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Up-regulation of Eukaryotic Elongation Factor-1 Subunits in Breast Carcinoma

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Abstract. *Background: Wide evidence suggests the involvement of translation elongation factors (EFs) at the onset of oncogenesis. To investigate the potential role of the EF-1 subunits (A, Bα and Bγ) in the formation and progression of breast cancer, we quantified their expression in human breast tissues and cell lines. Materials and Methods: The mRNA levels of EF-1A, -1Bα and -1Bγ in human breast tissues and cell lines were measured by semi-quantitative RT-PCR and Northern blotting, respectively. Results: The mRNA expression of the three EF-1 subunits was significantly higher in cancerous over normal tissues. However, there was no significant difference in their expression between tumor grades. Overexpression of EF-1 mRNA in breast cancer cell lines were not caused by increased mRNA stability. Conclusion: Although not indicative of tumor grading, the elevated levels of EF-1 subunits are suggestive of their early role in the pathogenesis of breast cancer, possibly through their increased rate of transcription.*

Breast cancer is the most widespread malignancy among women with 900,000 new cases occurring annually worldwide (1). It is thought to be the leading cause of death among women aged 35-54 years. Breast cancer is considered the most common cancer among Kuwaiti women, with an incidence rate of 32.8 per 100,000 women, and the disease is rising in incidence (2).

Protein synthesis is a central process in all living cells. Increasing evidence is emerging on the involvement of this process in cell growth and tumorigenesis (3). Increased protein synthesis is suggested to support proliferation of cancer cells and thus promote neo-angiogenesis for invasion of surrounding normal tissue. Recent studies showed that translation initiation factors (IF) and elongation factors (EF) have important regulatory roles in cell growth, apoptosis and tumorigenesis (4). EF-1A and IF4E are two translation factors that have been identified as important human oncogenes (5, 6). Translation elongation is carried out in the cell by two major elongation factors: EF-1 and EF-2. The multi-subunit EF-1 carries out the first step in elongation and consists of at least 4 subunits. EF-1A is an important GTP-binding protein that utilizes GTP to mediate the elongation step in protein synthesis by directing the aminoacyl-tRNA to the ribosomes (7). The guanine nucleotide exchange carried out by the EF-1Bαγ complex is rate-limiting for the catalytic function of EF-1A (8).

Several studies have reported on the involvement of the EF-1 family in oncogenesis. Constitutive expression of EF-1A causes fibroblast cell lines to become highly susceptible to transformation (5). Furthermore, overexpression of EF-1A mRNA has been correlated with increased metastatic potential in mammary adenocarcinoma, probably due to its interaction with the actin cytoskeleton (9). Enhanced expression of EF-1A has also been linked to transformation of rodent fibroblasts and increased growth rate of ovarian carcinoma cells (10). Overexpression of EF-1Bγ mRNA was observed in colorectal (11), gastric (12) and hepatocellular (13) carcinomas. However, the actual mechanism of EF-1 overexpression is still not known. No reports were found in the literature regarding the expression level of EF-1Bα mRNA at the time of this study.

We attempted to compare the mRNA expression of EF-1A, -1Bα and -1Bγ mRNA between cancerous and non-cancerous breast tissues and in human breast cell lines in order to investigate the pathophysiological significance of EF-1 subunits in breast cancer.

Materials and Methods

Cell culture. Human breast cell lines MCF-7, T47D, MDA-231 and MCF-10A were kindly donated by Dr. Yoshikuni Nagamine (Friedrich Miescher Institute, Switzerland). The cells were grown

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Key Words: Breast cancer, elongation factor-1, mRNA expression, RT-PCR.

