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**Estrogen receptor- $\alpha$  mediates human multidrug resistance associated protein 3 induction by 17 $\alpha$ -ethynylestradiol. Role of activator protein-1.**

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**ABSTRACT**

Previously, we have demonstrated that 17 $\alpha$ -ethynylestradiol (EE) induces rat multidrug-resistance associated protein 3 (Mrp3, Abcc3) expression transcriptionally through estrogen receptor- $\alpha$  (ER- $\alpha$ ) activation. We explored the effect of EE on MRP3 expression of human origin. HepG2 cells were transfected with ER- $\alpha$  and incubated with EE (1-10-50  $\mu$ M) for 48 h. MRP3 protein and mRNA levels were measured by Western blotting and Real time PCR, respectively. EE up-regulated MRP3 protein and mRNA at 50  $\mu$ M only in ER- $\alpha$ (+)-HepG2 cells. The in silico analysis of *mrp3* promoter region demonstrated absence of estrogen response elements, but showed several Ap-1 binding sites. We further evaluated the potential involvement of the transcription factors c-JUN and c-FOS (members of Ap-1) in MRP3 up-regulation. ER- $\alpha$ (+) HepG2 cells were incubated with EE and c-FOS and c-JUN levels measured by Western blotting in nuclear extracts. EE up-regulated only c-JUN. Experiments of overexpression and knock-down of c-JUN by siRNA further demonstrated that this transcription factor is indeed implicated in MRP3 upregulation by EE. Co-immunoprecipitation assay demonstrated that EE induces c-JUN/ER- $\alpha$  interaction, and chromatin immunoprecipitation assay showed that this complex is recruited to the AP-1 binding consensus element present at the position (-1300/-1078bp) of human *mrp3* promoter. We conclude that EE induces MRP3 expression through ER- $\alpha$ , with recruitment of ER- $\alpha$  in complex with c-JUN to the human *mrp3* promoter.

**Key words:** drug transporter, AP-1, estrogen, multidrug resistance, nuclear receptor, c-JUN.

## 1. INTRODUCTION.

Multidrug resistance-associated protein 3 (MRP3/ABCC3) is a member of the superfamily of adenosine tri-phosphate (ATP)-binding cassette (ABC) transporters. It is expressed in several epithelial cells including hepatocytes (1). Due to its basolateral localization it transports into blood a wide range of endogenous and exogenous compounds such as sulphated bile salts, bilirubin glucuronides, specific anti-cancer drugs such as methotrexate, etoposide, teniposide, etc (2-6). Under physiological conditions its expression is low, but it is highly inducible by drugs such as acetaminophen (7) and phenobarbital (8), or under several cholestatic situations such as later stages of primary biliary cirrhosis and extrahepatic cholestasis of different etiology (9). Induction of MRP3 activity results in re-directioning the secretion of common MRP substrates from the apical to the basolateral pole.

It is known that estrogens are involved in the pathogenesis of both oral contraceptive-induced cholestasis and cholestasis of pregnancy (10, 11). In previous work we demonstrated that 17 $\alpha$ -ethynylestradiol (EE), a synthetic estrogen widely used in contraceptive formulations and in estrogen replacement therapies, up-regulates hepatic Mrp3 in rats (12, 13). We later demonstrated that induction of Mrp3 by EE in rat liver is independent of cholestasis and occurs *via* activation of ER (14), though the underlying mechanism downstream ER remains unknown. The canonical model for ER-mediated regulation of gene expression involves the direct binding of dimeric ER to DNA sequences known as estrogen response elements (ERE), which are specific, inverted

palindromic sequences. In addition, ER can indirectly associate with promoters through protein-protein interactions with other DNA-binding transcription factors such as specificity protein 1 (Sp1) or activator protein-1 (AP-1), a complex composed of c-JUN protein homodimers or c-JUN/c-FOS heterodimers. In either case, interaction of ERs with estrogens leads to transcriptional activation of the associated genes *via* recruitment of co-activators and components of the basal transcriptional machinery (15). The liver expresses predominantly ER- $\alpha$  (16) and its expression is under multihormonal regulation.

Until present there are no reports about the effect of estrogens on human MRP3 expression. As interactions of nuclear receptors with selective ligands may vary between species and considering the differences between rat and human *mrp3* promoters (17, 18), we explored whether EE is also able to induce MRP3 in HepG2 cells, a human hepatoma cell line, and whether ER- $\alpha$  is involved. Additionally, we explored in these cells the intervention of potential candidates that can mediate the response downstream ER- $\alpha$ , such as the transcription factor AP-1.

## **2. MATERIALS AND METHODS.**

### **2.1. Chemicals.**

EE, leupeptin, phenylmethylsulfonyl fluoride, pepstatin A, were obtained from Sigma Aldrich Chemical Company (St. Louis, MO). All other chemicals were of analytical grade purity, and used as supplied.

### **2.2. Cell culture and treatment with EE.**

HepG2 cells were grown in monolayer culture and maintained in F-12 (Invitrogen, Carlsbad, CA, USA) and phenol-red DMEM (1:1) supplemented with 10% FBS (PAA, Pasching, Austria) (v/v) in a humidified atmosphere of 5% CO<sub>2</sub> and 37°C. Three days prior to harvesting, 3.5 x 10<sup>5</sup> cells were seeded into six-well plates and 24 h later, the medium was changed to phenol red-free DMEM/F-12 supplemented with 10% charcoal-dextran treated FBS (Hyclone Laboratories, Logan, UT), and treated with EE at 1, 10 or 50 µM for 48 h.

### **2.3. Transfection and treatment with EE.**

The expression vector encoding human ER-α (pCMV5-hERα) and the estrogen-responsive reporter plasmid 4XERE-TK-Luc, which contains four copies of ERE in front of a thymidine kinase promoter reporter construct, were a gift from Dr. Benita Katzenellenbogen (University of Illinois, Urbana-Champaign, IL). The expression vectors encoding c-JUN and the empty vector

