt, J.N. Saykally, B.M. Anose, K.R. Kalli, M.M. Sanders, Expression of the ZEB1 (deltaEF1) transcription factor in human: additional insights. *Mol. Cell Biochem.* 318 (2008) 89-99.
- [29] G. Fontemaggi, A. Gurtner, S. Strano, Y. Higashi, A. Sacchi, G. Piaggio, G. Blandino, The transcriptional repressor ZEB regulates p73 expression at the crossroad between proliferation and differentiation. *Mol. Cell Biol.* 21 (2001) 8461-8470.
- [30] G. Fontemaggi, A. Gurtner, A. Damalas, A. Costanzo, Y. Higashi, A. Sacchi, S. Strano, G. Piaggio, G. Blandino, deltaEF1 repressor controls selectively p53 family members during differentiation. *Oncogene* 24 (2005) 7273-7280.
- [31] T. Bui, J. Sequeira, T.C. Wen, A. Sola, Y. Higashi, H. Kondoh, T. Genetta, ZEB1 links p63 and p73 in a novel neuronal survival pathway rapidly induced in response to cortical ischemia. *PLoS One* 4 (2009) e4373.
- [32] R. Sekido, K. Murai, Y. Kamachi, H. Kondoh, Two mechanisms in the action of repressor deltaEF1: binding site competition with an activator and active repression. *Genes Cells* 2 (1997) 771-783.

- [33] R. Sekido, K. Murai, J. Funahashi, Y. Kamachi, A. Fujisawa-Sehara, Y. Nabeshima, H. Kondoh, The delta-crystallin enhancer-binding protein delta EF1 is a repressor of E2-box-mediated gene activation. *Mol. Cell Biol.* 14 (1994) 5692-5700.
- [34] G. Somlo, P. Chu, P. Frankel, W. Ye, S. Groshen, J.H. Doroshow, K. Danenberg, P. Danenberg, Molecular profiling including epidermal growth factor receptor and p21 expression in high-risk breast cancer patients as indicators of outcome. *Ann. Oncol.* 19 (2008) 1853-1859.
- [35] Y. Sasaki, H. Negishi, R. Koyama, N. Anbo, K. Ohori, M. Idogawa, H. Mita, M. Toyota, K. Imai, Y. Shinomura, T. Tokino, p53 family members regulate the expression of the apolipoprotein D gene. *J. Biol. Chem.* 284 (2009) 872-883.
- [36] N. Danilova, K.M. Sakamoto, S. Lin, p53 family in development. *Mech. Dev.* 125 (2008) 919-931.
- [37] G. Moreno-Bueno, F. Portillo, A. Cano, Transcriptional regulation of cell polarity in EMT and cancer. *Oncogene* 27 (2008) 6958-6969.
- [38] T. Blick, E. Widodo, H. Hugo, M. Waltham, M.E. Lenburg, R.M. Neve, E.W. Thompson, Epithelial mesenchymal transition traits in human breast cancer cell lines. *Clin. Exp. Metastasis* 25 (2008) 629-642.
- [39] S. Yang, J. Du, Z. Wang, J. Yan, W. Yuan, J. Zhang, T. Zhu, Dual mechanism of deltaEF1 expression regulated by bone morphogenetic protein-6 in breast cancer. *Int. J. Biochem. Cell Biol.* 41 (2009) 853-861.
- [40] Y. Xiong, G.J. Hannon, H. Zhang, D. Casso, R. Kobayashi, D. Beach, p21 is a universal inhibitor of cyclin kinases. *Nature* 366 (1993) 701-704.
- [41] M.N. Lee, E.Y. Jung, H.J. Kwun, H.K. Jun, D.Y. Yu, Y.H. Choi, K.L. Jang, Hepatitis C virus core protein represses the p21 promoter through inhibition of a TGF-beta pathway. *J. Gen. Virol.* 83 (2002) 2145-2151.
- [42] E. Stepniak, R. Ricci, R. Eferl, G. Sumara, I. Sumara, M. Rath, L. Hui, E.F. Wagner, c-Jun/AP-1 controls liver regeneration by repressing p53/p21 and p38 MAPK activity. *Genes Dev.* 20 (2006) 2306-2314.
- [43] A. Fritah, C. Saucier, J. Mester, G. Redeuilh, M. Sabbah, p21WAF1/CIP1 selectively controls the transcriptional activity of estrogen receptor alpha. *Mol. Cell Biol.* 25 (2005) 2419-2430.
- [44] R. Margueron, A. Licznar, G. Lazennec, F. Vignon, V. Cavaillès, Oestrogen receptor alpha increases p21(WAF1/CIP1) gene expression and the antiproliferative activity of histone deacetylase inhibitors in human breast cancer cells. *J. Endocrinol.* 179 (2003) 41-53.
- [45] D.L. Lazarova, M. Bordonaro, A.C. Sartorelli, Transcriptional regulation of the vitamin D(3) receptor gene by ZEB. *Cell Growth Differ.* 12 (2001) 319-326.
- [46] N.B. Dillner, M.M. Sanders, Transcriptional activation by the zinc-finger homeodomain protein delta EF1 in estrogen signaling cascades. *DNA Cell Biol.* 23 (2004) 25-34.
- [47] J. Du J, S. Yang, D. An D, F. Hu, W. Yuan, C. Zhai, T. Zhu, BMP-6 inhibits microRNA-21 expression in breast cancer through repressing deltaEF1 and AP-1. *Cell Res.* 19 (2009) 487-496.