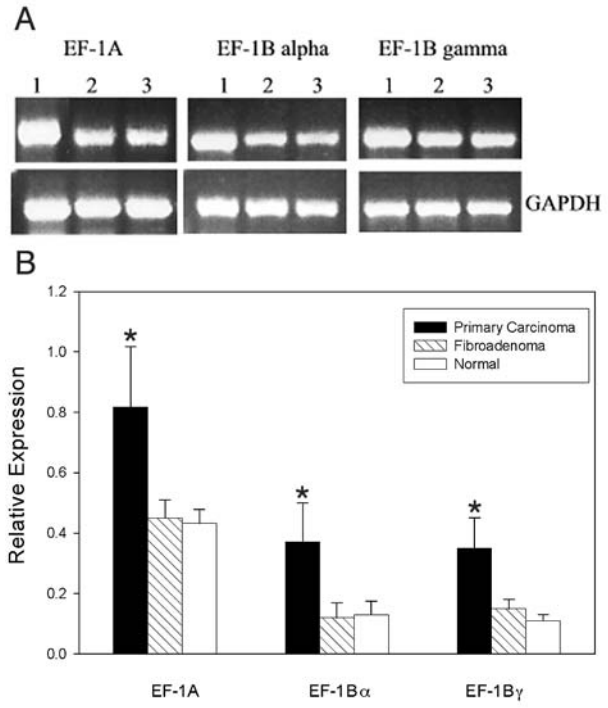

Figure 1. *Quantitative analysis of EF-1 (A, Bα and Bγ) transcripts in human breast tissues. A. Expression of EF-1 subunits (A, Bα and Bγ) in breast cancerous and non-cancerous tissues was measured using a semiquantitative RT-PCR. B. Expression of the three transcripts was quantified as a ratio to the levels of GAPDH mRNA. Error bars indicate the standard error of the mean from 3 independent experiments (*p<0.05).*

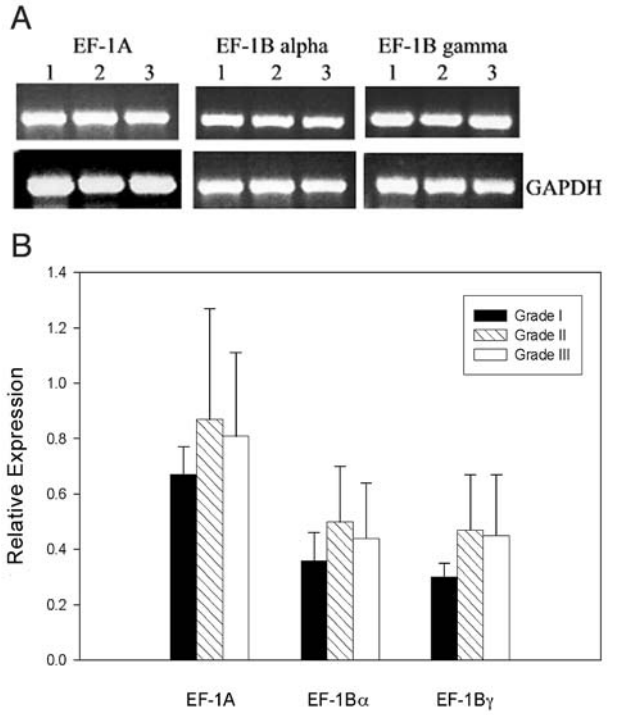

Figure 2. *Relationship between tumor grade and EF-1 (A, Bα and Bγ) mRNA expression in breast carcinoma. A. A representative RT-PCR experiment showing EF-1 mRNA expression. B. Relative mRNA expression of EF-1 subunits was calculated as a ratio to GAPDH mRNA expression. Error bars indicate the standard error of the mean from 3 independent experiments (*p<0.05).*

in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum, at 37˚C in a humidified atmosphere of 5% CO_2.

Patients and breast tissue specimens. The study included 58 breast samples consisting of 20 carcinomas, 9 fibroadenomas and 29 normal breast tissues. Tissue specimens were collected from patients undergoing surgery for tumor or whole breast removal (mastectomy), which were performed at H.M. Al-Jumaa Cancer Center, Kuwait. A portion of the surgical samples was fixed in 10% buffered formalin, processed into paraffin and used for pathological analysis to determine the histological diagnosis, tumor grade (using the modified Scarff-Bloom-Richardson tumor grading system for breast cancer), as well as the status of estrogen and progesterone receptors by immunohistochemistry. A second portion was used for mRNA analysis. The fresh tissues were kept at 4˚C in RNA Later reagent (Ambion, Austin, TX, USA) and stored at –80˚C until required for RNA extraction. Tissue samples were collected and processed with the approval of the local ethical committee at the Faculty of Medicine, Kuwait University.