were a gift from Dr. Beatriz Caputto (Universidad Nacional de Córdoba, Córdoba, Argentina). Plasmid DNA was purified by using EndoFree Plasmid Maxi kit from Qiagen Sciences (Maryland, USA). HepG2 cells were grown as described above. A day before transfection,  $3.5 \times 10^5$  cells were subcultured into 6 well-plates in phenol red-free DMEM/F12 supplemented with 10% charcoal stripped FBS and glutamine. Transfections were performed by using Lipofectamine2000 (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Cells were transfected with 5  $\mu\text{g}$  of pCMV5-hER $\alpha$  or pCMV5 (empty vector) as a negative control. In transcription factor over-expression experiments, cells were transfected with 4  $\mu\text{g}$  of pCMV5-hER $\alpha$  and 1  $\mu\text{g}$  of c-JUN plasmids or with their respective empty vectors. To monitor the estrogen-induced transcriptional activation, cells were subcultured into a 10 cm plate, and 24 h later, transfected with 5  $\mu\text{g}$  of the estrogen-responsive reporter plasmid 4X ERE-TK-Luc, and 5  $\mu\text{g}$  of pCMV5-hER $\alpha$  or 5  $\mu\text{g}$  of empty vector pCMV5 as a negative control. Eighteen h after transfection, the media were removed and the cells washed twice with PBS and re-plated in a 96-well plate. The cells were treated with vehicle (dimethyl sulfoxide) or estrogens (EE at 1, 10 or 50  $\mu\text{M}$  or 17 $\beta$ -estradiol at 1, 10 or 100 nM). The final concentration of dimethyl sulfoxide was adjusted to 0.08% (v/v). After 48 h, cells were washed with PBS and luciferase activity measured using Luciferase Assay System following the manufacturer's protocol (Promega, Madison, WI). For Western blot or real time PCR studies, the media were removed 18 h after transfection, the cells washed twice with PBS, and replaced with fresh media containing EE (1, 10 or 50  $\mu\text{M}$ ). After 48 h of incubation, cells were washed with PBS and harvested for preparation of whole-cell extract or total RNA extraction.



#### **2.4. Western blot studies.**

HepG2 cells were harvested and lysed in RIPA buffer (Pierce, Rockford, IL), 0.1  $\mu$ M phenylmethylsulfonyl fluoride and 35 nM leupeptin. Nuclear extracts were prepared for detection of c-JUN and c-FOS. Briefly, HepG2 cells were harvested in sucrose 0.3 M, 0.1  $\mu$ M phenylmethylsulfonyl fluoride and 35 nM leupeptin (Sigma Aldrich Chemical Company, St. Louis, MO), and samples sonicated and centrifuged at 500 g for 10 min. The pellets were resuspended in RIPA buffer plus protease and phosphatase inhibitors and protein concentration measured using bovine serum albumin as standard (19). Equal amounts of cell protein extract were resolved on an 8% (to detect MRP3) or 12% (to detect c-FOS and c-JUN) SDS-PAGE and transferred to nitrocellulose membranes. The blots were then exposed to primary antibodies: anti-MRP3 (M<sub>3</sub>II-21, Sigma, St. Louis, MO), anti-ER- $\alpha$ , anti c-JUN, anti c-FOS, anti-histone or anti- $\beta$ -Actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Immunoreactive bands were detected using a chemiluminescence kit (ECL + Plus, Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and quantified using the Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD) software.

#### **2.5. Quantitative Real Time PCR.**

Total RNA was isolated from HepG2 cells using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. cDNA was produced by using the SuperScript Preamplification System for first-strand cDNA synthesis

according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was performed on cDNA samples using the MX3000P system (Agilent Technologies, Santa Clara CA, USA) with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA). The amount of template was quantified with SYBR green (Invitrogen) (20). For MRP3 mRNA amplification the forward primer sequence was 5' gtccgcagaatggacttgat 3' while the reverse primer sequence was 5' tcaccactggggatcattt 3'. Results for MRP3 were normalized to 18S rRNA as housekeeping gene using the following primers: forward 5' cgccgctagagggtgaaattc 3' and reverse 5' ttggcaaatgctttcgctc 3'. All the primers were used at a final concentration of 1  $\mu$ M. The thermocycling regime was 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. The amplified product size was 120 and 62 bp for *mrp3* and 18S, respectively. PCR product sizes were confirmed by gel electrophoresis. Relative levels of MRP3 mRNA normalized to 18S rRNA were calculated based on the  $2^{-\Delta\Delta C_t}$  method. Specificity of the reaction was verified with a dissociation curve between 55°C and 95°C with continuous fluorescence measure (20).

## **2.6. RNA interference experiment.**

HepG2 cells ( $5 \times 10^4$  cells/well) were seeded in 24 well plates, incubated at 37°C and subjected to transfection 24 h later. Human c-JUN was transiently knocked down with c-JUN siRNA (Santa Cruz Biotechnology, sc-29223), targeting the human transcription factor mRNA. Control siRNA-A (Santa Cruz Biotechnology, sc-37007), a non-targeting siRNA, was used as a negative control. Both groups of cells were transfected also with ER- $\alpha$  plasmid.

Transfections were performed using Dharmafect4 Transfection Reaction (Dharmacon, Lafayette, CO, USA) according to the manufacturer's instructions. At 24 h post-transfection, the cells were exposed to EE (50  $\mu$ M) for additional 48 h, and then harvested and subject to Western blot analyses.

### **2.7. Co-immunoprecipitation assay.**

2.5x10<sup>6</sup> HepG2 cells were plated into a 100-mm plate, transfected with ER- $\alpha$ , and treated as described in section 2.2. They were harvested 48 h after EE treatment. Cell lysates and immunoprecipitation were performed using Protein A agarose (Roche Applied Science, Germany). The antibodies used for immunoprecipitation were anti ER- $\alpha$ , c-JUN or normal rabbit IgG (H-184, SC-45X, SC-2027, respectively, Santa Cruz Biotechnology, Inc.). Western blotting of immunoprecipitated ER- $\alpha$  and c-JUN was performed using anti ER- $\alpha$  or anti c-JUN (2Q418, SC-1694, respectively, Santa Cruz Biotechnology, Inc.).

### **2.8. Chromatin immunoprecipitation (ChIP) assay.**

2.5x10<sup>6</sup> HepG2 cells were plated into a 100-mm plate and transfected with ER- $\alpha$  as described above. ER- $\alpha$  transfected HepG2 cells were treated with EE (50  $\mu$ M, 48 h) or vehicle and cross-linked with 1% formaldehyde (37°C, 10 min). ChIP assay was performed following manufacturer protocol (Upstate Biotech, NY) using rabbit antibodies to c-JUN or normal rabbit IgG as a negative control (SC-45X, SC-2027, respectively, Santa Cruz Biotechnology, Inc.). An aliquot of lysates (20  $\mu$ l) was taken out as input control. DNA was

purified by phenol/chloroform extraction and ethanol precipitation. For PCR analysis, we used primers that amplify 222 and 183 bp fragments of the human *mrp3* promoter region from -1300 to -1078 and -501 to -318 bp, relative to the transcription start site, which include the two putative AP-1 binding sites ChIP-1: 5'gagtccgccgtccacacca3' and ChIP-2: 5'cttctgtcccgtgtcgcccta3' for AP-1 (A) and ChIP-3: 5'tagacatttacccttcccga3' and ChIP-4: 5'cacatagggtcaagggggaac3' for AP-1 (B) (Fig 4). The thermocycling regime was 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec.

## 2.9. Statistical Analysis.

Results are expressed as mean  $\pm$  S.D. Statistical analysis was performed using *Student t* test or one-way ANOVA followed by Newman Keuls test. Values of  $p < 0.05$  were considered to be statistically significant.