**Figure Legends**

**Fig. 1.**  $\delta$ EF1 overexpression promotes the proliferation of MDA-MB-231 breast cancer cells.

(a) Western blotting with anti-ZEB antibody was performed to show  $\delta$ EF1 expression in control ( $\Delta$ 6B) and  $\delta$ EF1-overexpressing ( $\Delta$  $\delta$ EF1) MDA-MB-231 cells. Actin was used as a loading control. (b)  $\delta$ EF1-specific siRNA plasmid (siRNA- $\delta$ EF1) was introduced into MDA-MB-231 cells. Control cells were treated with a scrambled siRNA. The efficiency of  $\delta$ EF1 protein knockdown was examined by western blot, using an anti-ZEB antibody. Actin was used as a loading control. (c)  $\delta$ EF1 or si- $\delta$ EF1 stable transfectants in MDA-MB-231 cells were cultured in regular growth medium for 4 days, respectively. A growth curve was generated by daily measurements of the cell number of each group. \* indicates  $p < 0.05$  in unpaired student t test when compared with vector alone. (d) MDA-MB-231 cells were transiently transfected with  $\delta$ EF1 or empty-vector control. At the indicated time points (0, 24, 48, and 72 h), cell proliferation assays were done with BrdU incorporation using flow cytometry assay. The upper panel represents the controls of BrdU-/PI-, BrdU+/PI-, and BrdU-/PI+, respectively. (e) MDA-MB-231 cells were transiently transfected with  $\delta$ EF1-specific siRNA plasmid (siRNA- $\delta$ EF1) or a scrambled siRNA control. The efficiency of  $\delta$ EF1 protein knockdown was examined by western blotting, using an anti-ZEB antibody. Actin was used as a loading control. At the indicated time points (0, 24, and 48 h), cell proliferation assays were done with BrdU incorporation using flow cytometry assay.

**Fig. 2.**  $\delta$ EF1 down-regulates p21 expression while promoting cell proliferation of MDA-MB-231 cells.

(a)  $\delta$ EF1-induced down-regulation of p21 mRNA in MDA-MB-231 cells was verified by quantitative RT-PCR. GAPDH was used to normalize the p21 level. Data represent three independent experiments. (b)  $\delta$ EF1-regulated expressions of p21 and p27 proteins in MDA-MB-231 cells were verified by western blotting. Actin was

used to normalize the p21 and p27 levels. (c)  $\delta$ EF1-specific siRNA plasmid (si- $\delta$ EF1) was introduced into MDA-MB-231 cells using transient transfection. Control cells were treated with a scrambled siRNA. The efficiency of  $\delta$ EF1 mRNA knockdown was examined by RT-PCR. The inhibition of p21 mRNA level was determined at different time points (0, 24, and 48 h) after transfection, using quantitative RT-PCR. GAPDH was used to normalize the p21 level. SIP1, a close homolog of  $\delta$ EF1, was used as an off-set control of siRNA interference. Data represent three independent experiments.