RNA extraction. Cells were cultured in 100-mm dishes (BD Labware, Franklin Lakes, NJ, USA) to sub-confluency. Total RNA was extracted by the acid guanidine phenol/chloroform method (14). Total RNA was extracted from frozen tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Contaminating chromosomal DNA was digested with DNAse I (Invitrogen), according to manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR analysis was performed in the Gene Amp 9700 thermocycler (Applied Biosystems, Warrington, UK). PCR conditions were optimized for annealing temperature and cycle number for the amplification of the PCR products EF-1A, -1Bα and -1Bγ. Specific primers for the three EF-1 subunits were custom-designed and used in PCR amplifications. The nucleotide sequences for the different primer pairs were as follows: EF-1A: sense 5' TGACTGTGCTGT CCTGA 3', antisense 5' GACCTTGCC AGCTCC 3'; EF-1Bα: sense 5' GGGTTTCGG AGACCTGA 3', antisense 5' CAGTGATCTGCTCCTCCA 3'; EF-1Bγ: sense 5' TTGCAGAGACCCAACCTA 3', antisense 5' CAGGTGCA GGCAGCTA 3'; GAPDH: sense 5'ATGCATGGCA CCGTC AAGGC 3', antisense 5' CGCCTGCTTCACCACCTT CT 3'. Total RNA (1 μg) was used for first strand cDNA synthesis using the Thermoscript II One-step RT-PCR Kit (Invitrogen). The conditions for one-step RT-PCR were as follows: 20 minutes at 55˚C for reverse transcription, 5 minutes at 95˚C for RT inactivation, and then 27 cycles of amplification for 30 seconds at 95˚C, 30 seconds at 52-58˚C and 30 seconds at 72˚C. The final

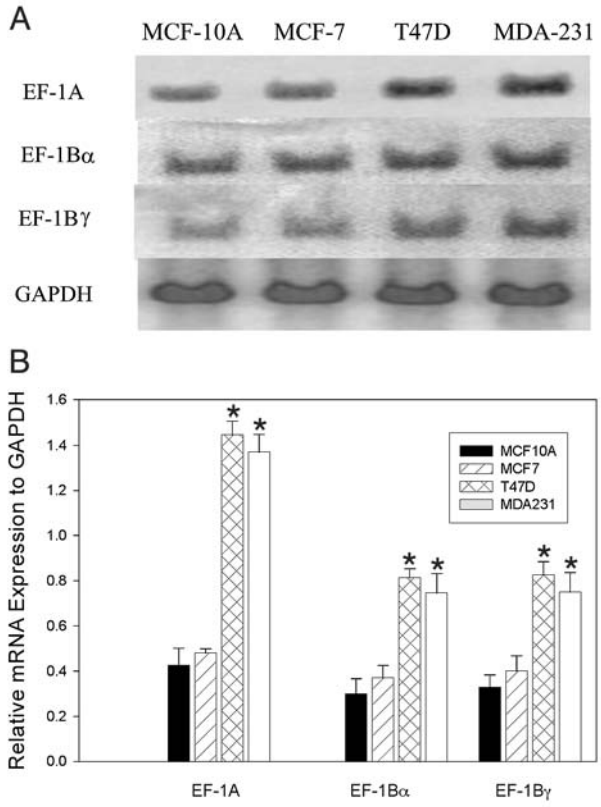

Figure 3. *Expression of EF-1 subunits (A, Bα and Bγ) in human breast cell lines. A. Northern blotting was performed to analyze the relative EF-1 (A, Bα and Bγ) mRNA expression in three breast cancer cell lines and in one normal breast tissue. The loading control was GAPDH mRNA. B. The mRNA ratio of EF-1 subunits to GAPDH. Error bars indicate the standard error of the mean from 3 independent experiments (*p<0.05).*