### 3. RESULTS.

#### ***3.1. ER- $\alpha$ is necessary for EE to up-regulate MRP3 in HepG2 cells.***

HepG2 are well differentiated cells, retain many hepatocyte-specific features, and represent a tool for understanding the regulation of MRP3 in a human liver cell line (21). We found no changes in MRP3 protein expression at any concentrations of EE analyzed (1, 10 or 50  $\mu$ M) (Fig 1). HepG2 cells express ER at low levels that are insufficient for ligands to induce transactivation of an ERE-containing synthetic target gene under transient transfection conditions (22, 23). We therefore transfected HepG2 cells with a plasmid encoding human ER- $\alpha$ , and confirmed ER- $\alpha$  expression by Western blotting (data not shown). The activity of ER- $\alpha$  was monitored by introducing an estrogen-responsive indicator in which four copies of the ERE were linked to a thymidine kinase promoter of a luciferase expression plasmid (4XERE-TK-Luc). As shown in Fig 2, EE (upper panel) and 17 $\beta$ -estradiol (positive control, lower panel) treatments activated 4XERE-TK-Luc in a dose dependent manner in HepG2 cells transfected with ER- $\alpha$  but not in those transfected with the empty vector. These data demonstrate that ER- $\alpha$  was active and able to transduce the estrogen signal in HepG2 cells. Western blot studies indicate that MRP3 expression significantly increased (270%) in cells transfected with ER- $\alpha$  and incubated with 50  $\mu$ M EE (Fig 3A), whereas it remained unchanged in the empty vector-transfected HepG2 cells at all concentrations of EE tested (Fig 3B). Real time PCR studies showed an increase in the content of MRP3 mRNA in ER- $\alpha$  transfected HepG2 cells incubated with 50  $\mu$ M EE (180%) with respect

to control cells, whereas no changes were observed in empty vector-transfected cells (Fig 3C).

### **3.2. Changes in the expression of AP-1 are responsible for MRP3 up-regulation by EE.**

To understand how ER- $\alpha$  might mediate MRP3 induction by EE, we performed an in silico analysis using the TFSearch database (<http://www.cbrc.jp/research/db/TFSEARCH.html>). Although we could not identify any ERE in the *mrp3* promoter sequence, the analysis located two binding sites for AP-1 (Fig 4). This led us to hypothesize an indirect action of the ER- $\alpha$  involving these transcription factors. To test this, we evaluated the effect of EE on expression of c-JUN and c-FOS. We found that c-JUN but not c-FOS protein level was increased in ER- $\alpha$  expressing HepG2 cells after treatment with 50  $\mu$ M EE (Fig 5A-B). To explore if c-JUN up-regulation may be a mediator of MRP3 induction, we analyzed the effect of c-JUN over-expression on MRP3 levels. HepG2 cells were co-transfected with c-JUN together with ER- $\alpha$  cDNA or their empty vectors as controls. As shown in Fig 5C, over-expression of c-JUN in ER- $\alpha$  expressing cells led to up-regulation of MRP3, even in the absence of incubation with EE, and to a similar extent to that produced by EE treatment in cells transfected with ER- $\alpha$  only. This same Fig also shows that EE treatment of cells overexpressing c-JUN did not further increase MRP3 expression when compared with these same cells incubated with EE vehicle. ER- $\alpha$  non-expressing HepG2 cells did not show any increase in MRP3 protein levels in response to transfection with c-JUN (Fig 5D). To

further confirm that c-JUN modulation is unambiguously involved in MRP3 up-regulation, we knocked down c-JUN and tested the effect of EE on MRP3 expression. Figure 6A demonstrates a decrease of 50% in c-JUN protein level in ER- $\alpha$ -transfected HepG2 cells after treatment with small interference RNA (siRNA), either in the presence or absence of EE in the incubation medium. As shown in Fig 6B, knock-down of c-JUN prevented from any MRP3 induction by EE. In contrast, treatment of control siRNA scrambled-transfected HepG2 cells with EE resulted in up-regulation of MRP3 by 230% (Fig 6B). This induction is similar in extent to that shown above for cells not transfected with siRNA or its control (Fig 3A).

### ***3.3. c-JUN interacts with ER $\alpha$ and binds to the mrp3 promoter after EE stimulus.***

The *mrp3* proximal promoter region lacks ERE elements but contains AP-1 binding consensus elements (Fig 4). Our results strongly suggest that ER- $\alpha$ -presence and c-JUN up-regulation are needed for EE to induce MRP3 expression. Because both actions are needed at the same time, we further investigated whether EE treatment promotes ER- $\alpha$ /AP-1 interaction. We performed co-immunoprecipitation studies in ER- $\alpha$ -transfected HepG2 cells. We observed that a greater fraction of ER- $\alpha$  co-precipitated with c-JUN in the EE treated cells (Fig 7A). This ER- $\alpha$ /c-JUN interaction was confirmed by reverse co-immunoprecipitation with ER- $\alpha$ . In this case, the amount of c-JUN associated with ER- $\alpha$  was greater in EE-treated cells than in control cells (Fig 7B).

To address whether c-JUN is recruited to the promoter region of *mrp3* after EE treatment, we performed ChIP assays using two pairs of primers that include the two putative AP-1 binding sites identified in the *mrp3* promoter region (Fig 4). The data show that specific anti c-JUN antibody, but not the non-immune IgG control, successfully co-immunoprecipitated c-JUN with significant quantities of the *mrp3* promoter (-1300/-1078 bp). Interestingly, in cells treated with EE, the amount of *mrp3* promoter immunoprecipitated with c-JUN increased significantly (Fig 7C), indicating that EE promotes the binding of AP-1 to the (-1300/-1078 bp) region of the endogenous *mrp3* promoter. No amplification product was detected with the primers ChIP 3 and ChIP 4 (-501/ -318 bp) (data not shown).

#### 4. DISCUSSION.

ER activated by ligand can regulate transcription through either a classical or a non-classical mechanisms (15). The in silico analysis of the *mrp3* promoter region did not show any ERE sequence but showed several potential binding sites for AP-1. Based on this information, we proposed that EE-induction of *mrp3* transcription is mediated by ER- $\alpha$  acting not on ERE sequences but on Sp1 or AP-1 elements (non-classical pathway). Regulation of AP-1 activity can be achieved through changes in transcription of genes encoding AP-1 subunits, independent of changes in its phosphorylation status (24, 25). We found that c-JUN protein expression was increased in nuclear extracts from HepG2 cells transfected with ER- $\alpha$  and incubated with EE, and



that over-expression of c-JUN led to induction of MRP3 expression to the same extent as found under EE treatment conditions. Experiments in c-JUN knock-down cells confirmed that this transcription factor is indeed involved in MRP3 up-regulation by EE. Whether AP-1 non-classical pathway is the only mechanism mediating MRP3 induction by EE is uncertain, however, the experiments showing that MRP3 induction by c-JUN over-expression was not further exacerbated by EE treatment (Fig 5C), strongly pointed to c-JUN induction as a key player.

