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## Title

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**The eukaryotic translation elongation factor eEF1A2 induces neoplastic properties and mediates tumorigenic effects of ZNF217 in precursor cells of human ovarian carcinomas**

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Running title: eEF1A2 and ZNF217 in ovarian cancer

Novelty and impact: (1) First report on direct oncogenic effects of eEF1A2 on ovarian cancer precursors; (2) evidence that eEF1A2 mediates or facilitates oncogenic effects of ZNF217 – a new example of oncogene interaction.

## ABSTRACT

Ovarian epithelial carcinomas (OEC) frequently exhibit amplifications at the 20q13 locus which is the site of several oncogenes, including the eukaryotic elongation factor *EEF1A2* and the transcription factor *ZNF217*. We reported previously that overexpressed *ZNF217* induces neoplastic characteristics in precursor cells of OEC. Unexpectedly, *ZNF217*, which is a transcriptional repressor, enhanced expression of eEF1A2. In this study, array comparative genomic hybridization, single nucleotide polymorphism and Affymetrix analysis of *ZNF217*-overexpressing cell lines confirmed consistently increased expression of eEF1A2 but not of other oncogenes, and revealed early changes in *EEF1A2* gene copy numbers and increased expression at crisis during immortalization. We defined the influence of eEF1A2 overexpression on immortalized ovarian surface epithelial cells, and investigated interrelationships between effects of *ZNF217* and eEF1A2 on cellular phenotypes. Lentivirally induced eEF1A2 overexpression caused delayed crisis, apoptosis resistance and increases in serum-independence, saturation densities, and anchorage independence. siRNA to eEF1A2 reversed apoptosis resistance and reduced anchorage independence in eEF1A2-overexpressing lines. Remarkably, siRNA to eEF1A2 was equally efficient in inhibiting both anchorage independence and resistance to apoptosis conferred by *ZNF217* overexpression. Our data define neoplastic properties that are caused by eEF1A2 in nontumorigenic ovarian cancer precursor cells, and suggest that eEF1A2 plays a role in mediating *ZNF217*-induced neoplastic progression.

**Keywords:** Ovarian cancer, ZNF217, EEF1A2, oncogene, ovarian epithelial cells, neoplastic progression.

## Introduction

The transcription factor *ZNF217* and the eukaryotic translation elongation factor *EEF1A2* are both located on chromosome 20q13, a locus which is frequently amplified in ovarian epithelial carcinomas. There is both clinical and experimental evidence that these two proteins act as oncogenes in ovarian carcinogenesis as well as in other forms of cancer. However, because of the coexistence of multiple putative oncogenes on chromosome 20q, it has been difficult to define their individual specific influence on malignant progression.

20q13 amplification and overexpression of *ZNF217* have been described in many types of human cancers, including ovarian carcinomas (1, 2). Recent evidence indicates that *ZNF217* is a transcriptional repressor (3, 4) and that it may contribute to neoplastic progression by attenuating apoptotic signals (5, 6). In experimental systems, overexpression of *ZNF217* has been shown to immortalize cell lines with limited life spans, such as human mammary epithelial cell lines and SV40 Tag/tag transduced ovarian surface epithelial cells (IOSE cells). In both of these cell types, *ZNF217* increased telomerase activity and stabilize telomere lengths (7, 8). *ZNF217*-transduced IOSE cells also acquired anchorage independence and reduced serum dependence.

The translation elongation factor eEF1A2 is one of two isoforms of eEF1A, eEF1A-1 and eEF1A-2. In contrast to eEF1A-1, which is expressed ubiquitously, eEF1A2 is normally present only in brain and muscle. The classical role of these proteins is the regulation of ribosomal polypeptide elongation, but eEF1A2 also has noncanonical functions such as cytoskeletal modifications (9, 10), targeting proteins for degradation (11) involvement in heat shock responses (12), phosphatidylinositol signaling (9) and apoptosis (13). There is increasing evidence that it also exerts oncogenic functions. eEF1A2 is amplified and overexpressed in ovarian cancers (14, 15), and is overexpressed in cancers of the breast and lung (16, 17, 18, 19). It also induces neoplastic

properties in NIH3T3 cells (14).

eEF1A2 has been reported to be antiapoptotic (20) and to regulate filopodia formation and migration through Akt/PI3-dependent cytoskeletal remodeling (9). The precise mode of action through which this elongation factor exerts its oncogenic properties remains unclear (21), though its effects on cytoskeletal modifications, apoptosis and AKT signaling likely play a role.

We recently reported that virally transduced, overexpressed ZNF217 induces neoplastic characteristics in nontumorigenic precursor cells of ovarian carcinomas (IOSE cells), derived from ovarian surface epithelial (OSE) cells (8). As in other systems, the effects of ZNF217 on IOSE cells were predominantly gene-suppressive. However, among the few genes that were upregulated, *EEF1A2* stood out as the only oncogene that was consistently overexpressed, over long periods of time and many passages, in two IOSE cell lines (lines I-144RZ and I-80RZ) that were derived from two different women.

The present study was undertaken to further examine the relationship between ZNF217 and eEF1A2, and, in particular, to determine whether *ZNF217*-induced neoplastic changes require interactions with *EEF1A2*. Importantly, Krieg et al. (3) recently carried out a comprehensive identification of all genes that are directly regulated by the gene suppressor ZNF217, using ChiP-chip assays. There was negligible interaction between ZNF217 and *EEF1A2*, indicating that the effects of ZNF217 on *EEF1A2* expression, as observed by Li et al. (8), were likely indirect, perhaps through inhibition by ZNF217 of negative regulator(s) of eEF1A2 expression (13, 22).

In the present study, we created the first stable preneoplastic ovarian cell lines that overexpress eEF1A2. These lines allowed us to provide the first direct evidence showing that eEF1A2 overexpression contributes neoplastic characteristics to precursor cells of ovarian carcinomas, and thus support previous evidence pointing to an oncogenic role of this protein in

ovarian carcinogenesis. The results suggest further that eEF1A2 may play an important role in mediating and/or enhancing the oncogenic effects of ZNF217.

## **Material and methods**

### *Cell culture and infection*

Normal ovarian surface epithelial cells (OSE) were obtained at surgery for nonmalignant gynecological disorders. Ethical permits were obtained as required by the University of B.C. OSE cells in low passage were immortalized with SV40 large T and small t antigen to give rise to IOSE (“immortalized OSE”) lines (23). To obtain IOSE lines constitutively overexpressing eEF1A2, two IOSE lines that were derived from two different women, IOSE-80 and IOSE-144, were infected with the ViraPower Lentiviral Expression System (Invitrogen, Carlsbad, CA), using constructs encoding lentiviral *EEF1A2-V5* and *β-galactosidase* as control that were amplified in STBL3 competent cells (Invitrogen). High titer stocks of *EEF1A2-V5* and control lentivirus were prepared using a transient packaging kit (Invitrogen). Starting at 48 hours after infection, cells were selected with 2 μg/ml blasticidin, and infection efficiency was evaluated cytochemically in cultures with the recombinant lentivirus encoding β-galactosidase.

As described previously (8), two ZNF217-overexpressing IOSE-derived cell lines, I-80RZ and I-144RZ, were obtained from different women, and two isogenic sublines were derived from each of these two lines at the time of crisis: one subline was maintained with, and one without 10 ng/ml of EGF. All cells were grown under the conditions described previously (8).

To monitor the timing of changes in EEF1A2 at the genomic and transcriptional level, IOSE-144 cells, infected with HA-tagged *ZNF217* as described above, were examined by array CGH and Affymetrix array analysis as described previously (8), both prior to and after crisis.

Furthermore, ZNF217-HA-infected IOSE-144 cultures entering crisis were monitored for the appearance of growing colonies. When such colonies had reached a size representing approximately 20,000 to 40,000 cells, they were scraped off, lysed and analysed for eEF1A2 expression by CGH–SNP arrays.

#### *CGH–Single nucleotide polymorphism (SNP) arrays*

DNA was extracted from cells using a Puregene protocol (Gentra Systems, Minneapolis, MN). Genomic DNA was amplified with phi29 enzyme using the REPLI-g Midi kit (Qiagen, Valencia, CA). The DNA concentration was determined using a spectrophotometer and diluted to a concentration of 50 ng/μL. Preparation of DNA for hybridization to the GeneChip® Human Mapping 50K Array Xba 240 was done according to the manufacturer's instructions (100K Mapping Assay Manual; Affymetrix, Santa Clara, CA). Briefly, 250 ng of genomic DNA was digested with *Xba*I and a common adapter oligo ligated to the ends of the digestion fragments and amplified using a universal primer set. The amplified product was fragmented and labeled with biotin and hybridized to the Mapping 50K Xba array overnight. After removal of the hybridization cocktail, the chip was stained with streptavidin-R-phycoerythrin conjugate and scanned using a GeneChip® Scanner 3000 7G, and genotyped and signals were detected by Affymetrix GeneChip® Operating Software (GCOS). DNA copy number and LOH calls were generated using dSNPChip software from Harvard (<http://biosun1.harvard.edu/complab/dchip/>). The SNPs are positioned using the May 2004 assembly (NCBI Build 35) from the UCSC genome browser.

#### *Reverse Transcription PCR*



Total RNA was extracted and purified from cells grown to 70-80% confluency with the RNeasy kit from Qiagen by following manufacturer's instructions (Qiagen, Mississauga, ON, Canada), while 1st-strand cDNA synthesis was performed with a first-strand cDNA synthesis kit (Amersham Biosciences, Baie d'Urfe, Quebec). RNA denaturation, reaction setup and sample treatment were performed as previously reported (8). A pair of primers spanning the 3' terminal of *EEF1A2* gene and 5' terminal of V5 tag (5'—CCGGCGGGGCAACGTGTGTGGGGAC—3' and 5'—CGAGGAGAGGGTTAGGGATAGGCTTAC—3', respectively) was used to amplify the transgene *EEF1A2-V5*. PCR amplification was carried out as reported previously (8), with PCR products of *EEF1A2-V5* analyzed by agarose gel electrophoresis.

#### *Western blot analysis*

Cells were lysed as described previously (8) with protein concentrations measured by Bio-Rad protein assay kit (Bio-Rad Laboratories, Mississauga, ON, Canada). To analyse the expression of recombinant *EEF1A2-V5*, total proteins (30ug) were separated on 8% SDS-PAGE gels followed by transfer onto nitrocellulose membranes. After blocking for 30 min with 5% nonfat dried milk, blots were probed at 4 °C for overnight with mouse monoclonal anti-*EEF1A2* (Cell Signaling, Danvers, MA) at 1:10000. Upon extensive washing with TBST, the immune complexes were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody at 1:3000 (Bio-Rad Laboratories, Hercules, CA) for 1 hr followed by enhanced chemiluminescence (ECL) detection system (BioLynx, Brockville, ON, Canada).