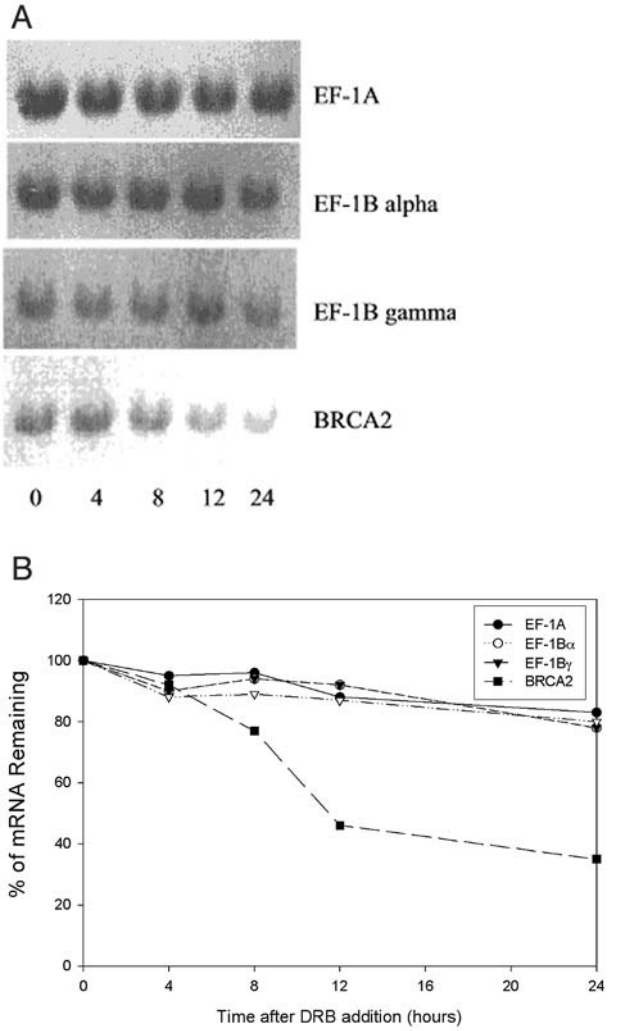

Figure 4. *Decay rates of EF-1 mRNAs in human breast cancer cell line T47D. A. After the addition of 5, 6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB), cells were collected at the times indicated, and 5 μg of total mRNA was analyzed for EF-1 subunits and BRCA2 mRNAs by Northern blot hybridization. B. After hybridization, the signals were quantified by a gel documentation system, and their values were plotted. Similar results were obtained from two independent experiments.*

RT-PCR products (10 μl) were run on 1% agarose gel containing ethidium bromide. The specific bands were quantified by the Gene Genius gel documentation system (Bio Imaging System-Syngene, MD, USA). Expression of EF-1 subunits in a sample was indicated relative to the internal standard GAPDH mRNA.

Northern blot analysis. Ten μg of total RNA from each cell line was separated on a 1% formaldehyde-agarose gel then transferred to a nylon membrane. The membrane was stained with methylene blue for rRNA detection to confirm equal RNA loading and transfer. Hybridization was performed with the quickHyb hybridization solution (Stratagene, Amsterdam, The Netherlands) and ^{32}P-labelled cDNAs as probes for EF-1A, -1Bα and -1Bγ. The same membrane was rehybridized with the radiolabelled cDNA probe for GAPDH. The radioactivity of EF-1A, -1Bα and -1Bγ bands was normalized based on that of the internal standard glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Quantitative analyses were performed using Image Quant software (Molecular Dynamics Phosphoimager, Sunnyvale, CA, USA).

mRNA stability. Stability was measured by the RNA synthesis inhibitor-chase method, as described previously (15). Briefly, cells were treated with 5, 6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) (20 μg/ml) to inhibit transcription, and total RNA was isolated at several points thereafter. RNA was analyzed by Northern blot hybridization. Graphs were created by plotting values of mRNA levels with the Sigma plot program (Jandel Scientific, USA).

Statistical analysis. Differences in EF-1 subunit expression between tumor and normal breast tissues as determined by RT-PCR were evaluated using a two-tailed unpaired *t*-test. *P* values <0.05 were considered significant. The software package SPSS version 10.1 was employed for all statistical analyses (SPSS Inc., Chicago, IL, USA).

Results

We have determined the relative amount of EF-1A, -1Bα and -1Bγ mRNA expression in human breast tissues by quantitative RT-PCR. Relative to the GAPDH expression, all tissues examined expressed reduced levels of EF-1Bα and -1Bγ mRNAs as compared to EF-1A. As shown in Figure 1, the expression of EF-1A mRNA was significantly elevated (0.817±0.2) in breast cancers compared to non-cancerous tissues (0.36±0.1, $p<0.05$). EF-1Bα and -1Bγ mRNA levels were found to be comparable in all tissues. Although their expression was lower than EF-1A, a significant increase was measured in their mRNA levels in cancerous rather than in non-cancerous tissues (EF-1Bα/GAPDH ratio 0.45±0.06 *vs* 0.12±0.05; EF-1Bγ/GAPDH ratio 0.433±0.04 *vs* 0.12±0.02, $p<0.05$). However, no clear relationship was obtained between the mRNA levels of the three EF-1 subunits examined and several clinicopathological features including age, hormone receptor status, lymph node metastasis, disease stage and tumor histology (data not shown). There was also no significant difference in the expression of the studied EF-1 subunits between grades I (n=4), II (n=10) and III (n=6) (Figure 2). Because 5-year survival data were not available for some patients, we were not able to evaluate survival outcome.