Several other studies indirectly implicate AP-1 as a key mediator in increasing Mrp3/MRP3 expression. AP-1 transcriptional activity is increased 300% in HepG2 cells after exposure to tauro lithocholic acid (26), and could explain findings of Teng et al. (27) demonstrating that tauro lithocholic acid increased MRP3 mRNA in human hepatoma HuH7 cell line. Bile duct ligation in mice increases AP-1 binding activity in liver (28), which could be linked to studies demonstrating that Mrp3 is up-regulated in liver of bile-duct ligated rats (29). Liver of mice treated with lipopolysaccharide showed an increase of AP-1 binding activity detected by gel shift assays (30), whereas independent studies described an up-regulation of Mrp3 after similar treatment in rats (31, 32). It was reported that binding of c-Jun to electrophile responsive elements (EpRE) sequences increased subsequent to 4-hydroxy-2-nonenal exposure in HepG2 cells (33), and at the same time produces a marked up-regulation of MRP3 in human bronchial epithelial and Keap1 wild-type non-small-cell lung carcinoma (NSCLC) cells (34). Appropriate experiments are needed to establish whether AP-1 activation and Mrp3 up-regulation are causally or casually related under

conditions different from that of EE treatment. MRP3/Mrp3 up-regulation was demonstrated to be an adaptive response to deal with liver toxicity of harmful endogenous metabolites, xenobiotics and drugs, either under cholestatic or non-cholestatic conditions (7, 35). We proposed AP-1 activation to be a significant participant in this adaptive response.

The mechanisms to explain the increased expression of c-JUN by EE, and the subsequent interaction with the promoter region of *mrp3*, remain poorly understood. It was demonstrated that human *c-Jun* promoter region has several transcription factor binding sites including AP-1 sites (36), indicating that *c-Jun* could be positively auto-regulated. Oxidative metabolites of EE, i.e., the 2-OH and 4-OH catechols, produce reactive oxygen species in rat liver (37), which in turn up-regulate AP-1 (38). It is thus possible that these same species contribute to AP-1 induction in our experimental conditions. Further studies are necessary to elucidate the mechanism of AP-1 up-regulation by EE. Our findings demonstrating that the over-expression of AP-1 requires the presence of ER- $\alpha$  to induce MRP3 suggest that they might need to interact with each other. This hypothesis was confirmed by co-immunoprecipitation assays, which further demonstrated that EE likely exacerbated this interaction. Moreover, CHIP analysis shows that from the two putative *mrp3* promoters containing AP-1 binding sites, only the distal one (-1300/-1078 bp) binds the complex c-JUN/ER- $\alpha$ .

In conclusion, the present data demonstrate for the first time that EE induce MRP3 in HepG2 cells via ER- $\alpha$  through the non-classical pathway

mediated by AP-1. A graphical scheme summarizes the proposed mechanism (Fig 8).

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#### **AUTHOR CONTRIBUTIONS.**

Participated in research design: Ruiz, Banchio, Mottino, Catania.

Conducted experiments: Ruiz, Rigalli, Arias, Villanueva.

Contributed analytic tools: Banchio, Vore.

Performed data analysis: Ruiz, Rigalli, Mottino, Catania.

Wrote or contributed to the writing of the manuscript: Ruiz, Rigalli, Vore, Mottino, Catania.



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**FIGURE LEGENDS**

**Figure 1: MRP3 protein expression in wild type HepG2 cells.** Cells were incubated with 17 $\alpha$ -ethynylestradiol (EE 1, 10 or 50  $\mu$ M, 48 h) or vehicle (C). MRP3 was detected by Western blotting. Equal amounts of protein (15  $\mu$ g) were loaded in all lanes. Uniformity of protein loading and transfer from gel to nitrocellulose membrane were controlled with Ponceau S and detection of  $\beta$ -actin. Densitometric analyses represent means  $\pm$  SD of 4 independent experiments.

**Figure 2: Activity of estrogen receptor- $\alpha$  (ER- $\alpha$ ) in transfected HepG2 cells.** HepG2 cells were transfected with expression plasmid vectors for ER- $\alpha$  or empty vector together with an estrogen reporter gene (4XERE-TK-Luc), and treated with the indicated concentrations of 17 $\alpha$ -ethynylestradiol (EE, upper panel) or 17 $\beta$ -estradiol (E2, lower panel). Data represent mean  $\pm$  SD of 4 independent experiments. a: Significantly different from control,  $p < 0.05$ .

**Figure 3: MRP3 protein expression in ER- $\alpha$ -transfected HepG2 cells.**

**A-** MRP3 protein expression in HepG2 transfected with estrogen receptor- $\alpha$  (ER- $\alpha$ ) vector.

**B-** MRP3 protein expression in HepG2 transfected with empty vector. Cells were incubated with 17 $\alpha$ -ethynylestradiol (EE) or vehicle [control (C)] for 48 h. Equal amounts of protein (15  $\mu$ g) were loaded in all lanes. Uniformity of protein loading and transfer from gel to nitrocellulose membrane were controlled with Ponceau S and detection of  $\beta$ -actin. Densitometric analyses represent means  $\pm$

SD of 4 independent experiments. a: significantly different from C,  $p < 0.05$ . b: significantly different from 17 $\alpha$ -ethynylestradiol (EE) 1  $\mu$ M,  $p < 0.05$ .

**C-** MRP3 mRNA expression in HepG2 cells transfected with ER- $\alpha$  or empty vector. Cells were incubated with EE (50  $\mu$ M) or vehicle (C) for 48 h and mRNA levels were detected by real time PCR. Data are presented as a percent of control (100%) and were expressed as means  $\pm$  SD of 4-6 independent experiments. 18S rRNA was used as an internal control. a: significantly different from C,  $p < 0.05$

**Figure 4: MRP3 proximal promoter region analysis.** Consensus elements for AP-1 nuclear factors identified by TF-Search database are underlined. Numbers represent the positions of nucleotides in relationship to the transcription initiation site +1 indicated by arrows. ChIP 1, ChIP 2, ChIP 3 and ChIP 4 represents the primers utilized in ChIP assay.

**Figure 5: Involvement of c-JUN in MRP3 induction.**

**A-** c-JUN, and **B-** c-FOS, protein expression in nuclear extracts from HepG2 cells transfected with estrogen receptor- $\alpha$  (ER- $\alpha$ ) and incubated with EE (50  $\mu$ M) or vehicle (C).