#### *Anchorage independence assay*

Anchorage independent cell growth was determined by analyzing colony formation of cells in soft agar. Cells ( $1.3 \times 10^4$ ) were suspended in 5 ml 0.3% agarose in M199:105 medium containing 10% FBS and plated on pre-solidified agarose (0.5%) in 60 mm dishes with grids for colony quantification. Five milliliters of medium were added on top of the agarose and changed once per

week. After 3 weeks, colonies of different sizes were counted microscopically, with data obtained from triplicate experiments.

#### *Cell growth and serum dependence assays*

Cells in the exponential growth phase were trypsinized and plated in 96-well strip plates (Corning Inc., Corning, NY) at  $2 \times 10^3$  cells/well. For proliferation assays, cells were incubated in 199:105 medium (5% FBS). Six replicates were collected on days 0, 1, 3, 5, 7, 9, 11, 13, respectively. For the serum dependence assays, cells were incubated in 199:105 medium with serum concentrations ranging from 0 to 5%, and collected after 7-9 days. For both assays, at the end of the growth period, cells were washed with serum-free medium and fixed with  $-20^{\circ}\text{C}$  methanol 20 min followed by 3 washes with PBS. Cells were then stained with 5.0 ug/ml Hoechst 33258 (Sigma) for 2 minutes, washed to remove unbound dye, and analyzed on a fluorescence plate reader with filters of wavelength 360/40 and 460/40.

#### *Apoptosis assays*

Cells were either treated with 1  $\mu\text{M}$  camptothecin for 16-20 hr at  $37^{\circ}\text{C}$  to induce apoptosis or left untreated as controls. Both induced and control cells were then harvested, lysed and assayed as follows:

##### (1) EnzChek caspase-3 assay

Cells were harvested after being incubated with apoptosis inducer for optimized lengths of time and washed in phosphate-buffered saline (PBS). Each cell sample was resuspended in 50  $\mu\text{l}$  of the  $1\times$  lysis buffer, and cells were subject to lysis on ice for 30 min. At the same time, a  $2\times$  reaction buffer was prepared by adding 400  $\mu\text{l}$  of the  $5\times$  reaction buffer and 10  $\mu\text{l}$  of 1M DTT to 590  $\mu\text{l}$  of  $\text{dH}_2\text{O}$ , and a  $2\times$  substrate working solution was made by mixing 10  $\mu\text{l}$  of the 5mM Z-DEVD-R110 substrate with 990  $\mu\text{l}$  of the  $2\times$  reaction buffer. The lysed cells were centrifuged at 500 rpm for 5 min at room temperature to pellet the cellular debris. Fifty microliters of  $2\times$  substrate working

solution was added to each sample. The microplate was covered and samples incubated at room temperature for 30 min. Fluorescence was measured in a microplate reader with the excitation filter at  $485\pm 20\text{nm}$  and emission detection at  $530\pm 20\text{nm}$  after the indicated amount of time.

## (2) Cell death detection ELISA

Quantification of apoptotic cell death was determined by an ELISA assay that measures cytoplasmic histone-complexed DNA fragments, using the one-step sandwich immunoassay to detect nucleosomes released from the nuclei (Roche Diagnostics, Indianapolis, IN). After dilution to  $1\times 10^5$  cells/ml, 100  $\mu\text{l}$  of suspended cells were added into individual wells of a 96-well plate and incubated for 4 hr at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ . The 96-well plate was then centrifuged, cell pellets resuspended and mixtures incubated according to the manufacturer's instructions (Roche Diagnostics).. The solutions were removed thoroughly by tapping or suction, and each well was given 100  $\mu\text{l}$  ABTS solution. The reactions were incubated on a shaker at 250 rpm until the color development was sufficient for photometric analysis. Absorbance was measured at 405 nm against ABTS solution as a blank.

## *Migration assays*

Cells were grown to confluency (95%) in 60-mm cell culture dishes. A scratch was made through the cell monolayer using a 200  $\mu\text{l}$  pipette tip. After two washes to remove floating cells and debris, fresh culture medium with 5% FBS was added. Distances between the edges of the wounds were measured at specific time points at 0, 12 and 24 hr after wounding, and the changes from time 0 were halved to calculate the distance that the cells had migrated.

## *siRNA assays*

Exponentially growing cells were seeded at 70% density for 24h. Then, the cells were

transfected with *EEF1A2*-specific siRNA and negative control (NC) siRNA (Qiagen) at varying concentrations using HiPerFect (Qiagen). The transfected cells were incubated overnight before being trypsinized, counted, and assayed for anchorage independent growth as described above. After 7 days of incubation, colonies were counted. Changes in growth and morphology associated with the presence of *EEF1A2* siRNA assay were also examined with cells that were transfected with siRNA and maintained under standard conditions for 1-4 days posttransfection.

## **Results**

### ***Generation of IOSE cell lines overexpressing eEF1A2***

IOSE-144 cells (see Materials and Methods for definition) at passage 6~7 were lentivirally infected with *EEF1A2-V5* or a *LacZ-V5* control vector. Thirteen independent cell lines infected with *EEF1A2-V5* (LE-lines) and 2 lines infected with *LacZ-V5* control vector (LL lines) were established. Expression of  $\beta$ -galactosidase from the control construct confirmed that infection efficiency was near 80% (not shown). Infection with either lentiviral *EEF1A2-V5* or vector control caused no obvious phenotypic changes other than nonspecific transient damage such as detachment and vacuolation, which was similar in both groups.

The *EEF1A2-V5* infected cells were resistant to blasticidin, were positive for the construct RNA by RT-PCR and showed enhanced expression of eEF1A2 by Western blots with anti-eEF1A2 antibody (Fig. 1A, B). The overexpressed eEF1A2 appeared as a double band with a slower upper band, representing the tagged form of the molecule.

### ***Phenotypic changes in lines overexpressing eEF1A2***

eEF1A2 overexpressing lines were significantly less serum dependent ( $p < 0.05$ ) than the parent line, and cells proliferated even in the complete absence of serum (Fig. 2). In contrast, the control  $\beta$ -Gal overexpressing line I-144LL did not exhibit enhanced serum-independence.

Short term proliferation assays showed that I-144LE cells grew relatively slowly at low densities as compared with the IOSE-144 and I-144LL cells, but they overtook the latter by 7 days of continued growth and reached saturation densities ranging from 10% to 33% higher, as compared with the parent IOSE-144 line and the I-144LL line (Fig. 2B). Proliferation over long periods demonstrated a significant increase in population doubling potential, increased lifespan and delayed crisis of eEF1A2-overexpressing LE cell lines, when compared with the parent IOSE line (Fig. 2C). The control cells ceased proliferating and entered crisis at approximately 80 days (52 PDs), while I-144LE cells reached 120-130 days (60 PDs) before undergoing crisis. Delayed crisis of a cell line is indicative of an increase in population doubling potential, which leads to an increased lifespan of precrisis cells. This change usually results from an increase in proliferative capacity and/or enhanced resistance to apoptosis. However, overexpression of eEF1A2 alone was insufficient to induce further immortalization and circumvention of crisis

Upon 3 weeks incubation in soft agar, anchorage independence assays demonstrated that the I-144LE line had 6 to 7 times more cell colonies (Fig. 2D), as compared with either the IOSE-144 parent line or I-144LL line, indicating decreased anchorage dependence with increased eEF1A2 expression. However, the total number of colonies was small, suggesting that the change took place in a minor subpopulation of cells.

Scratch assays indicated that the migration capability of I-144LE cells into wounds in cell monolayers was different, which is in contrast to the parent line-IOSE144 cells (Fig. 3 a-f), with the migration rate of the former increased significantly ( $p < 0.05$ ) as compared to the latter (Fig. 3g).

#### ***EEF1A2 gene expression and gene copy numbers in ZNF217-transformed lines***

We examined two ZNF217-transformed isogenic sublines derived from I-144RZ and two ZNF217-transformed isogenic sublines derived from I-80RZ (8): medium in one of each pair of

sublines was supplemented with 10ng EGF/ml which had been shown previously to be required transiently for immortalization (8). As shown in the present report, EGF had no influence on gene expression or other properties of the cell lines. eEF1A2 expression was consistently amplified in all 4 sublines (Table 1). Importantly, in line I-80RZ, where the 20q13 locus is amplified, eEF1A2 was significantly more overexpressed (23-27- fold) than in line I-144RZ (4.8- 6.6 fold) where 20q13 is not amplified. Of 11 established or putative ovarian epithelial oncogenes, the only ones that were sporadically overexpressed were STAT1, STAT3 and PI3KCA in the two I-144RZ lines and STAT1, STAT3 and RAB6B in the I-80RZ lines; all other oncogenes were either downregulated or unchanged, and none besides eEF1A2 were consistently overexpressed in all 4 sublines.

Early stages of ZNF217-induced immortalization in line IOSE-144 were studied in colonies that appeared in ZNF217-infected cultures at the time of crisis. As shown in Table 1, eEF1A2 expression increased dramatically at the time of crisis and remained continuously upregulated thereafter. To determine whether *EEF1A2* gene copy changes could be detected very early in the immortalization process, IOSE-144 cultures were infected with ZNF217 in passage 15, i.e. just prior to crisis, and when growing colonies, estimated to contain 20,000 to 40,000 cells, appeared during crisis, they were scraped off the culture dishes, lysed and analysed by CGH by Affymetrix SNP chip.

Of 9 such colonies analysed, one *EEF1A2* gene copy was lost in five of the colonies, and the gene was amplified to 3-4 copies in 3 other colonies (Table 2). In Table 2, we included SNPs surrounding SNP\_A-1757091 rs1406 and SNP\_A-1698559 rs6089982, where eEF1A2 genetically resides, to emphasize the point that the observed variability in copy numbers is not limited to *EEF1A2* but is a generalized phenomenon. Multiple other factors determine which variants will have a selective advantage that will allow the cells to overcome crisis. Cells with *EEF1A2*

amplification appear to be such variants. The most consistent feature among these colonies and fully transformed I-144RZ cell lines was gene loss at chromosome 18 (Table 3), which has also been reported as a frequent change in benign and neoplastic ovarian tumors (Tibiletti et al., 2003) (23).

### ***Phenotypic characteristics***

To determine whether the phenotypic changes in I-144LE lines were caused specifically by eEF1A2, and whether eEF1A2 mediated effects of ZNF217, we transiently transfected I-144LE and I-144RZ cells with siRNA against eEF1A2 and studied the effects on protein expression, anchorage independence and apoptosis.

Upon siRNA transfections, RT-PCR revealed diminishing eEF1A2 mRNA signals in eEF1A2 overexpressing cell lines (Fig. 4A), and Western blots indicated reduced eEF1A2 protein expression, in both I-144LE cells and I-144RZ cells (Fig. 4B).