**Fig. 3.**  $\delta$ EF1 concurrently up-regulates the expressions of CDK2 and CDK4 while promoting cell proliferation of MDA-MB-231 cells.

(a)  $\delta$ EF1-induced up-regulation of CDK2 and CDK4 mRNA levels in MDA-MB-231 cells were verified by quantitative RT-PCR. GAPDH was used to normalize the CDK2 and CDK4 levels. Data represent three independent experiments. (b)  $\delta$ EF1-specific siRNA plasmid (si- $\delta$ EF1) was introduced into MDA-MB-231 cells using transient transfection. Control cells were treated with a scrambled siRNA. The efficiency of  $\delta$ EF1 mRNA knockdown was examined by RT-PCR. The inhibition of CDK2 and CDK4 mRNA levels were determined at different time points (0, 24, and 48 h) after transfection, using quantitative RT-PCR. GAPDH was used to normalize the p21 level. SIP1 was used as an off-set control of siRNA interference. Data represent three independent experiments. (c)  $\delta$ EF1-induced up-regulation of CDK2 and CDK4 protein levels in MDA-MB-231 cells was verified by western blotting. Actin was used to normalize the CDK2 and CDK4 levels.

**Fig. 4.**  $\delta$ EF1 represses the transcriptional activity of p21 promoter by binding to E<sub>2</sub> box.

(a) Sequential deletion and mutation of the human p21 promoter were fused to the luciferase reporter. (b) MDA-MB-231 cells on 24-well plate were co-transfected with the  $\delta$ EF1 expression plasmid (1  $\mu$ g/well) and wild-type (p21-wt), or truncated (p21-

tE2), or mutated (p21-mE2) p21 promoter luciferase reporters (1  $\mu$ g/well), respectively. The luciferase activity of the extracts was determined 24 h after transfection using a Betascope analyzer. Luciferase values are normalized with Renilla activities. \* indicates  $p < 0.05$  in unpaired student t test when compared with vector alone. Data represent three independent experiments. (c) MDA-MB-231 cells on 24-well plate were co-transfected with different amount of  $\delta$ EF1 expression plasmid (0, 0.5, 1, or 2  $\mu$ g/well) and wild-type (p21-wt) p21 promoter luciferase reporter (1  $\mu$ g/well). The luciferase activity of the extracts was determined 24 h after transfection using a Betascope analyzer. Luciferase values are normalized with Renilla activities. \* indicates  $p < 0.05$  in unpaired student t test when compared with vector alone. \*\* indicates  $p < 0.05$  in one-way analysis of variance followed by Dunnett's test when compared with vector alone. Data represent three independent experiments. (d)  $\delta$ EF1-specific siRNA plasmid (si- $\delta$ EF1) and wild-type (p21-wt) p21 promoter luciferase reporter were co-transfected into MDA-MB-231 cells. The inhibition of  $\delta$ EF1 mRNA level was determined at different time points (0, 24, and 48 h) after transfection, using RT-PCR. SIP1 was used as an off-set control of siRNA interference. The luciferase activity of the extracts was determined 24 h after transfection using a Betascope analyzer. Luciferase values are normalized with Renilla activities. \* indicates  $p < 0.05$  in unpaired student t test when compared with vector alone. \*\* indicates  $p < 0.05$  in one-way analysis of variance followed by Dunnett's test when compared with vector alone. Data represent three independent experiments.

**Fig. 5.**  $\delta$ EF1 overexpression increases its recruitment to endogenous p21 promoter in an E<sub>2</sub>-box-dependent manner.