**C-** HepG2 cells transfected with ER- $\alpha$  vector (ER $\alpha$  (+)), and **D-** HepG2 cells transfected with empty vector (ER $\alpha$  (-)), were transfected with c-JUN (c-JUN (+)) or empty vector (c-JUN (-)), and incubated with EE (50  $\mu$ M) or vehicle (C). MRP3 protein expression was then assessed by Western blotting in cell lysates. Equal amounts of protein (5  $\mu$ g for nuclear extracts and 15  $\mu$ g for total lysates) were loaded in all lanes. Uniformity of protein loading and transfer from gel to

nitrocellulose membrane were controlled with Ponceau S and detection of histone or  $\beta$ -actin. Densitometric analyses represent means  $\pm$  SD of 4 independent experiments. a: significantly different from c-JUN(-) C,  $p < 0.05$ .

**Figure 6: MRP3 and c-JUN protein expression in c-JUN knock down HepG2 cells.**

**A-** c-JUN protein expression in nuclear extracts from HepG2 cells transfected with ER- $\alpha$  and c-JUN siRNA (c-JUN-) or siRNA scramble (c-JUN+).

**B-** MRP3 protein expression in cell lysates from HepG2 cells transfected with ER- $\alpha$  and c-JUN siRNA (c-JUN-) or siRNA scramble (c-JUN+).

Cells were incubated with 17 $\alpha$ -ethynylestradiol (EE, 50  $\mu$ M for 48 h) or vehicle (control (C)).

Equal amounts of protein (5  $\mu$ g for nuclear extracts and 15  $\mu$ g for total lysate) were loaded in all lanes. Uniformity of protein loading and transfer from gel to nitrocellulose membrane were controlled with Ponceau S and detection of histone or  $\beta$ -actin. Densitometric analyses represent means  $\pm$  SD of 4 independent experiments. a: significantly different from c-JUN (+) and c-JUN (-) EE,  $p < 0.05$ . b: significantly different from c-JUN (+) C, c-JUN (-) C and c-JUN (-) EE,  $p < 0.05$ .

**Figure 7: Interaction of ER- $\alpha$  with c-JUN and recruitment of c-JUN to MRP3 promoter.** ER- $\alpha$ -transfected HepG2 cells were treated with EE (50  $\mu$ M, 48 h) or vehicle (control (C)). Co-immunoprecipitation assays were performed by treating cell lysates with either c-JUN antibody or normal rabbit IgG followed by Western blot detection of ER- $\alpha$  (**A**) or by treating cell lysates with either ER- $\alpha$

antibody or normal rabbit IgG followed by Western blot detection of c-JUN **(B)**. The inputs represent 2% of total proteins in the cell lysates. **(C)** ChIP assay for detection of c-JUN association to *MRP3* promoter. An unrelated antibody, anti IgG, was used as control. Similar results were obtained in a separated experiment.

IP: immunoprecipitation.

**Figure 8: ER- $\alpha$  and c-JUN mediation of human MRP3 up-regulation by 17- $\alpha$ -ethynylestradiol (EE).** The graphical scheme represents a sequence of events starting with EE treatment of the HepG2 cells and leading ultimately to induction of human MRP3 protein expression. As a first step of this sequence, EE induces up-regulation of a major component of AP-1, c-JUN. Secondly, in order to induce the expression of MRP3, c-JUN interacts with ER- $\alpha$  and the resulting complex is recruited to a specific promoter region of the human *mrp3* gene. On this regard, two putative regions were found to be potential targets for the complex to bind to, but only one of them showed to be involved in the current experimental conditions.

**FOOTNOTES.**

These experiments were partially presented as a poster in the 47<sup>th</sup> International Liver Congress 2012 in Barcelona, Spain. The presentation was selected as TOP 10% by the EASL committee.

**Abbreviations:** MRP3, multidrug resistance-associated protein 3; EE, 17 $\alpha$ -ethynylestradiol; ER, estrogen receptor; AP-1, activating protein-1; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; ERE, estrogen response elements.