Expression of EF-1α, -1Bα and -1Bγ was studied in three human breast cancer cell lines MCF-7, T47D and MDA-231 and one non-transformed human breast epithelial cell line, MCF-10A, using Northern blot analysis. As shown in Figure 3, the expression of EF-1 subunits is ubiquitous in all breast cell lines. Metastatic cell lines had significantly increased levels of the three EF-1 mRNAs. An average of 2.3- and 2.9-fold increase was observed in EF-1A and EF-1Bαγ mRNA expression, respectively, in breast cancer cell lines T47D, and MDA-231 when compared to a normal cell line MCF-10A and the non-metastatic MCF-7. The similar results for the overexpression of the EF-1 mRNAs in breast tissues and breast cancer cell lines demonstrate the reliability of cell lines as more practical models than tissues.

To establish whether the increased mRNA levels of EF-1A, -1Bα and -1Bγ were due to increased transcription or to slower turnover, we examined mRNA stability during incubation in the presence of the transcription inhibitor, DRB. The decay rates of EF-1 mRNAs were determined in the T47D breast cancer cell line by Northern blotting. As shown in Figure 4, the three mRNAs are relatively stable, and no significant differences in their turnover was observed as compared to the internal control, BRCA2 mRNA ($t_{1/2}$= 10 hours) (16).

Discussion

Several studies have shown that the components of EF-1 are possible oncogenes. EF-1A was first implicated in cell transformation by Tatsuka and colleagues (5). They showed that the mouse homolog of EF-1A enhanced the rate of both spontaneous and chemically-induced transformation. A mutated form of EF-1A-named prostate tumor-inducing gene (PTI), was also found to be expressed in primary prostate carcinomas but not in normal prostate (17). The GTPase activity of EF-1A is dependent on the loading of GTP, which is carried out by the exchange complex EF-1Bαγ. Thus, it is suggested that EF1-Bαγ possesses, similar to EF-1A, oncogenic activity. EF-1Bα is not shown yet to have transforming ability or to be up-regulated in any cancer and, although EF-1Bγ was found to be over-expressed in hepatic, gastric and colorectal carcinomas (11-13), it too was not shown to transform cells.

As a G-protein, it is thought that EF-1A is regulated through a common G-protein signaling pathway that is stimulated during oncogenesis or upon external stimuli (18), which might explain the difference in expression levels when compared to EF-1Bαγ. The lack of correlation between the overexpression of EF-1A, -1Bα and -1Bγ and tumor grade suggests that these factors are up-regulated at an early stage of cancer development and represent an important and necessary event in cancer progression. However, the exact mechanism of this overexpression is unclear.

In our study, we tried to identify initial clues to the nature of EF-1 regulation and possible implications in the development of breast cancer. Genes can be regulated at the transcriptional or post-transcriptional levels. Using a transcription inhibitor, DRB, steady state levels of EF-1 subunits and their decay rate were measured over a 24-hour period. Stable turnover rates for the EF-1 subunits in normal breast and cancer cell lines suggest that regulation of EF-1 subunits occurs at the transcriptional level and not at the post-transcriptional level. Transcriptional regulation can be either increased by transcription rate or gene amplification. EF-1A is mapped to chromosome 6q14, a region reported to be amplified in some human cancers (4). Thus, EF-1A might be a potential candidate for DNA amplification, which could lead to its overexpression. In colorectal and pancreatic cancer, mutations in the coding sequences of EF-1Bγ, gene amplification and gene rearrangement were not responsible for its high frequency of overexpression (11). As for EF-1Bα, this is the first report of its overexpression in a type of carcinoma. Further investigations on the role of EF-1Bα in carcinogenesis are required. Several growth-promoting factors can also lead to the transcriptional activation of EF-1 subunits such as growth factors (19), insulin (20), PMA stimulation (21) or other oncogenes like Myc and Ras.

Overexpression of EF-1 could lead to increased translation rate and overall protein synthesis. This may enhance cellular proliferation and reduce the time required for protein production, particularly in stimulated and

cancerous cells. Due to their important cellular function in controlling cell growth and cancer, the EF-1 complex can be targeted by translation inhibitors or chemotherapeutic agents in rapidly growing tumor tissues.