Anchorage independence assays showed that I-144RZ cells formed approximately 100 times more colonies than I-144LE cells. However, importantly, not only the I-144LE cells but also the I-144RZ cells formed significantly fewer colonies in the presence of siRNA to eEF1A2 (Fig. 4C,D).

The susceptibility to apoptosis of lines I-144LE and I-144RZ was examined by treatment with the DNA topoisomerase I inhibitor camptothecin (CAM). After treatment for 16-20 hr, dramatic cell death was observed in the parent line IOSE-144 (data not shown). In contrast, the damaging effects were alleviated significantly in I-144LE cells, although both I-144RZ cells and SKOV3 cells, representing a camptothecin resistant established ovarian cancer line, exhibited relatively more robust resistance against this cytotoxic drug (Fig. 5A,B). In parallel, the EnzChek Caspase-3 Assay and Cell Death Detection ELISA also demonstrated greatly reduced apoptosis in lines I-144LE and I-144RZ compared with the parent line IOSE-144 (Fig.5A,B).. These assays measure caspase-3 activity and increased histone-associated DNA fragmentation into mono- and

oligonucleosomes, respectively, as markers of these responses to apoptosis-inducing drugs among known drug-resistant cell lines. Caspase-3 activity and DNA fragmentation were significantly increased upon apoptosis induction in the presence of eEF1A2-siRNA in both I-144LE and I-144RZ cells (Figs.5C, D). Importantly, the anti-apoptotic effects of both eEF1A2 and ZNF217 were reduced to a similar degree by siRNA to eEF1A2. These results confirmed the specific role of eEF1A2 in regulating anchorage independence and apoptosis resistance in I-144LE cells, and support the hypothesis that eEF1A2 mediates proneoplastic features resulting from overexpression of ZNF217.

## Discussion

While increased eEF1A2 expression has previously been demonstrated in ovarian carcinomas (14), our study defines, for the first time, direct proneoplastic effects of eEF1A2 on the nontumorigenic precursors of these neoplasms. Furthermore, our results suggest that the two known ovarian oncogenes, *ZNF217* and *EEF1A2*, may act interdependently in ovarian neoplastic progression, thus revealing a possible mechanism by which *ZNF217*, a gene suppressor protein, may exert its oncogenic effects. Krig et al. (3) recently carried out a comprehensive identification of all genes that are directly regulated by the gene suppressor *ZNF217*, using ChiP-chip assays (3). They found that there is negligible direct interaction between *ZNF217* and *EEF1A2*. This conclusion is consistent with our present and previous results which indicate that, in contrast to a large number of genes eEF1A2 expression is not downregulated by overexpressed *ZNF217*. The results of the present study, which show that eEF1A2 is in fact overexpressed in the presence of overexpressed *ZNF217*, suggests that the interrelationships between *ZNF217* and *EEF1A2* must be indirect, perhaps through inhibition by *ZNF217* of physiologic negative regulator(s) of eEF1A2 expression (13, 20).



Overexpressed eEF1A2 rendered IOSE cells resistant to apoptosis and resulted in increased anchorage- and serum independence, suggestive of altered production of growth factors and/or extracellular matrix components. It also resulted in increased saturation densities, which indicate potential transition from normalcy toward malignancy. Normal cell populations become growth arrested when they reach specific levels of confluency (saturation density) through inhibition of further proliferation by intercellular contact. This regulatory process, named contact inhibition of growth, is compromised in neoplastic cells, thus allowing the cells to continue growing to increased saturation densities. Interestingly, the changes induced by eEF1A2 resemble the neoplastic characteristics that were induced in IOSE by ZNF217 (8), but in eEF1A2-overexpressing cells, they were subtle compared to the more robust effects of ZNF217. Both of these genes exert some of their oncogenic effects by activating Akt2 and thereby increasing its antiapoptotic and anchorage independence-enhancing action (5, 9). Most importantly, siRNA to eEF1A2 reversed the anchorage independence and the resistance to apoptosis induced by overexpression of either eEF1A2 or of ZNF217.

Our results indicate that eEF1A2 may mediate the proneoplastic changes caused by ZNF217, and/or that ZNF217 may enhance the proneoplastic effects of eEF1A2 by upregulating its expression. In addition, eEF1A2 may complex with ZNF217, since this protein has been reported to function through other protein-protein interactions: it forms complexes with Prdx-1 which protect cells from apoptosis more efficiently than either protein alone (25) and it forms complexes with the zinc finger protein ZRP1, which translocate to the nucleus to maintain cell cycling (26). However, at present there is no evidence for such an interaction between eEF1A2 and ZNF217.

In our studies of ZNF217-transduced IOSE cells, most genes were downregulated (8), as would be expected from a transcriptional gene suppressor (3). In our past and present studies, *EEF1A2* stood out as the only oncogene that was consistently overexpressed, independently of source,

duration in culture or EGF treatment. Although eEF1A2 was consistently overexpressed in all four I-144RZ and I-80RZ lines, the q20.13 locus where the *EEF1A2* gene is located was amplified only in the I-80RZ lines but not in the I-144RZ lines (8). It has been reported previously that copy numbers of *EEF1A2* do not always correlate with expression levels of the gene, indicating that eEF1A2 expression and/or protein levels must also be regulated at other levels (15). The fact that, in our studies, eEF1A2 was overexpressed in lines with and without 20q13 amplification indicates that, similar to Tomlinson's observations (15, 16), mechanisms besides gene dosage were responsible for its overexpression. Furthermore, eEF1A2 overexpressing IOSE cells appeared to have a selective advantage regardless of the means by which overexpression was achieved, since they were selected for in every line. Interestingly, the degree of overexpression was approximately 5-fold greater in the lines with 20q13 gene amplification compared to the lines with normal gene dosage. This observation suggests that 20q13 amplification may have enhanced the overexpression of eEF1A2 and thus the selective advantage of these cell populations.

Using SNP analysis of very small colonies consisting of approximately 20,000-40,000 cells, which appeared during senescence and crisis of ZNF217-transfected IOSE cultures, we observed considerable variation in gene copy numbers, indicative of genetic instability. The number of *EEF1A2* copies ranged from 1-4 in various colonies. Since the colonies had to be lysed for analysis, and recovery from crisis is a rare event, it was impossible to determine whether this variability had any relationship to the establishment of permanent immortal lines – however, the results do show that the genetic instability at the time of crisis affects *EEF1A2* gene expression which may be a factor influencing the success of crisis survival (27).

In summary, this study further defines the role of eEF1A2 in ovarian carcinogenesis and shows that this protein confers neoplastic characteristics on preneoplastic, nontumorigenic precursors of

ovarian cancer. eEF1A2 thus has the potential to exert some of its transforming effects early in ovarian neoplastic progression. Another important point raised by the results of this study is the possibility that at least some of the proneoplastic effects of the transcriptional gene suppression protein ZNF217 may be conveyed indirectly by upregulated EEF1A2. In view of the complexity of the transcriptional regulation of eukaryotic ribosomal protein genes (22), the detailed molecular mechanisms of such an interaction remain to be determined.

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## FIGURE LEGENDS

Fig. 1. **A**, RT-PCR, using a primer spanning the 3' terminal of *EEF1A2* gene and 5' terminal of V5 tag, demonstrates *EEF1A2*-V5 expression in I-144LE cell lines, but not in the parent line. From the left to right: GeneRuler DNA ladder mix, IOSE-144 (parent line), I-144LE(9) 9, I-144LE (10), I-144LE(11), I-144LE(13) all at passage 9. The numbers in parentheses indicate independently created lines. **B**, Western blot: monoclonal anti-eEF1A2 reveals eEF1A2 overexpression in *EEF1A2*-V5-infected cultures of the 4 LE lines shown in **A**.

Fig. 2. **A**. Serum dependence. At low serum concentrations the eEF1A2 overexpressing line I-144LE was significantly less serum dependent ( $p < 0.05$ ) than either the parent line or the  $\beta$ -Gal overexpressing line I-144LL, and proliferated even in the complete absence of serum. **B**. Short term growth assay. In medium with 5% FBS I-144LE cells grew slowly at low densities but reached saturation densities ranging from 10% to 33% above the control lines IOSE-144 and I-144LL by 13 days. **C**. Proliferation potential. I-144LE cell lines gained a prolonged lifespan, with the population doublings increased significantly as compared to controls. The times when Tag/tag and *EEF1A2* were introduced into the cultures are indicated on the graph. Crisis occurred as proliferation ceased. **D**. Anchorage independence assay. Upon 3-week incubation in soft agar, the I-144LE line had 6 to 7 times more colonies per 60 mm dish, as compared with either the IOSE-144 parent line or the I-144LL line, though the absolute number of colonies was small, suggesting that it represented a minor subpopulation. **E**. Cell migration. **a-c**: IOSE144 cells at 0 hrs (**a**), 12 hrs (**b**) and 24 hrs (**c**). after the monolayers were scratched. **d-f**: I-144LE

cells at 0 hrs (**d**) 12 hrs (**e**) and 24 hrs (**f**) after the monolayers were scratched. **G**. Migration distances of two sets of IOSE-144 and I-144LE cells during the wound healing assay, measured at different time points shows significant differences ( $p < 0.05$ ).

Fig. 3. Effects of siRNA against eEF1A2 on protein expression and anchorage independence. NC is control siRNA. **A**, RT-PCR demonstrated reduced eEF1A2 mRNA levels in the eEF1A2-overexpressing cell line I-144LE upon transfection with increasing concentrations of siRNA. **B**, Western blot: siRNA significantly reduced eEF1A2 expression in both I-144LE and I-144RZ cells. GAPDH was a loading control. **C, D**. Effects of eEF1A2-siRNA on anchorage independence. In both the I-144LE line and the I-144RZ line, siRNA significantly inhibited colony formation in agarose compared to controls and NC siRNA ( $p < 0.01$ ). Note that the scale bars differ between the two cell lines: I-144RZ cells form approximately 100 –fold more colonies than I-144LE cells.

Fig. 4. **A, B**, apoptosis resistance of lines IOSE-144, I-144LE, I-144RZ and SKOV3 prior to and after camptothecin (CAM) treatment. **A** EnzCheck caspase-3 activity assay of the above cell lines. **B**. Cell death detection ELISA assay for histone-associated DNA fragmentation into mono- and oligonucleosomes. **C, D**, Effects of eEF1A2-siRNA on apoptosis resistance in lines I-144LE and I-144RZ before and after CAM treatment. **C**. EnzChek caspase-3 activity assay. **D**. Cell death detection ELISA.

**Table 1. Changes in gene expression in two isogenic lines I-144RZ and two isogenic lines I-80RZ, infected with retroviral ZNF217 in passage 9, and maintained with (+EGF) or without(-EGF) 10ng EGF/ml medium. Crisis occurred around passages 14-20.**

The genes listed are known or putative ovarian oncogenes and related genes, followed by HOXA genes that influence ovarian carcinogenesis.