Figure 1

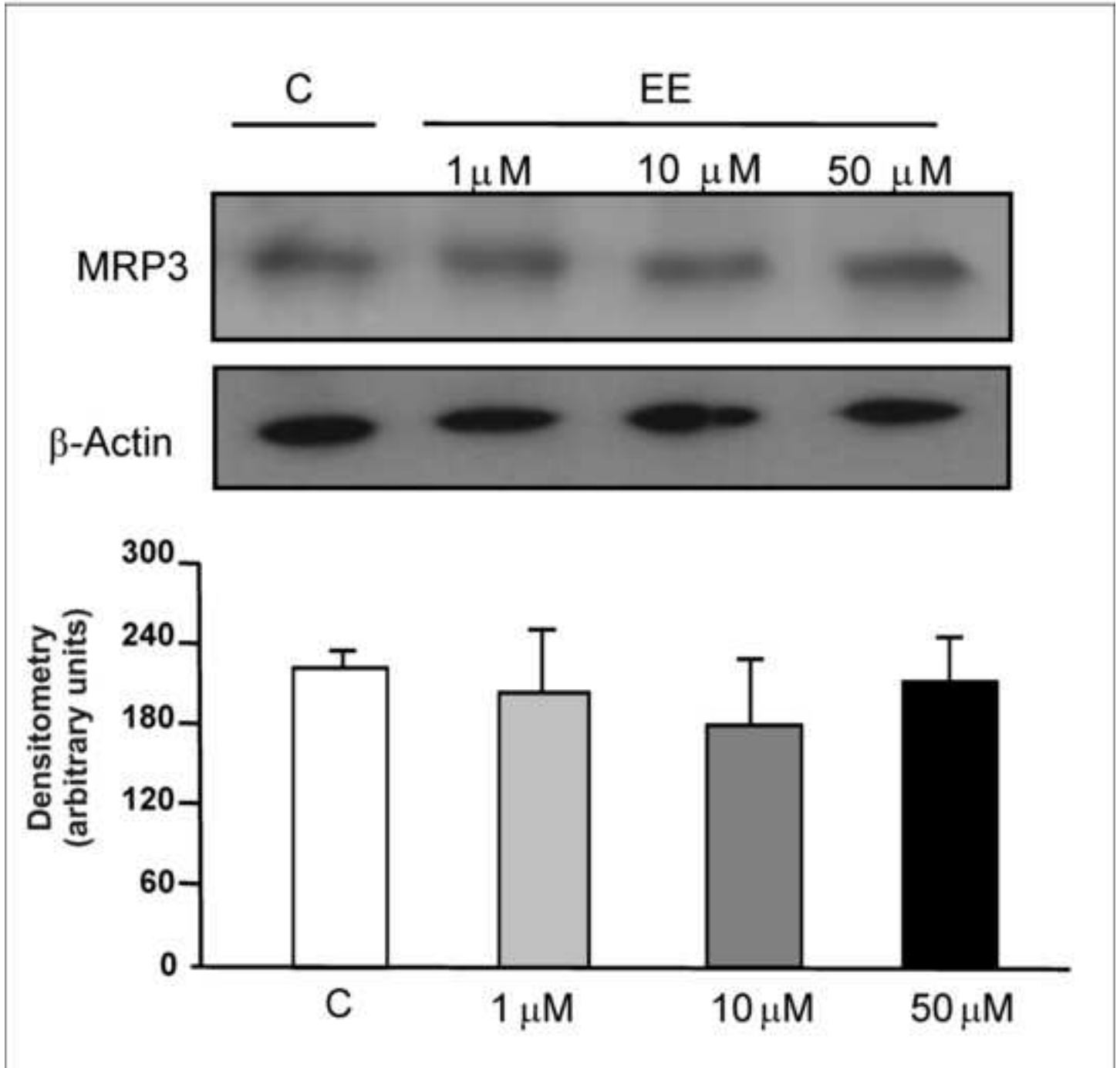


Figure 2

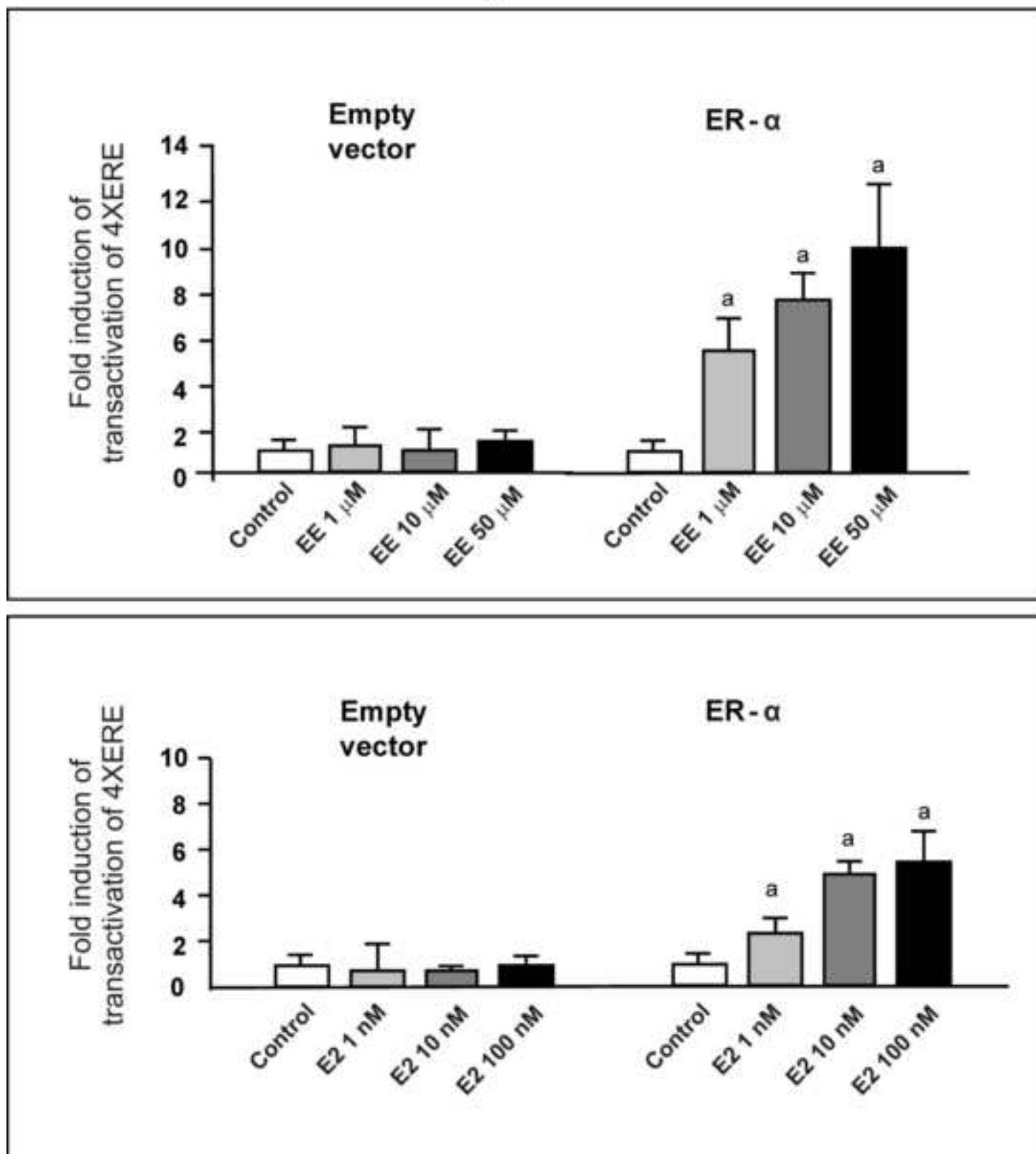


Figure 3