(a) Association of  $\delta$ EF1 with the proximal human p21 promoter was analyzed by CHIP analyses in MDA-MB-231 cells, using polyclonal antibody against  $\delta$ EF1 or unrelated IgG antibody. Amplified human p21 promoter fragment of E<sub>2</sub> box containing sequence is shown. Amount of DNA in input is confirms equal loading of

chromatin. (b)  $\delta$ EF1 transfection to MDA-MB-231 cells significantly enhanced its recruitment to endogenous p21 promoter by quantitative CHIP assay. \* indicates  $p < 0.05$  in unpaired student t test when compared with vector alone. Data represent three independent experiments.

**Fig. 6.** Expressions of  $\delta$ EF1 and p21 are inversely correlated in breast cancer.

(a) mRNA and protein levels of  $\delta$ EF1 and p21 in MDA-MB-231, MCF-7, T47D, and ZR-75-1 breast cancer cells were detected by quantitative RT-PCR and western blotting, respectively. GAPDH and Actin were used to normalize the individual expression levels. (b) Transcript levels of the two genes were determined in tumor samples from 22 breast cancer specimens by quantitative RT-PCR. GAPDH was used to normalize the individual expression levels. (c) Transcript levels of the two genes were determined in the tumor tissues (T) and the corresponding normal tissues (N), respectively, using quantitative RT-PCR.  $\delta$ EF1 expression level is much higher in the tumor tissues than that in the corresponding normal tissues. Oppositely, p21 expression level exhibited higher in normal tissues. The expression level of  $\delta$ EF1 and p21 in normal breast tissue was normalized to 1.