Cell Line	Growth stage: Precrisis			Crisis						Postcrisis		
	I-144RZ			I-144RZ			I-80RZ			I-80RZ		
EGF*				- EGF			+EGF			-EGF +EGF		
Passage	14	15	20	29	51	29	62	59	48			
<b>Gene</b>												
<b>ANXA10</b>	<b>3.57</b>	<b>3.34</b>	<u>0.35</u>	<u>0.42</u>	<u>0.29</u>	<u>0.41</u>	<u>0.39</u>	<u>0.02</u>	<u>0.06</u>			
<b>THBS1</b>	1.22	0.42	<u>0.11</u>	<u>0.13</u>	<u>0.12</u>	<u>0.12</u>	<u>0.26</u>	<u>0.05</u>	<u>0.12</u>			
<b>CDH11</b>	<b>6.93</b>	<b>4.28</b>	0.97	<u>0.35</u>	<u>0.41</u>	<u>0.34</u>	<u>0.36</u>	<u>0.10</u>	<u>0.29</u>			
<b>DSC3</b>	<b>3.31</b>	1.90	1.00	<u>0.27</u>	<u>0.32</u>	<u>0.29</u>	<u>0.31</u>	<u>0.09</u>	<u>0.09</u>			
<b>CDH2</b>	<b>3.81</b>	<b>3.19</b>	1.31	<u>0.31</u>	<u>0.44</u>	<u>0.32</u>	<u>0.36</u>	<u>0.23</u>	<u>0.24</u>			
<b>KRAS2</b>	<b>4.05</b>	<b>3.88</b>	<b>3.88</b>	0.63	0.79	0.69	0.76	<u>0.42</u>	<u>0.35</u>			
<b>ERBB2</b>	1.59	1.93	<b>2.51</b>	1.13	1.25	1.19	1.17	1.32	0.54			
<b>BRAF</b>	0.57	0.65	0.57	0.88	1.17	1.45	0.92	0.57	1.00			
<b>EVI1</b>	1.39	1.39	1.52	1.09	1.37	1.06	1.15	1.28	1.05			
<b>RAB25</b>	1.80	1.90	1.90	1.35	1.28	1.05	1.24	0.95	1.14			
<b>MYC</b>	<b>6.77</b>	<b>7.33</b>	<b>10.71</b>	1.30	0.90	1.43	1.42	0.92	1.51			
<b>MYB</b>	0.76	0.79	0.71	1.09	1.07	1.00	1.03	0.79	0.68			
<b>ETS1</b>	0.75	0.75	0.62	1.00	0.73	1.06	0.96	0.81	0.87			
<b>EGFR</b>	1.96	1.44	<b>2.06</b>	1.55	1.63	1.56	1.55	0.67	1.23			

<b>MET</b>	1.77	1.15	0.94	1.05	1.09	0.96	1.26	0.53	0.88
<b>PIK3CA</b>	<b>2.10</b>	<b>0.49</b>	0.81	1.71	<b>2.07</b>	1.41	1.47	0.94	0.88
<b>RAB6B</b>	0.57	0.57	0.57	0.87	0.80	0.80	1.04	<b>2.37</b>	<b>2.55</b>
<b>STAT1</b>	<b>2.35</b>	<b>3.32</b>	<b>10.02</b>	<b>3.32</b>	1.40	<b>3.33</b>	<u>0.45</u>	<b>5.13</b>	<b>2.20</b>
<b>STAT3</b>	<b>2.00</b>	<b>2.56</b>	<b>4.03</b>	1.02	1.14	1.05	1.12	0.79	<b>2.00</b>
<b>EEF1A2</b>	1.67	<b>2.31</b>	<b>33.14</b>	<b>6.57</b>	<b>6.57</b>	<b>5.23</b>	<b>4.82</b>	<b>23.18</b>	<b>36.99</b>
<b>HOXA4</b>	<b>4.62</b>	<b>7.27</b>	<b>4.16</b>	0.88	0.75	0.88	0.70	0.59	<u>0.36</u>
<b>HOXA7</b>	1.27	1.32	1.31	1.11	1.18	1.08	0.98	0.64	<u>0.48</u>
<b>HOXA9</b>	<u>0.37</u>	<u>0.40</u>	<u>0.36</u>	<u>0.22</u>	<u>0.21</u>	<u>0.20</u>	<u>0.20</u>	<u>0.25</u>	<u>0.16</u>
<b>HOX10</b>	<u>0.45</u>	0.52	<u>0.45</u>	1.12	0.85	1.15	0.70	<u>0.26</u>	<u>0.31</u>
<b>HOX11</b>	0.66	0.67	0.66	0.78	0.80	0.72	0.83	0.53	0.59

Bold = overexpressed genes (2.0 or more), underlined = underexpressed genes (0.5 or less);

EGF\*, parallel lines were maintained with and without 10 ng/ml EGF (Li et al., 2007)

Note: ANXA10, DSC3 and CDH2 are tumor suppressors.

**Table 2. GENE COPY NUMBER VARIATION ON CHROMOSOME 20 AMONG IOSE-144B COLONIES DURING CRISIS**

						COLONY NO.									
						7	8	9	10	11	12	13	14	15	
SNP_A-1655213	rs6061845	20	61.0842	61.0842	NA	2	3	2	3	2	2	3	3	3	
SNP_A-1655949	rs10485827	20	61.0852	61.0852	NA	2	3	2	3	2	2	3	3	3	
SNP_A-1674859	rs4308178	20	61.0862	61.0862	NA	2	3	2	3	2	2	3	3	3	
SNP_A-1748779	rs6121489	20	61.1719	61.1719	NA	1	1	1	1	1	2	3	3	3	
SNP_A-1692074	rs708629	20	62.1332	62.1332	NA	1	1	1	1	1	2	3	3	3	
SNP_A-1702295	rs3746765	20	62.2471	62.2471	NA	1	1	1	1	1	2	3	3	3	
SNP_A-1749637	rs2295001	20	62.2674	62.2674	NA	1	1	1	1	1	2	3	3	3	
SNP_A-1660585	rs2427462	20	62.3174	62.3174	NA	1	1	1	1	1	2	3	3	3	
SNP_A-1659348	rs910933	20	62.3177	62.3177	NA	1	1	1	1	1	2	3	3	3	
<b>SNP_A-1757091</b>	<b>rs1406961*</b>	<b>20</b>	<b>62.6223</b>	<b>62.6223</b>	<b>NA</b>	<b>1</b>	<b>1</b>	<b>1</b>	<b>1</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>3</b>	<b>3</b>	
<b>SNP_A-1698559</b>	<b>rs6089982*</b>	<b>20</b>	<b>63.1275</b>	<b>63.1275</b>	<b>NA</b>	<b>1</b>	<b>1</b>	<b>1</b>	<b>1</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>3</b>	<b>3</b>	
SNP_A-1758391	rs2427625	20	63.5986	63.5986	NA	1	1	1	1	1	2	3	3	3	