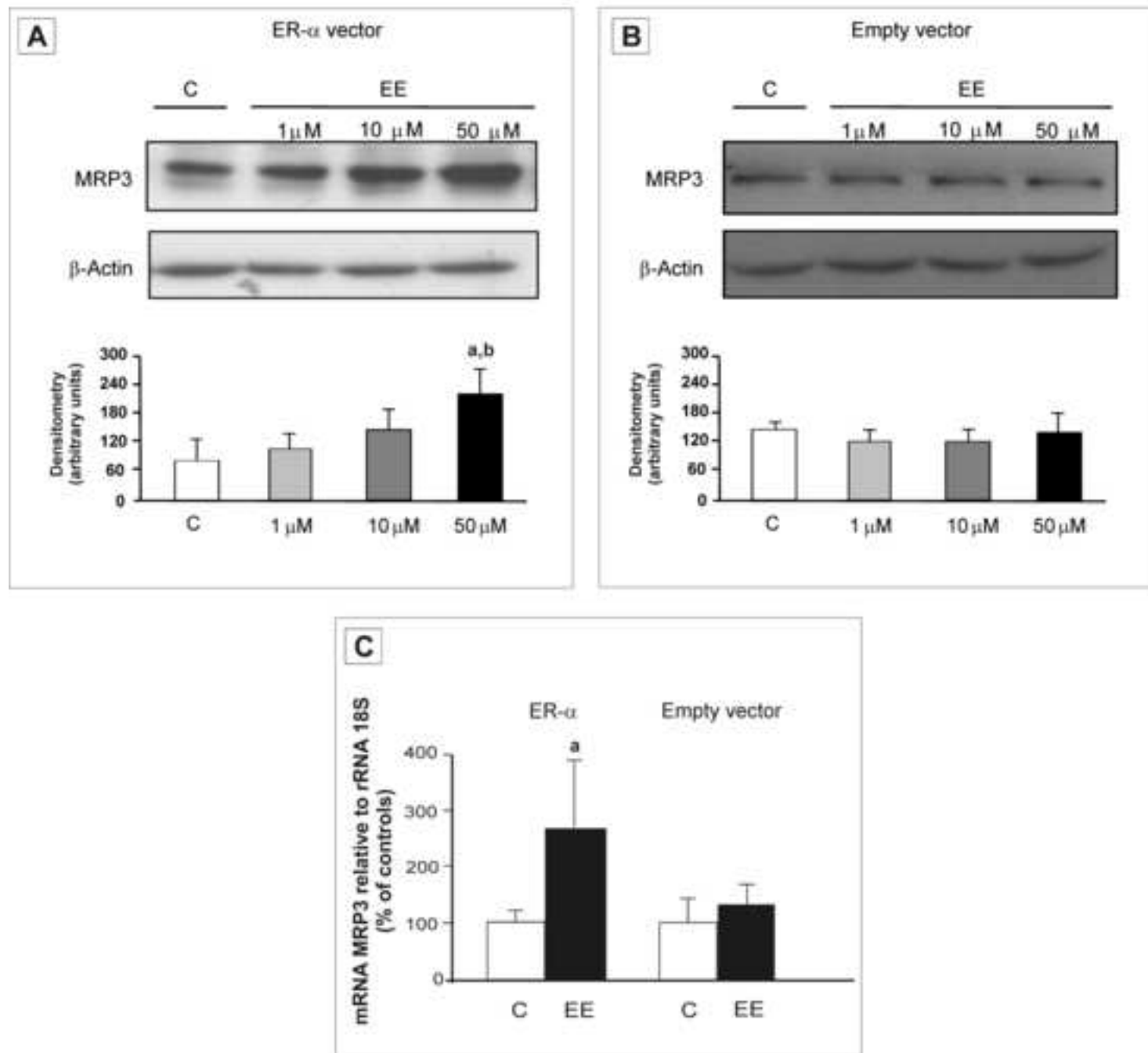




Figure 4



Figure 5

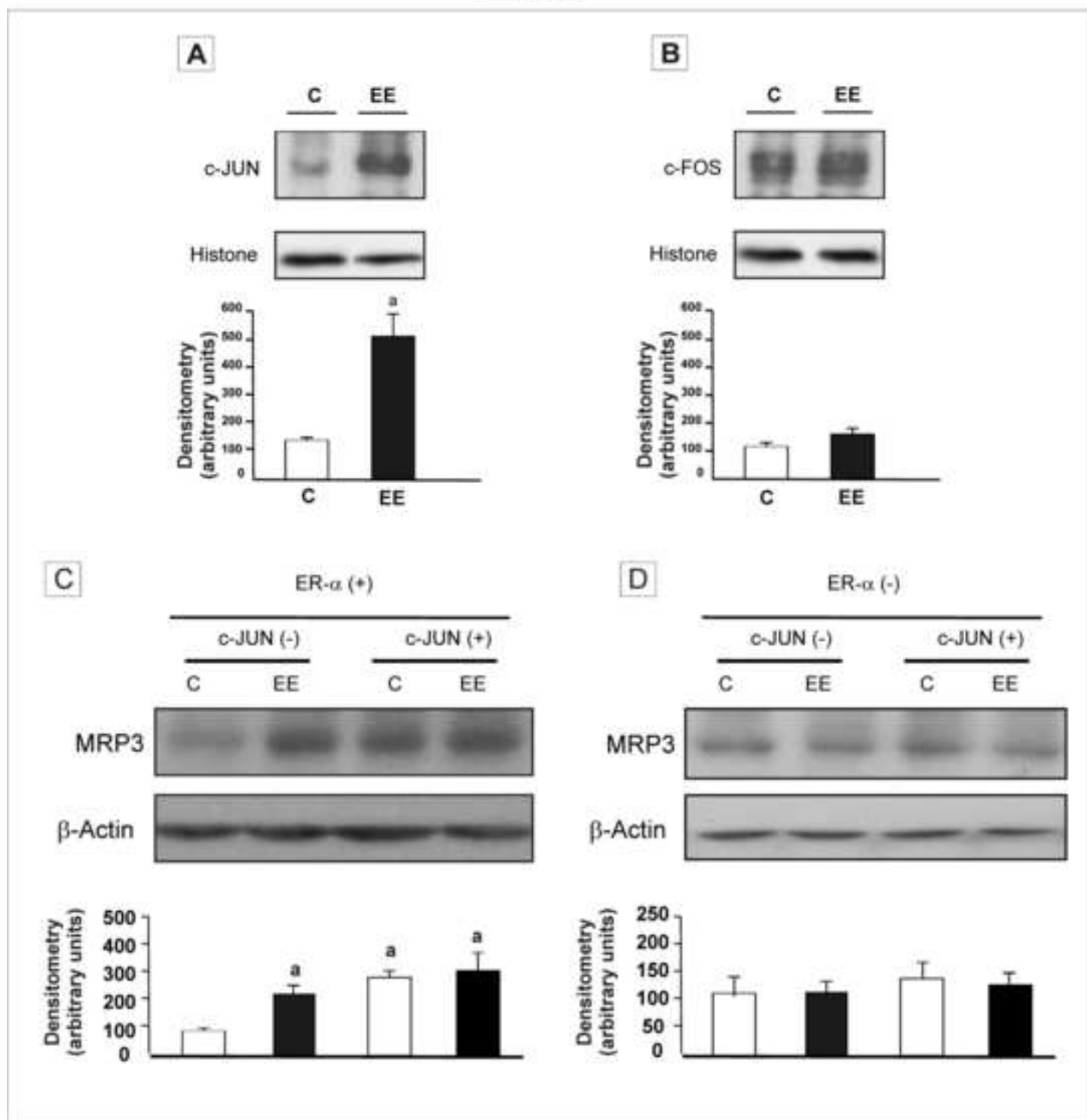