Figure 1a

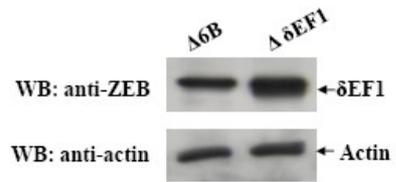


Figure 1A

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Figure 1b

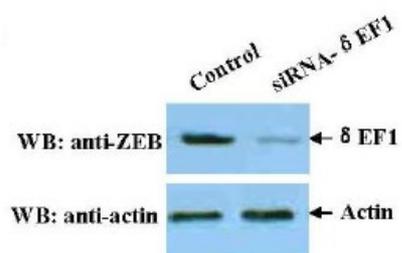


Figure 1B

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Figure 1c

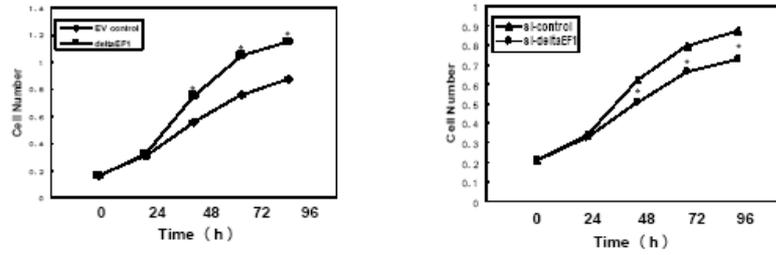


Figure 1C

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Figure 1d

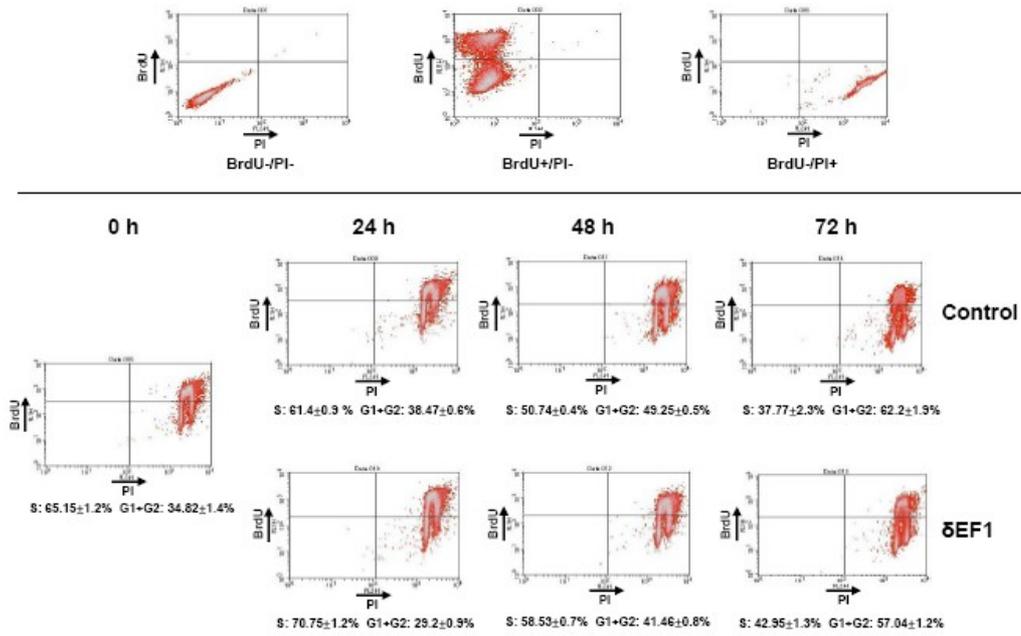