\* location of EEF1A2

**Table 3. aCGH: Common features of individual colonies and lines.**

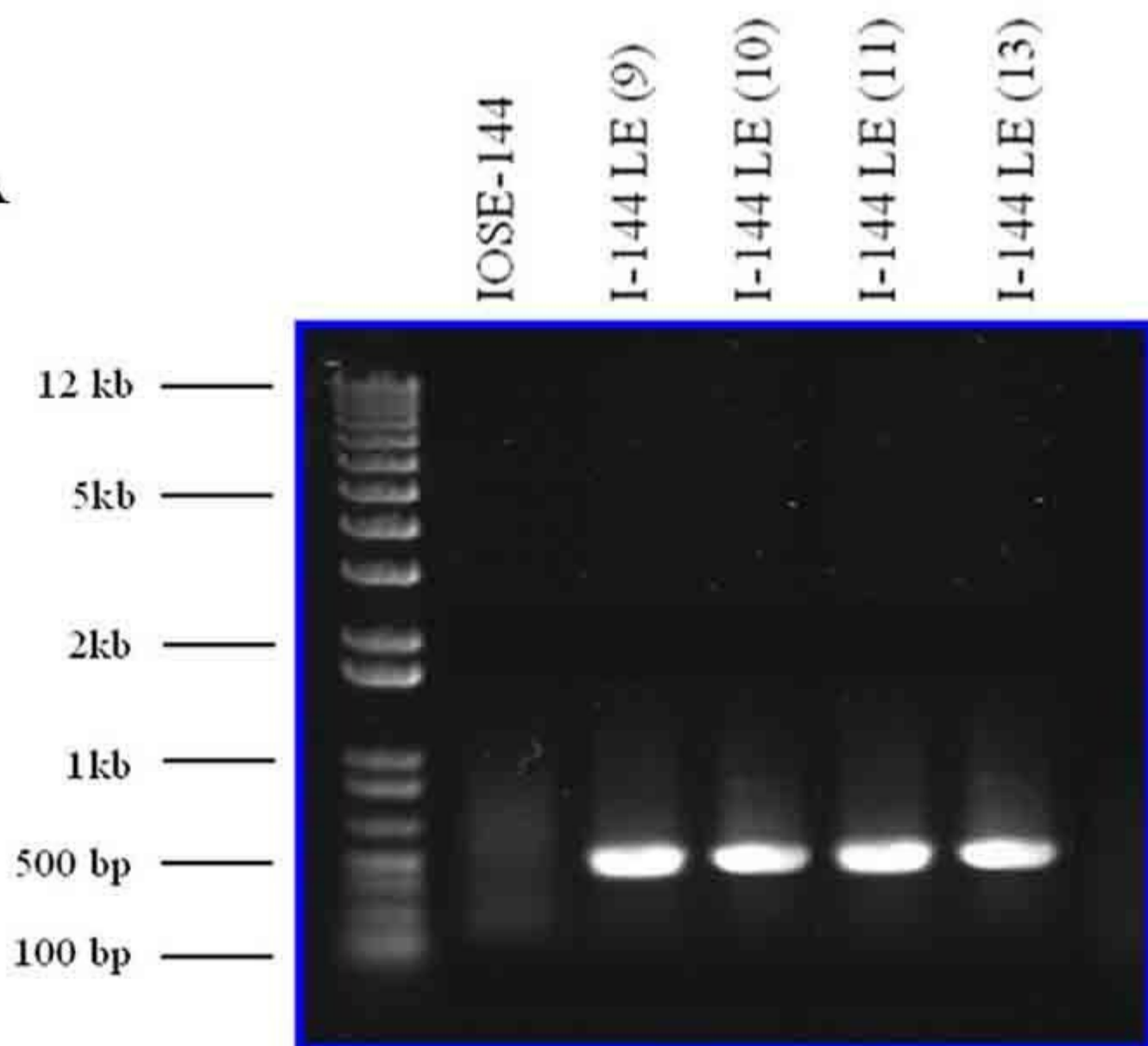
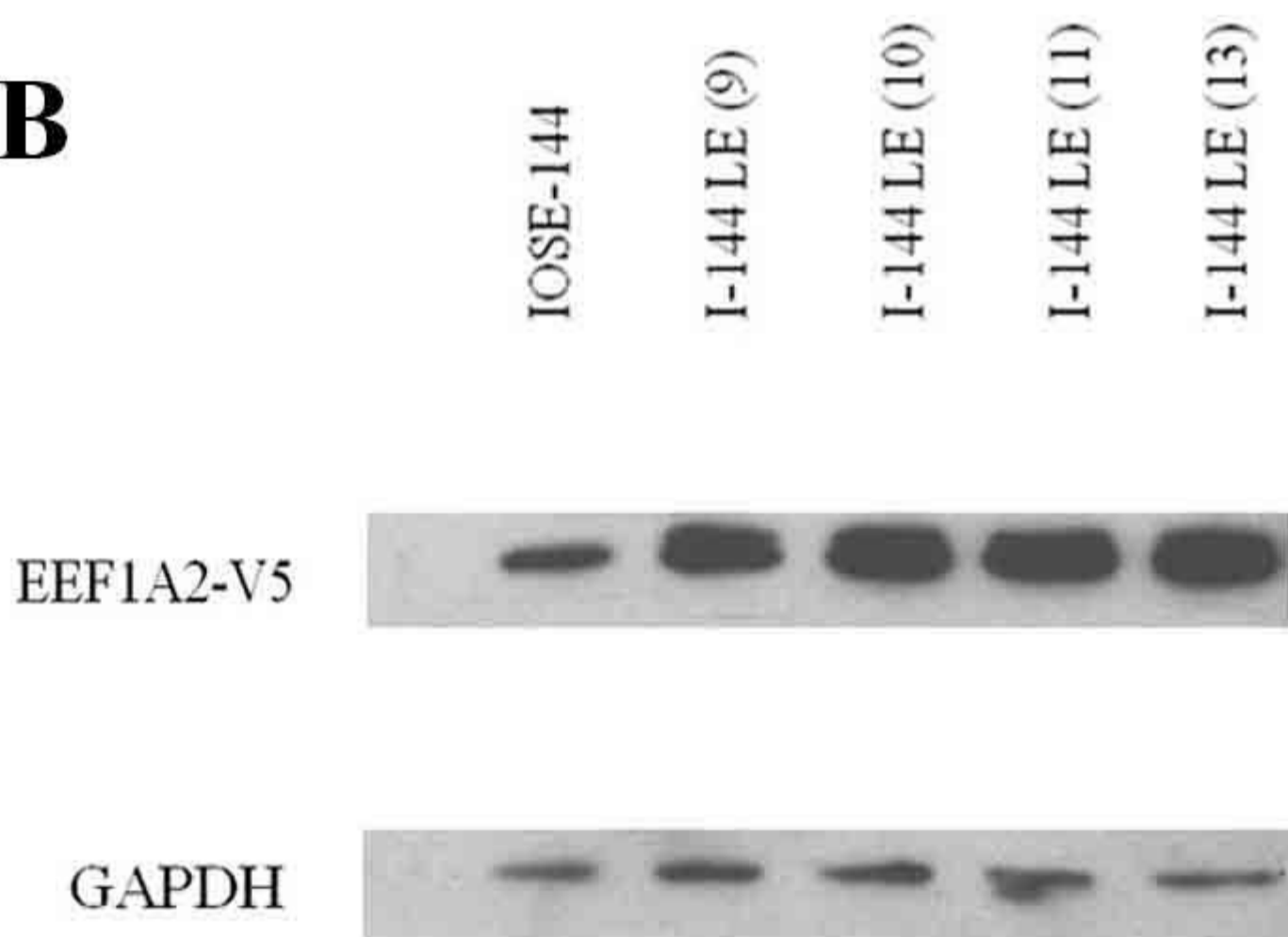
I-144RZ and I-144RZ-B are lines obtained from independently ZNF217-infected IOSE-144; ↑ and ↓ indicate gene gains and losses. Colonies of 20,000 to 40,000 cells, appearing during crisis, were scraped off and lysed. Only changes that occurred in 4 or more colonies are listed.

I-144RZ-B colonies:

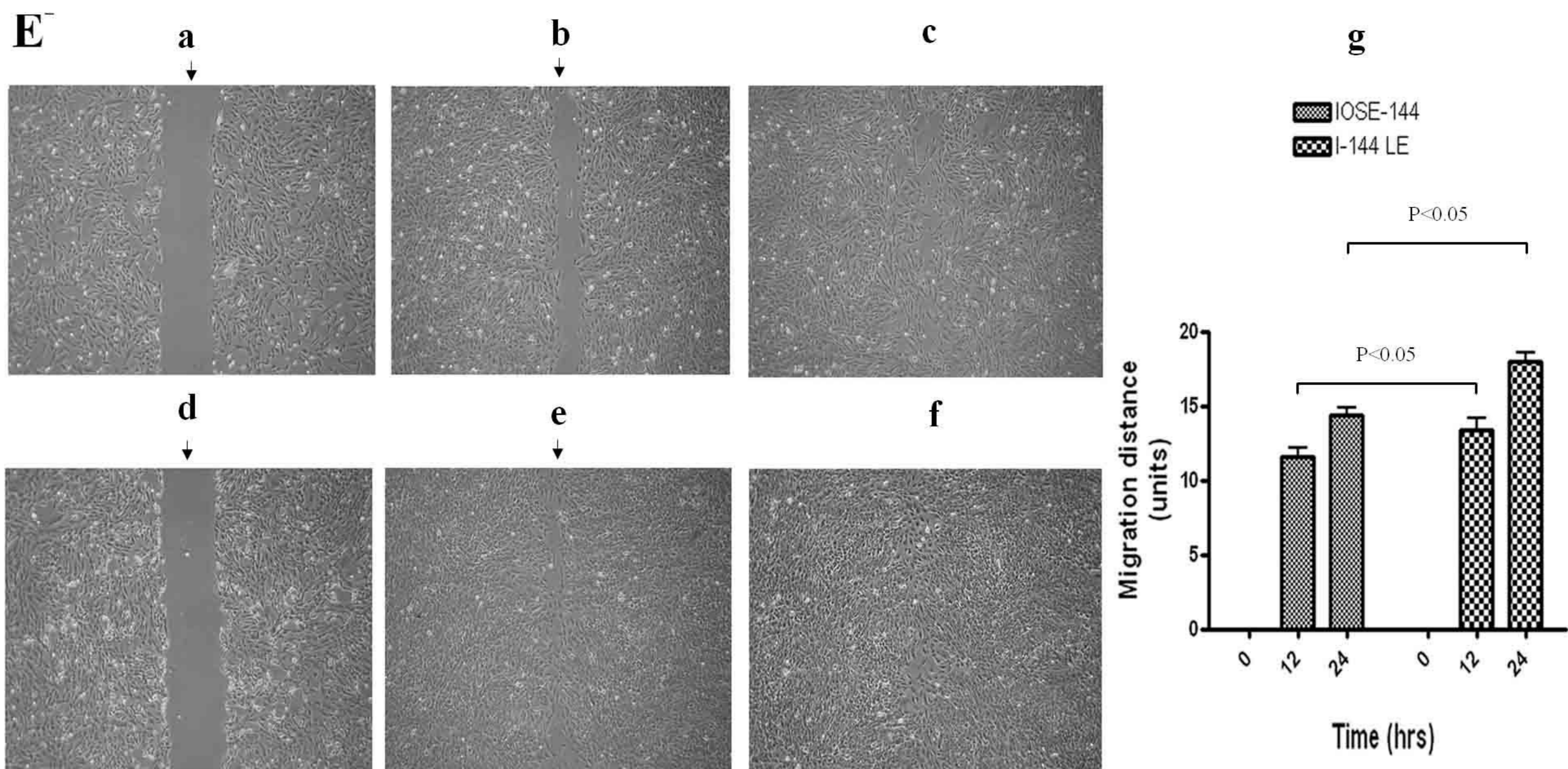
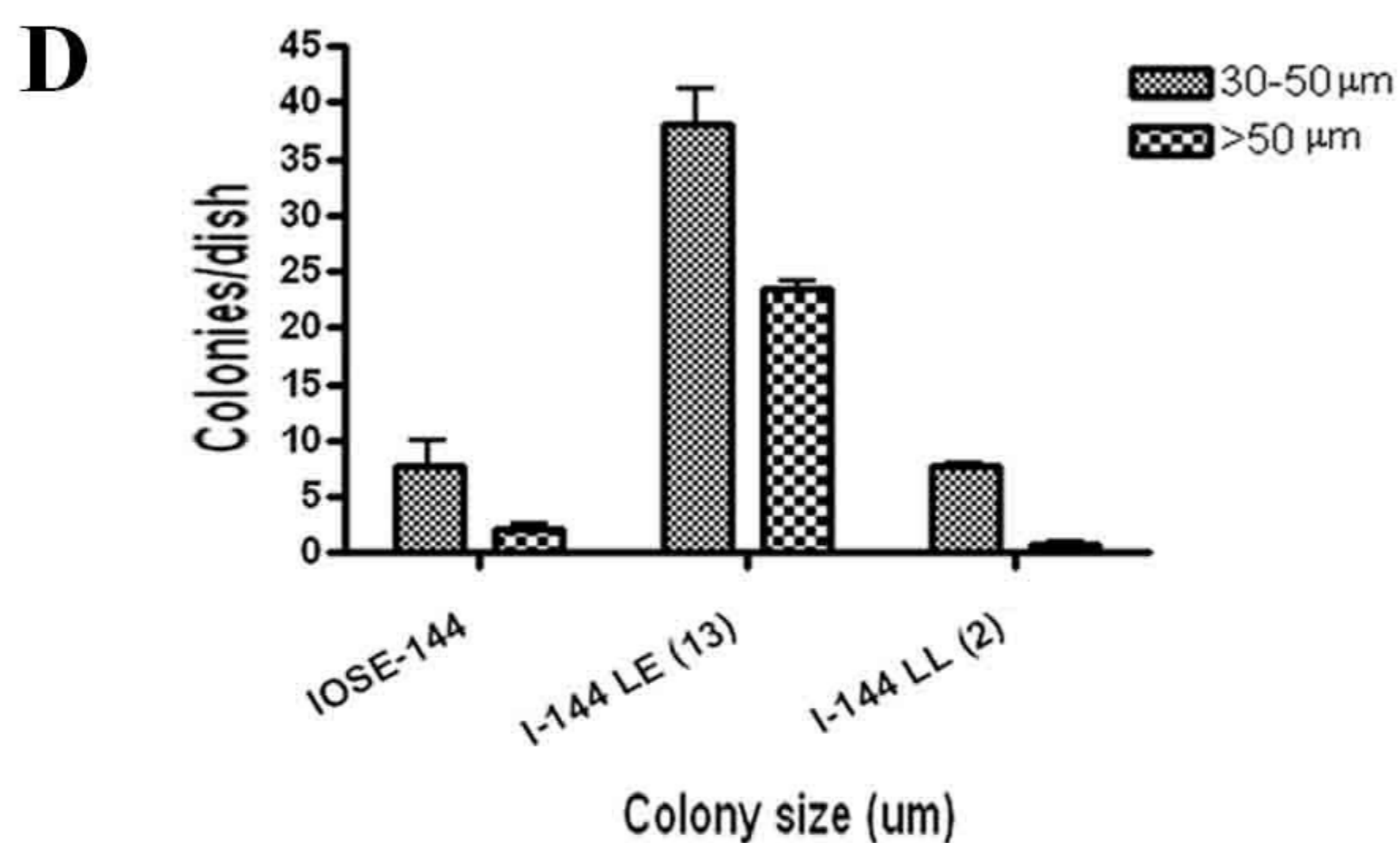
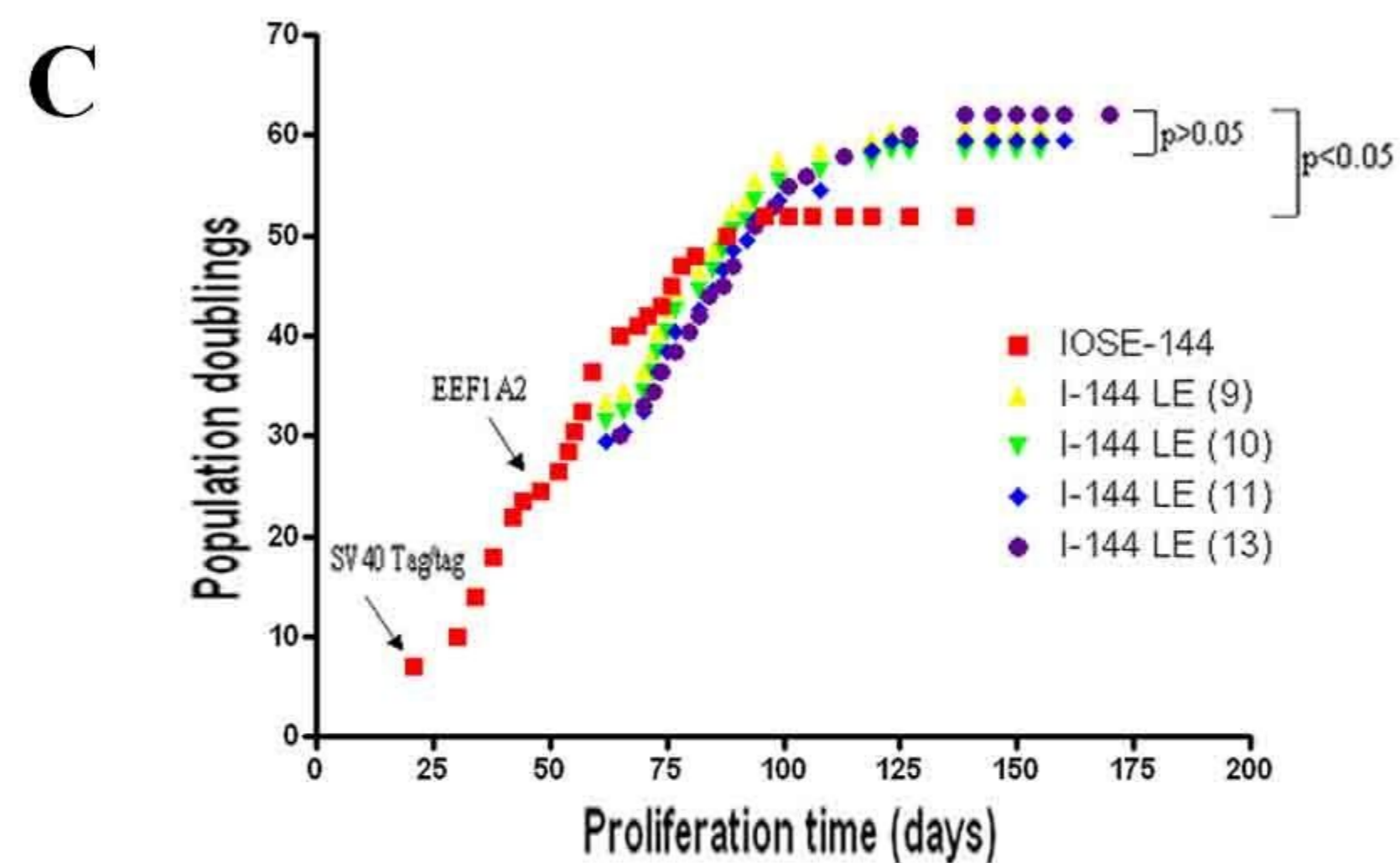
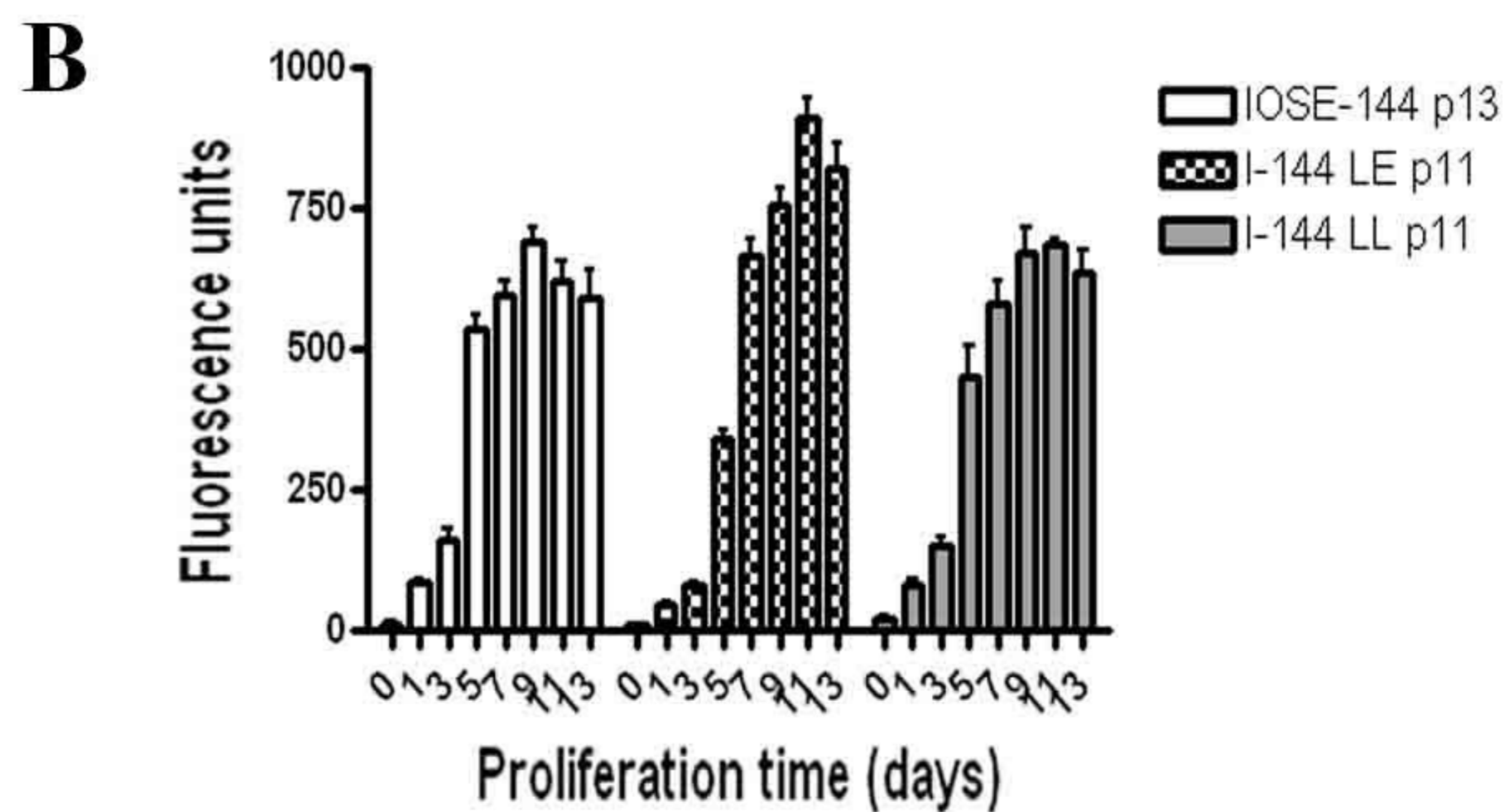
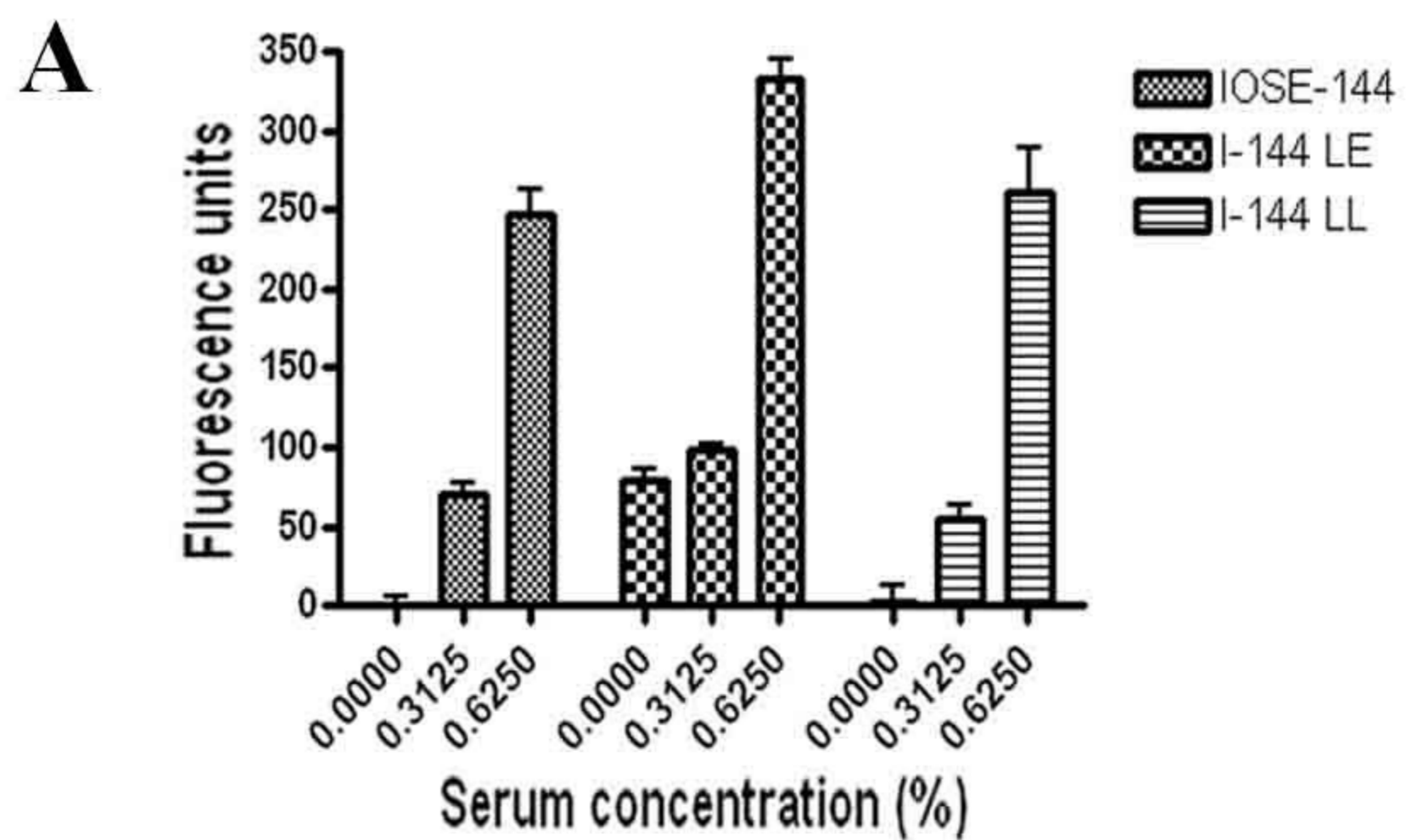
7	↓: 1	7	18	↑:	5	8	15	20	
8	↓: 1q	7	18	↑:	5	8	14	15	20
9	↓: 1	11	18	↑:	5	8	14	15	20
10	↓: 1		18	↑:	5				20
11	↓: 3	11	18	↑:	5		14		20
12	↓: 1		18	↑:	5	8	14	15	20
13	↓: 3	11	18	↑: 1p					
14	↓: 1	11	18	↑: 1p	5				
15	↓: 1	11	18	↑: 1p					