Figure 6

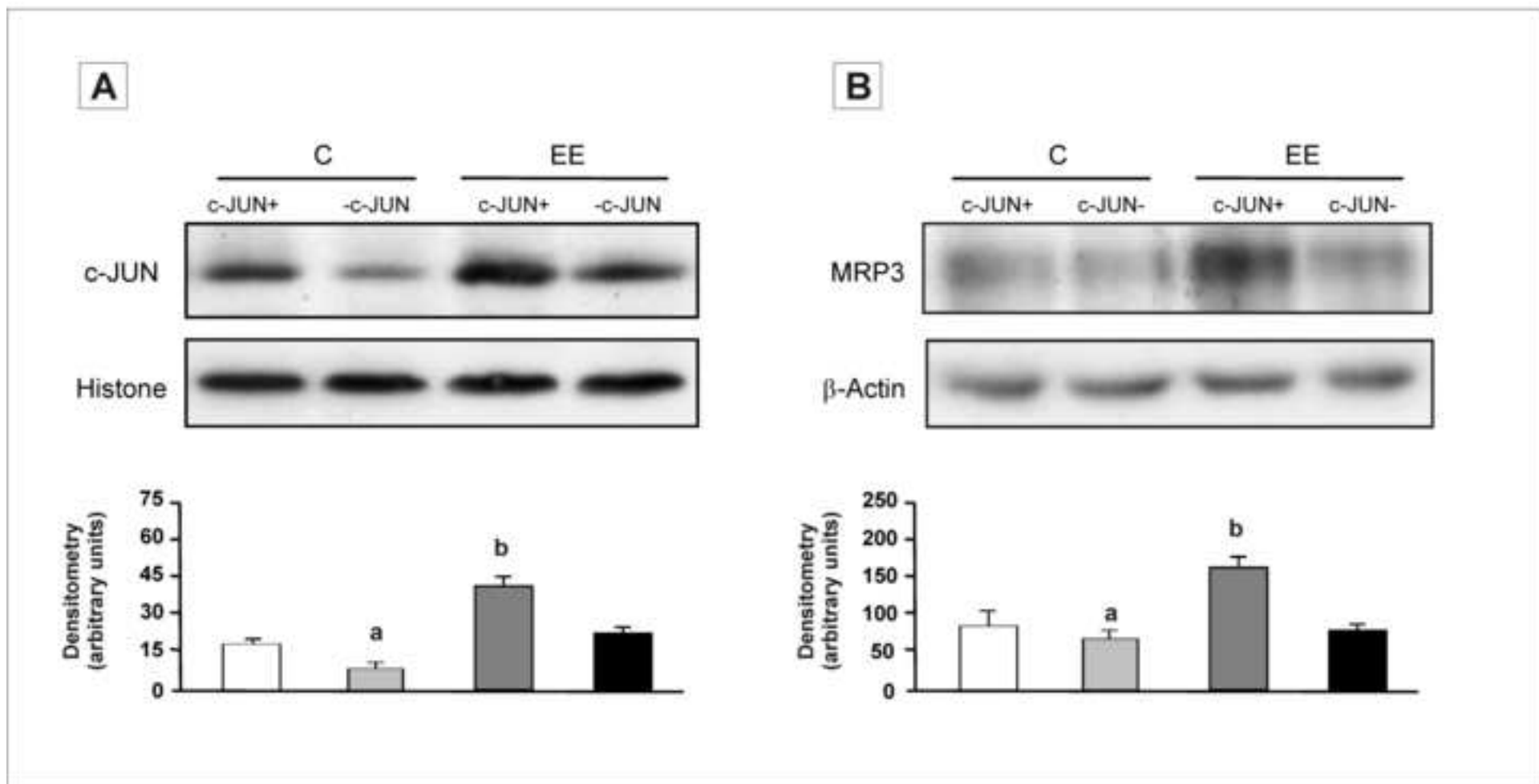


Figure 7

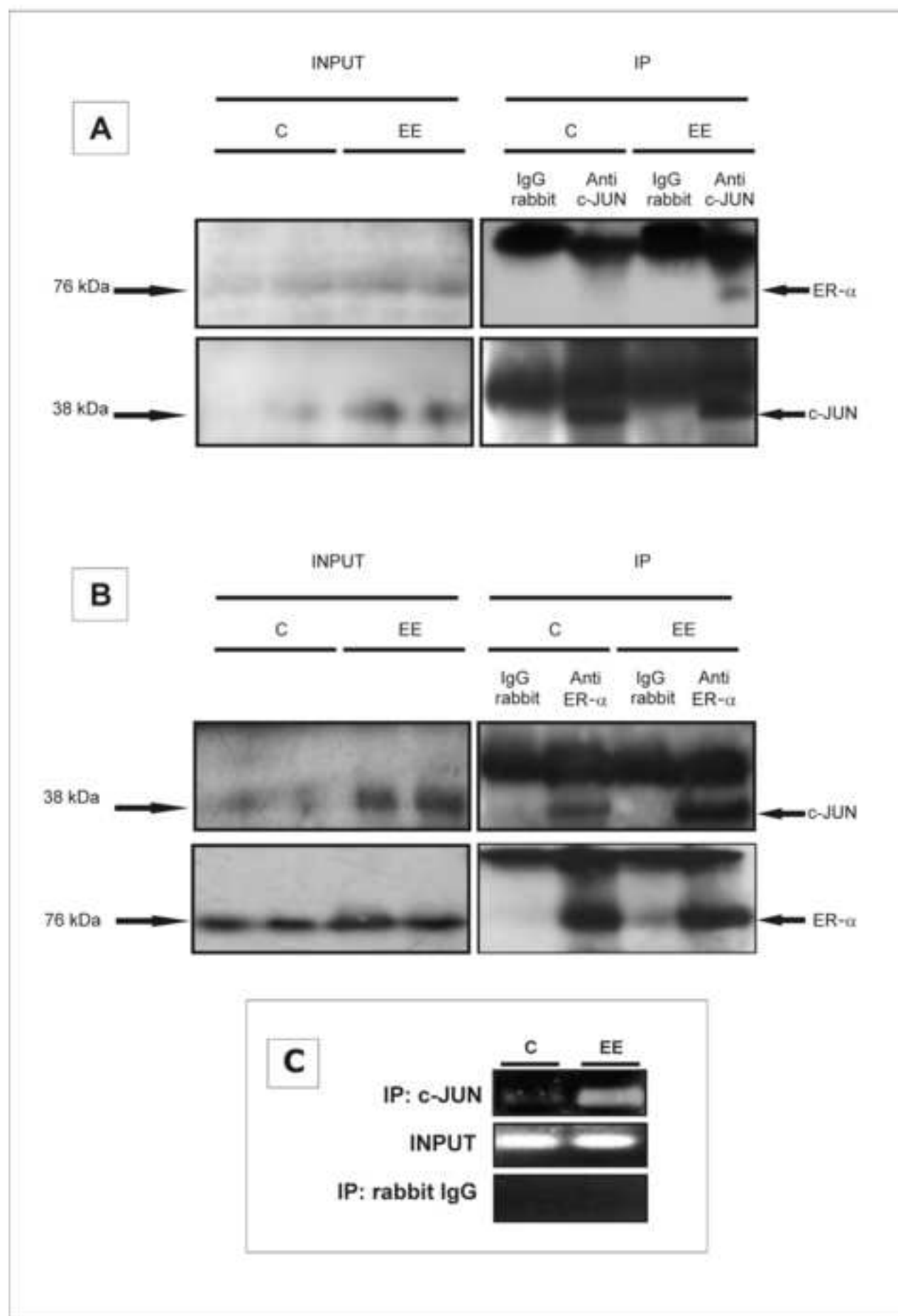


Figure 8