Figure 1D

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Figure 1e

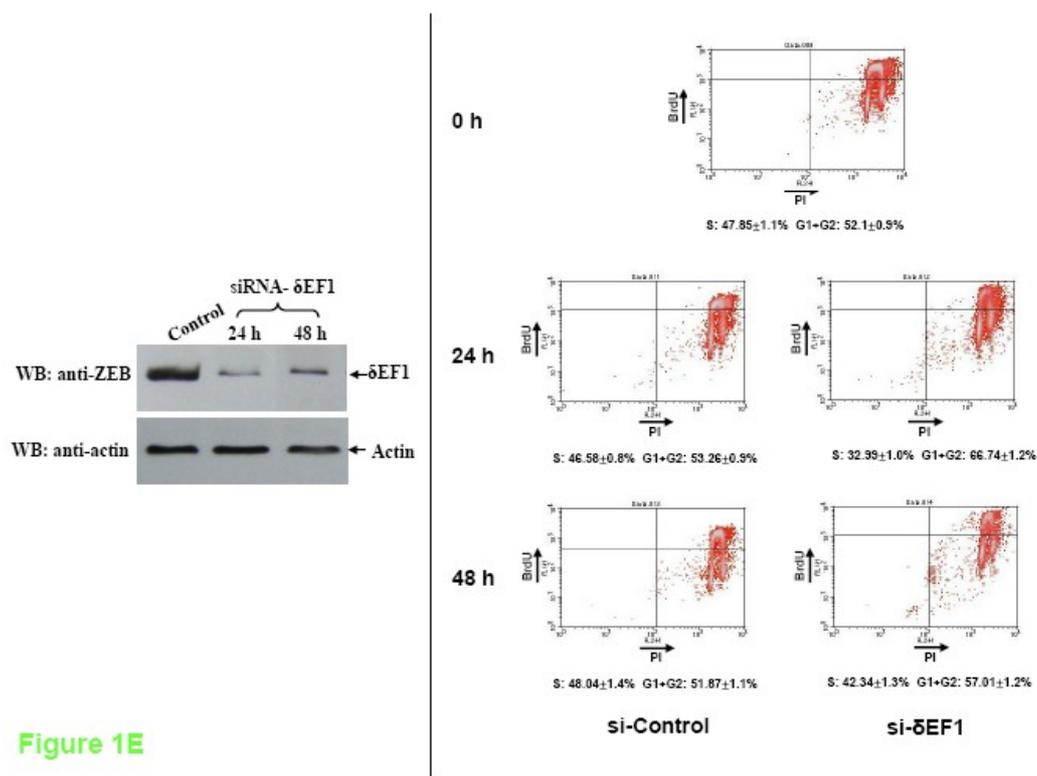


Figure 1E

ACCEPTED

Figure 2a

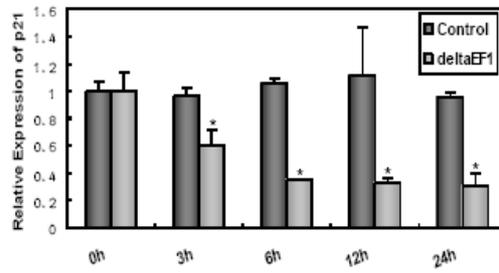


Figure 2A

EF

Figure 2b

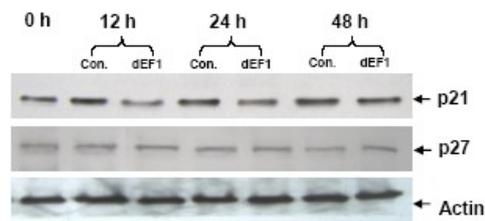


Figure 2B

Figure 2c

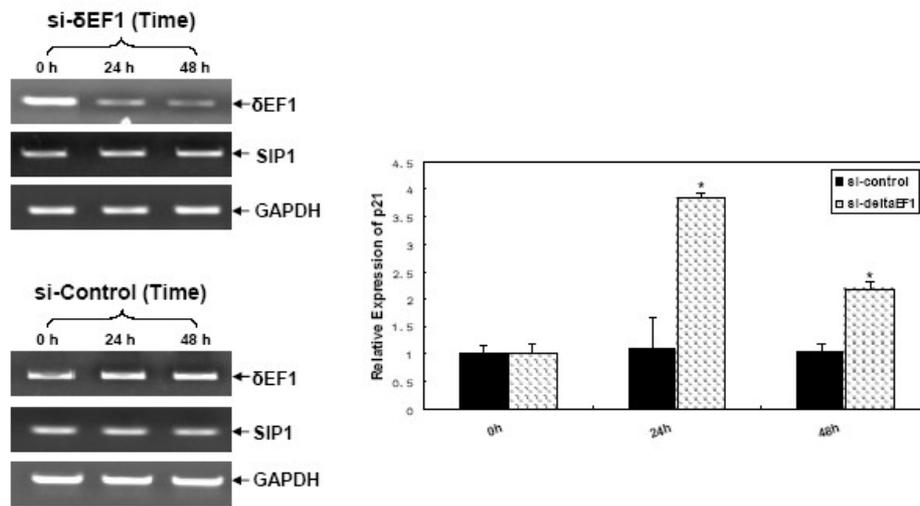


Figure 2C

ACCEPTED

Figure 3a

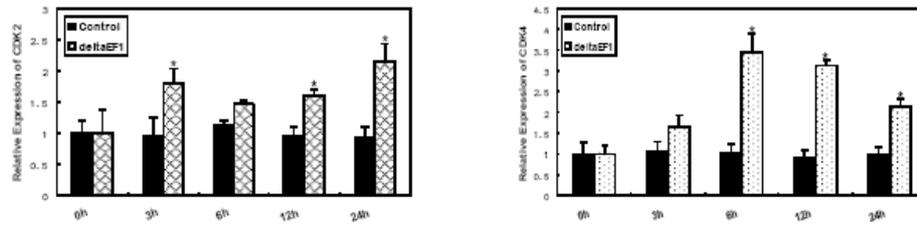