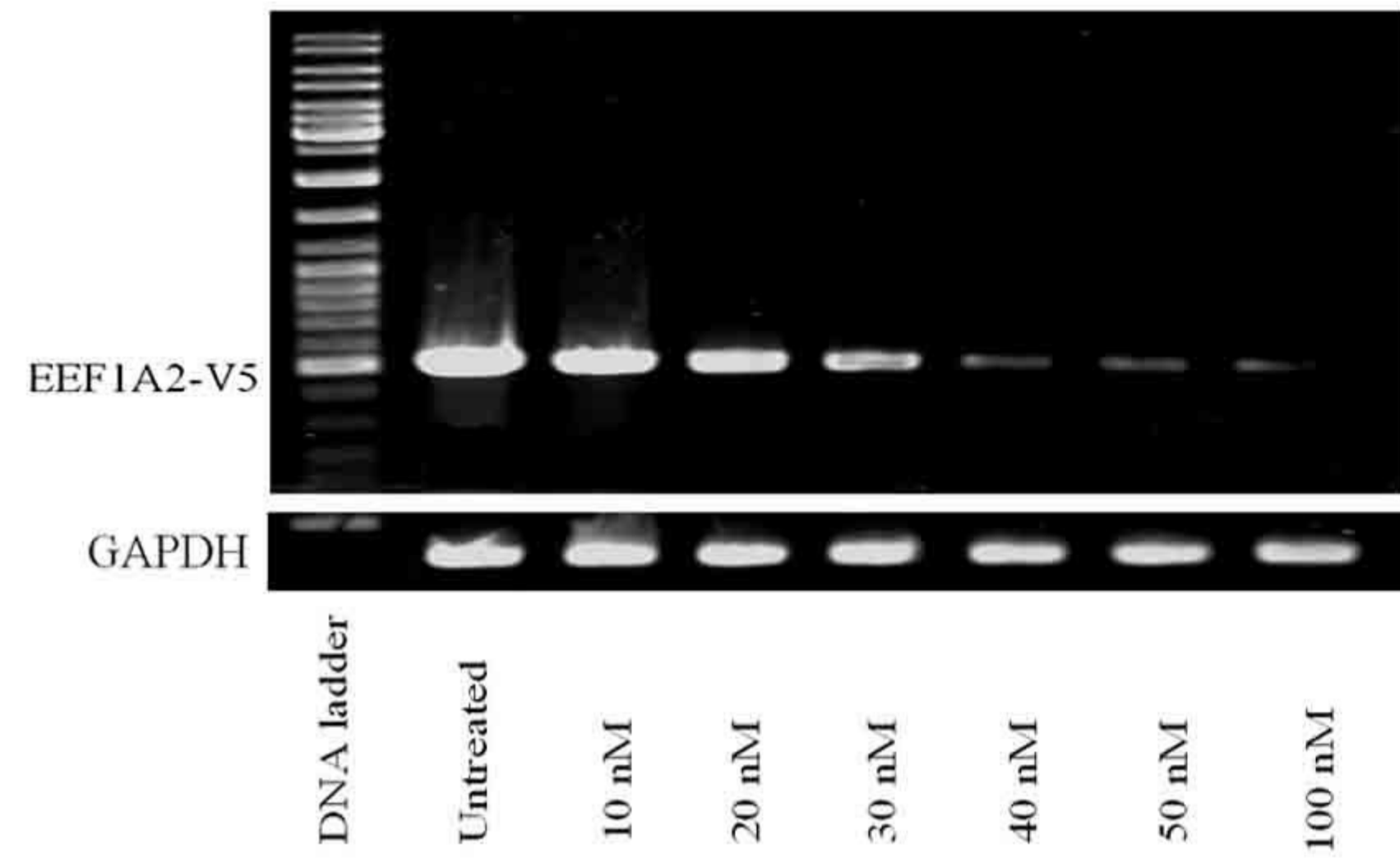
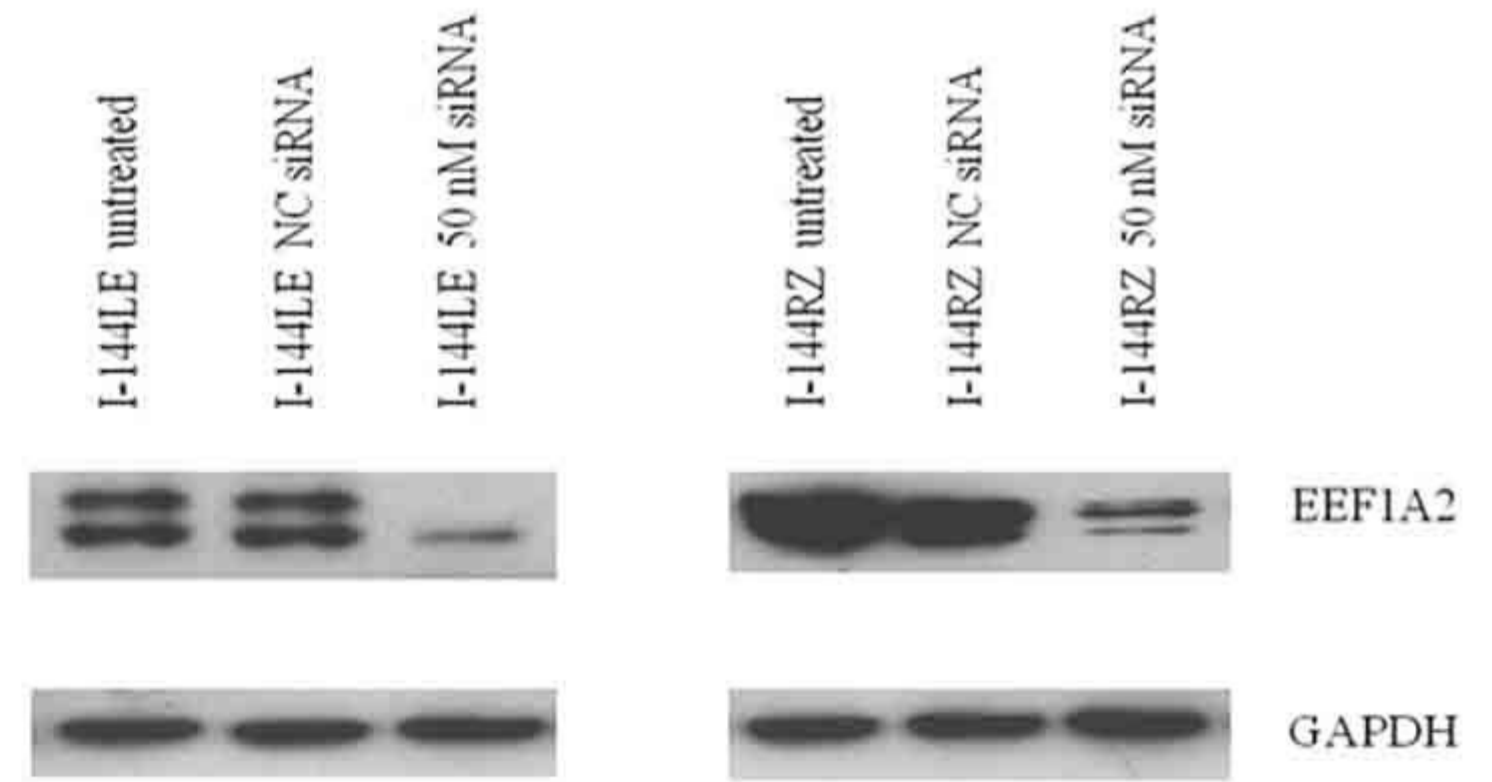
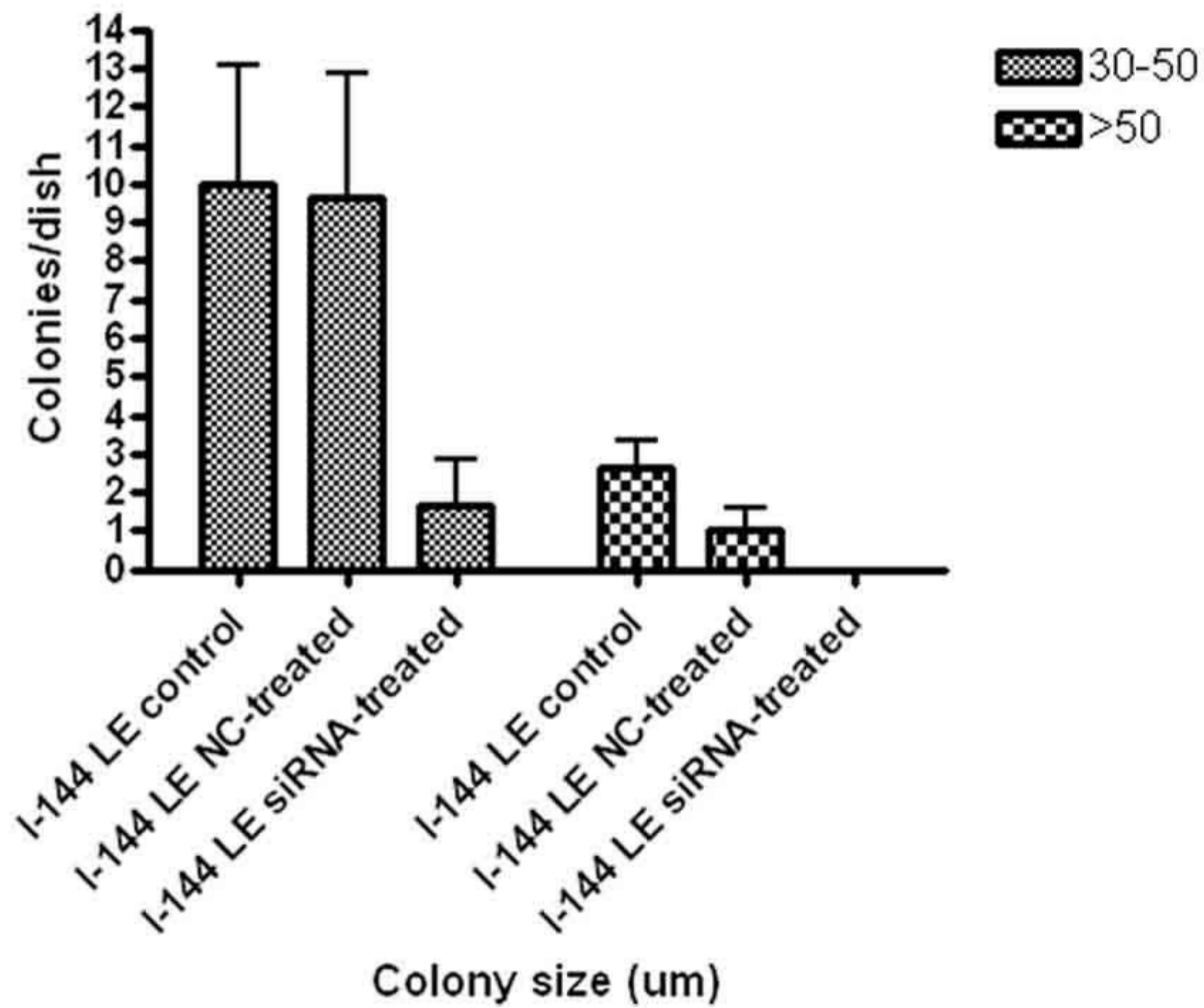
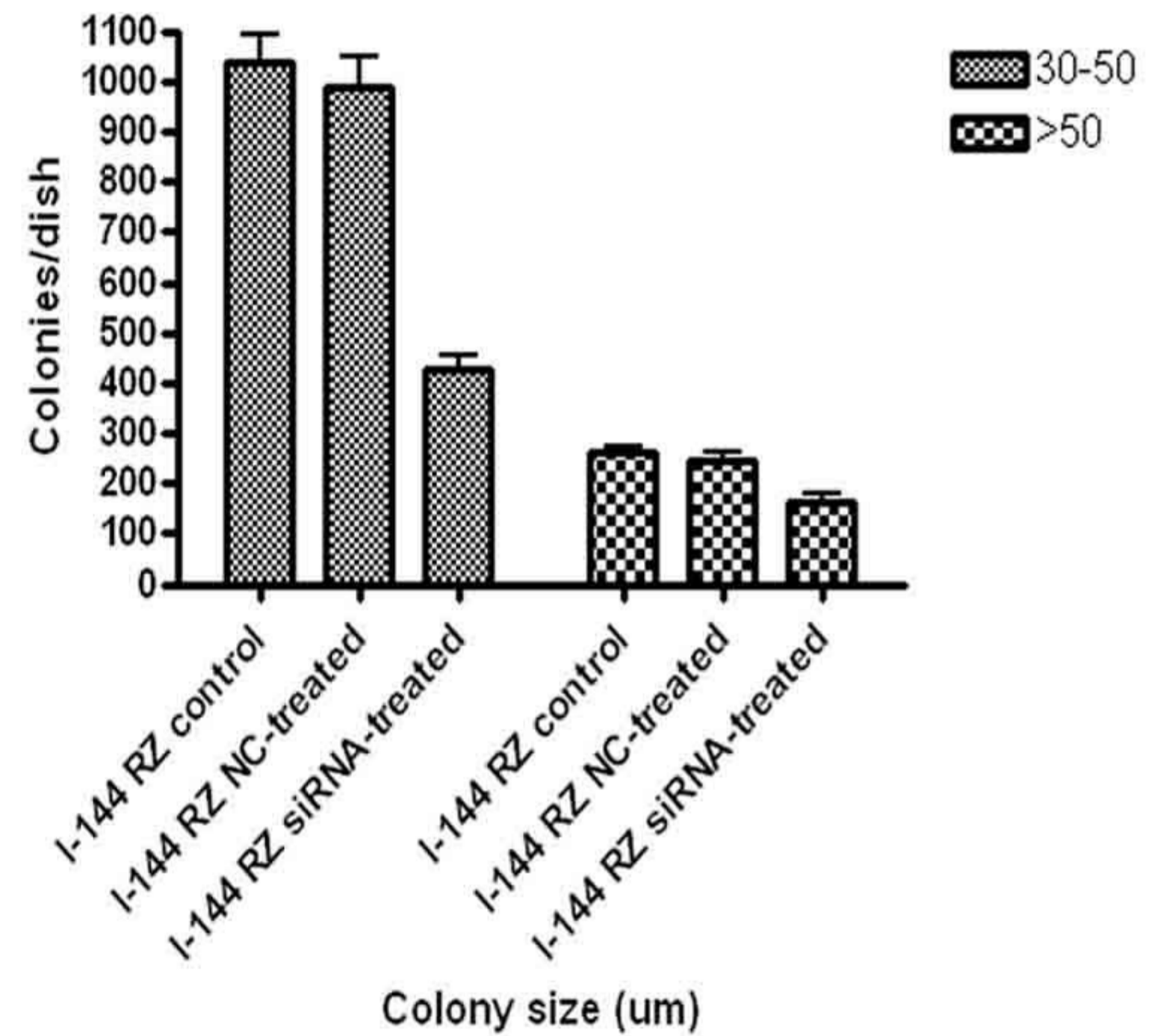
Postcrisis and long-term changes, respectively