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References

1 The World Health Report 1997. Conquering suffering, enriching humanity. Geneva, World Health Organization, 1997.
2 Kahan E, Ibrahim AS, El Najjar K, Ron E, Al-Agha EH, Polliack A and El-Bolkainy MN: Cancer patterns in the Middle East. Acta Oncologica *36*: 631-636, 1997.
3 Caraglia M, Budillon A, Vitale G, Lupoli G, Tagliaferri P and Abbruzzes A: Modulation of molecular mechanisms involved in protein synthesis machinery as a new tool for the control of cell proliferation. Eur J Biochem *267*: 3919-3936, 2000.
4 Thornton S, Anand N, Purcell D and Lee J: Not just for housekeeping: protein initiation and elongation factors in cell growth and carcinogenesis. J Mol Med *81*: 536-548, 2003.
5 Tatsuka M, Mitsui H, Wada M, Nagata A, Nojima H and Okayama H: Elongation factor-1α gene determines susceptibility to transformation. Nature *359*: 333-336, 1992.
6 Lazaris-Karatzas A, Montine KS and Sonenberg N: Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. Nature *345*: 544-547, 1990.
7 Proud CG: Peptide-chain elongation in eukaryotes. Mol Biol Reports *19*: 161-170, 1994.
8 Janssen GMC and Moller W: Kinetic studies on the role of elongation factors 1β and 1γ in protein synthesis. J Biol Chem *263(4)*: 1773-1778, 1988.
9 Edmonds BT, Wyckoff J, Yeung YG and Wang Y: Elongation factor-1 alpha is an overexpressed actin binding protein in metastatic rat mammary adenocarcinoma. J Cell Sci *109*: 2705-2714, 1996.
10 Anand N, Murthy S, Amman G, Wernick M, Porter LA, Cukier IH and Collins C: Protein elongation factor EEF1A2 is a putative oncogene in ovarian cancer. Nat Genet *31(3)*: 301-305, 2002.
11 Chi K, Jones DV and Frazier ML: Expression of an elongation factor-1 gamma related sequence in adenocarcinomas of the colon. Gastroenterology *103*: 98-102, 1992.
12 Frazier ML, Inamdar N, Alvula S, Wu E and Kim YH: Few point mutations in elongation factor-1 gamma gene in gastrointestinal carcinoma. Mol Carcinog *22*: 9-15, 1998.
13 Shuda M, Kondoh N, Tanaka K, Ryo A and Wakatsuk T: Enhanced expression of translation factor mRNAs in hepatocellular carcinoma. Anticancer Res *20*: 2489-2494, 2000.
14 Chomczynski P and Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem *162*: 156-159, 1987.
15 Ziegler A, Hagmann J, Kiefer B and Nagamine Y: Ca^{+2} potentiates cAMP-dependent expression of urokinase-type plasminogen activator gene through a calmodulin-and protein kinase C-independent mechanism. J Biol Chem *265*: 21194-21201, 1994.
16 Wu K, Jiang S and Couch FJ: p53 Mediates repression of the *BRCA2* promoter and down-regulation of BRCA2 mRNA and protein levels in response to DNA damage. J Biol Chem *268*: 15652-15660, 2003.
17 Shen R, Su ZZ, Olsen CA and Fisher PB: Identification of the human prostatic carcinoma oncogene PTI-1 by rapid expression cloning and differential RNA display. Proc Natl Acad Sci USA *92*: 6778-6782, 1995.
18 Reynet C and Khan CR: Unbalanced expression of the different subunits of elongation factor 1 in diabetic skeletal muscle. Proc Natl Acad Sci USA *98*: 3422-3427, 2001.
19 Hassell JA and Englhardt DL: The regulation of protein synthesis in animal cells by serum factors. Biochemistry *15*: 1375-1381, 1976.
20 Chang YW and Traugh JA: Phosphorylation of elongation factor 1 and ribosomal protein S6 kinase by multipotential S6 kinase and insulin stimulation of translational elongation. J Biol Chem *272*: 28252-28257, 1997.
21 Venema RC, Peters HI and Traugh JA: Phosphorylation of valyl-tRNA synthetase and elongation factor 1 in response to phorbol esters is associated with stimulation of both activities. J Biol Chem *266*: 11993-11998, 1991.

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