Figure 3A



Figure 3b

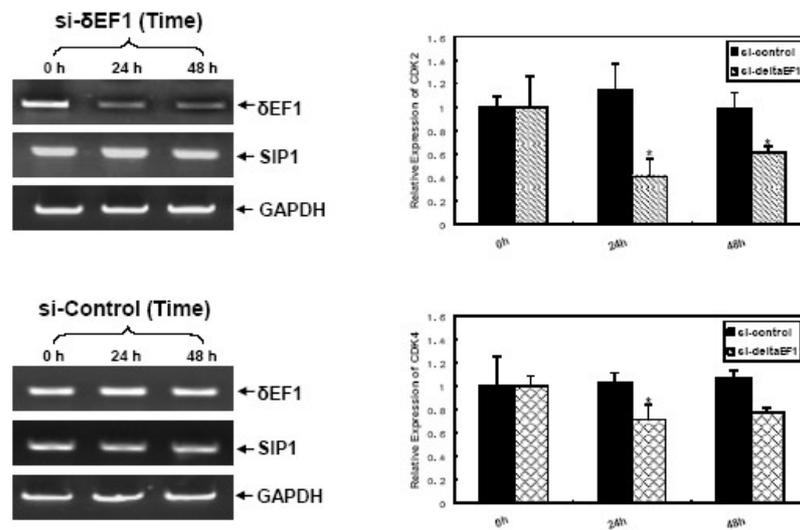


Figure 3B

Figure 3c

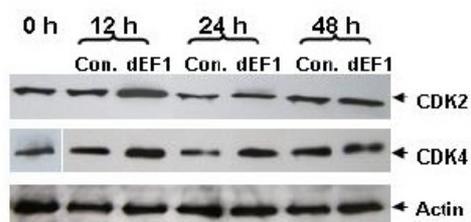


Figure 3C

EF

Figure 4a

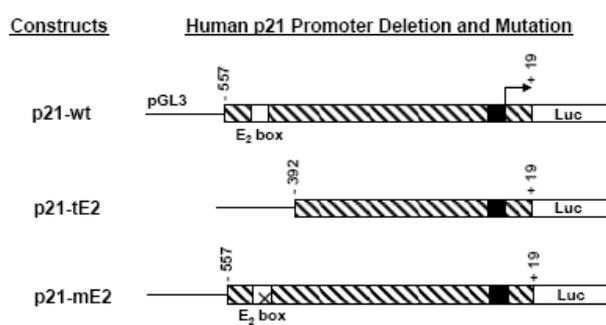


Figure 4A

Figure 4b

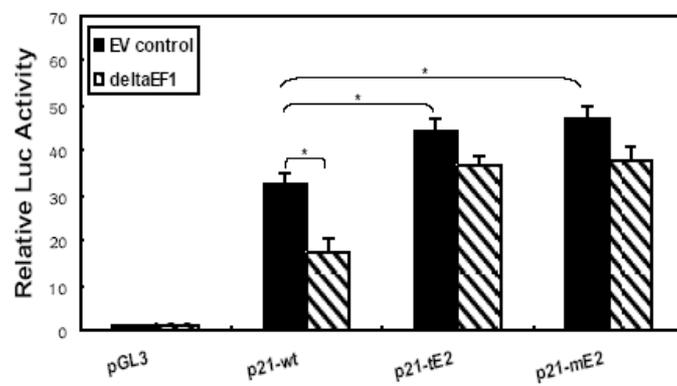


Figure 4B

ACCEPTED

Figure 4c

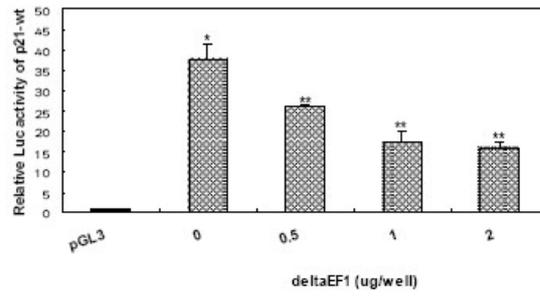


Figure 4C

ACCEPTED

Figure 4d

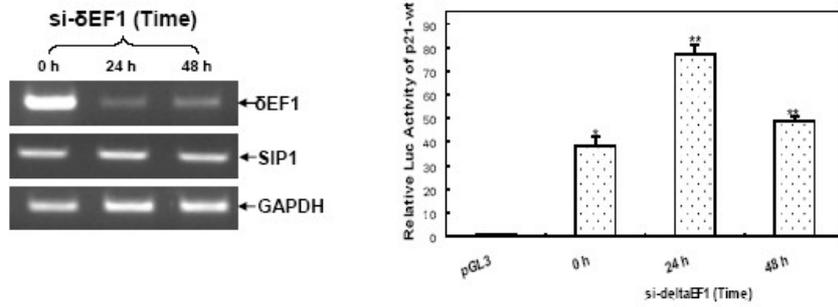