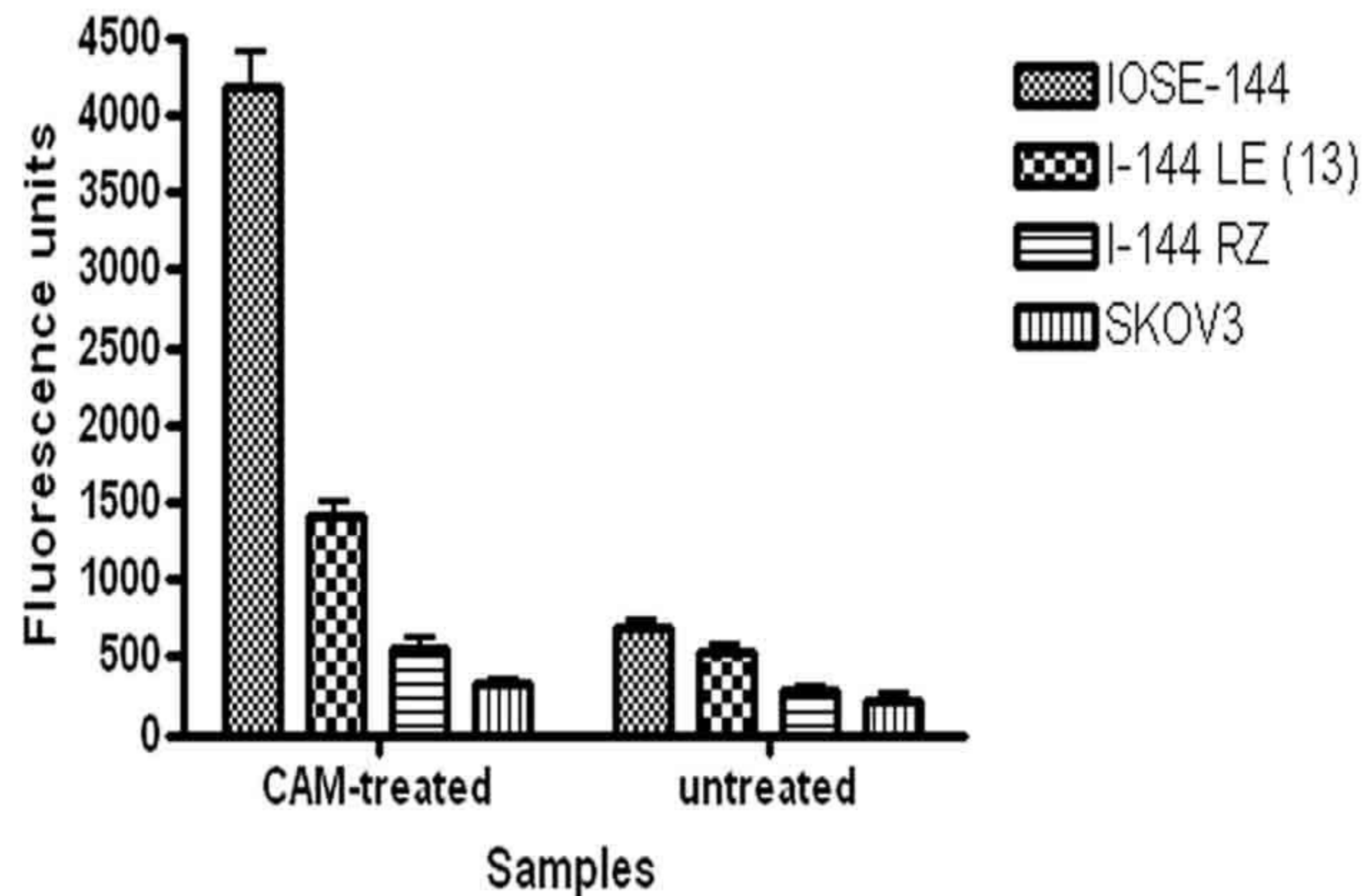
I-144RZ passage 20:	↓: 1q 3p 7q	18 X	↑: 3q	8q	14	
I-144RZ passage 61:	↓: 3p 7q	18 X	↑: 3q	5	8q	14

**A****B**





**A****B****C****D**

**A****B**