Figure 4D

ACCEPTED

Figure 5a

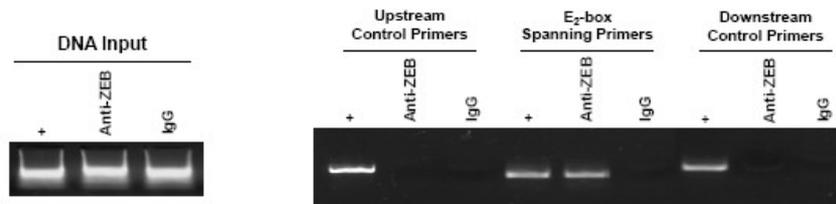


Figure 5A

ACCEPTED

Figure 5b

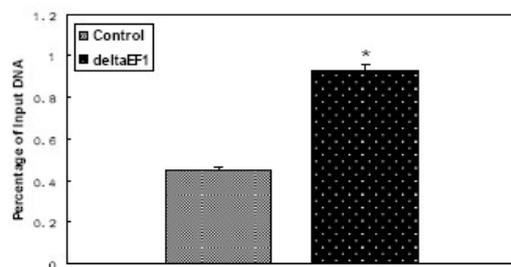


Figure 5B

ACCEPTED

Figure 6a

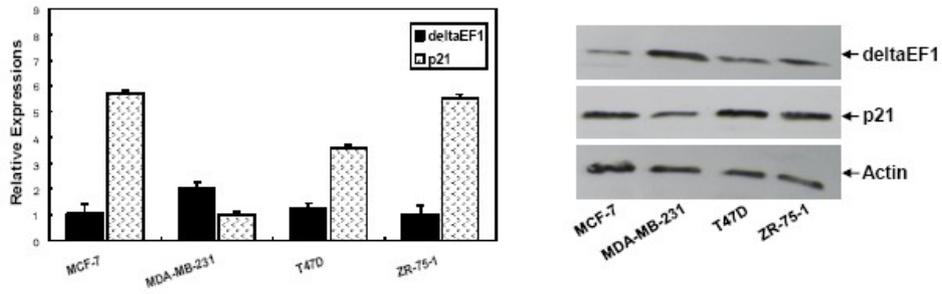


Figure 6A

ACCEPTED

Figure 6b

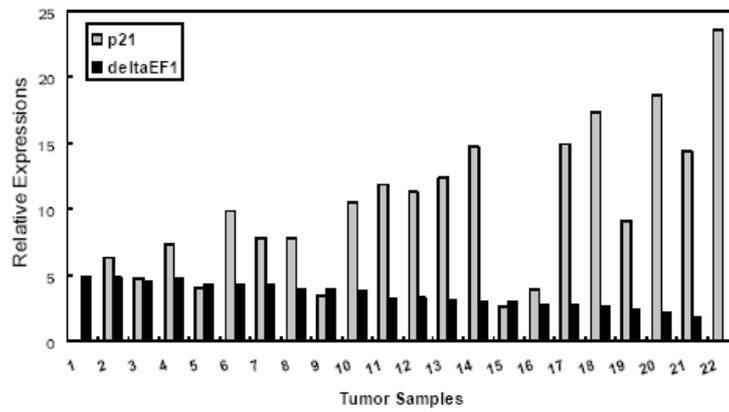


Figure 6B

ACCEPTED

Figure 6c

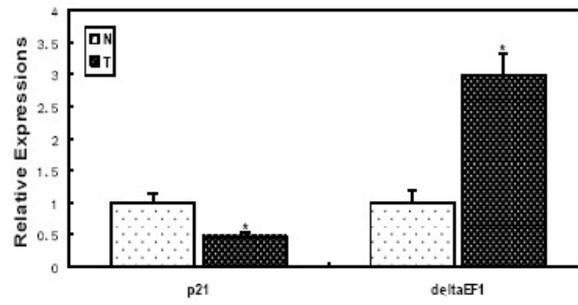


Figure 6C

ACCEPTED