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HAL Authorization

**17 $\beta$ -estradiol enhances  $\alpha_5$  integrin subunit gene expression through ER $\alpha$ -Sp1 interaction and reduces cell motility and invasion of ER $\alpha$  positive breast cancer cells.**

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**Running head:** Estradiol enhances  $\alpha_5\beta_1$  integrin expression.

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1 **Abstract**

2 In breast tumors the expression of estrogen receptor alpha ( $ER\alpha$ ) is known to be associated with  
3 a more favorable prognosis.  $ER\alpha$  expression has been reported to reduce the metastatic potential of  
4 breast cancer cells. Recently, we have observed that extracellular matrix proteins activate  $ER\alpha$  and that  
5 both liganded and unliganded receptor modulates cell invasiveness acting at nuclear level. To explain  
6 the mechanisms by which  $ER\alpha$  regulates cell adhesion, we have evaluated the expression of  $\alpha_5\beta_1$   
7 integrin, prevalently expressed in stationary cells, in response to  $17\beta$ -estradiol (E2). Here we show that  
8 E2/ $ER\alpha$  increases the expression of integrin  $\alpha_5\beta_1$  through Sp1 mediated binding to a GC rich region  
9 located upstream of an ERE half-site in the 5' flanking region of the  $\alpha_5$  gene forming a ternary  $ER\alpha$ -  
10 Sp1-DNA complex. Estrogen responsiveness of the  $\alpha_5$  gene promoter, as observed in HeLa cells,  
11 underlies a general mechanism of regulation which is not strictly linked to the cell type.

12 Our data reveal novel insight into the molecular mechanisms sustaining the reduced  
13 invasiveness of  $ER\alpha$  expressing cells demonstrating that  $\alpha_5\beta_1$  integrin expression is related to the  
14 maintenance of the stationary status of the cells, counteracting E2/ $ER\alpha$  capability to enhance breast  
15 cancer cell migration and invasion.

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## 1 **Introduction**

2 In the mammary gland estrogen receptor alpha ( $ER\alpha$ ) functions as a hormone-dependent transcription  
3 factor regulating the expression of proto-oncogenes which control cell growth and differentiation [1-5].

4 In breast cancer, however, the stimulatory effect of  $17\beta$ -estradiol (E2)/ $ER\alpha$  signaling on tumor cell  
5 growth strongly correlates with a lower invasiveness and reduced metastatic potential [6-8]. For  
6 instance, previous studies and our recent findings demonstrate how the exposure of breast cancer cells  
7 to extracellular matrix (ECM) proteins increases cell proliferation [9], enhances functional  
8 transactivation of  $ER\alpha$  and reduces cell invasiveness in  $ER\alpha$  positive breast cancer cells [10].

9 On the other hand, it is well documented how E2/ $ER\alpha$  up-regulates genes which reduce cell  
10 invasiveness like  $\alpha$ 1antichymotrypsin [11] and down-regulates genes involved in the enzymatic  
11 remodeling and degradation of ECM proteins like metalloproteinase-9 [12] and Collagenase type IV  
12 [13].

13 Cell adhesion to ECM is mainly mediated by integrins which mostly control the physical strength of  
14 cell substratum attachment [14]. Several studies have demonstrated that variations in cell surface  
15 expression levels of integrin [15], or in adsorbed concentration of its substratum, or its ligand [16], or  
16 in the integrin-ligand affinity [17], could affect the adhesion strength and the speed of cell movement  
17 [14].

18 A role for E2/ $ER\alpha$  in integrin expression derives from physiological circumstances consistent with  
19 evidence indicating that  $\alpha_5\beta_1$  levels double in breast tissues between puberty and sexual maturity and  
20 drop dramatically after ovariectomy [9]. So, E2 seems to exert a specific modulatory role on integrin  
21 expression which mostly regulates the adhesion of tumor cells to substrates.

22 Fibronectin (Fn) mediated attachment and signaling in cells occurs in response to Fn binding to cell  
23 surface integrins. Fn has been demonstrated to bind to different Fn specific integrins including  
24  $\alpha_4\beta_1$ ,  $\alpha_5\beta_1$ ,  $\alpha_v\beta_1$ ,  $\alpha_v\beta_3$ , and  $\alpha_v\beta_6$  [18]. However previous studies have shown that the  $\alpha_4$  subunit is not

1 present in mammary cells [19]. Furthermore immunohistochemical analyses revealed little or no  $\alpha_v$   
2 protein in mammary epithelia or in myoepithelial cells [20] at any stage of development examined.  
3 Other authors have reported that the  $\alpha_5$  is the key receptor subunit for Fn and that  $\alpha_5$  knockout mice  
4 most closely resemble Fn knockout mice [18, 21].

5 On the basis of these findings we reasonably focused our attention on the effects of E2 on the  
6  $\alpha_5$  integrin subunit being this subunit fully representative of the  $\alpha_5\beta_1$  integrin expression.

7 In the present study we elucidated the molecular mechanism by which E2/ER $\alpha$  signaling up-regulates  
8 the expression of the  $\alpha_5$  integrin subunit either in MCF-7 or HeLa cells ectopically expressing ER $\alpha$ .  
9 This event appears to occur through the formation of a transcriptionally-active ER $\alpha$ -Sp1 complex that  
10 binds to the GC-rich (Sp1) (-420 to -370) region located up-stream of ATG and very likely indicates a  
11 general modulatory mechanism induced by E2 which is not tightly linked to the cell type investigated.

12

## 13 **Materials and Methods**

### 14 ***Cell lines and cell culture conditions***

15 Two ER $\alpha$  positive (ER+) human breast cancer epithelial cell lines, MCF-7 and T47D and an  
16 ER $\alpha$  negative (ER-) *cervicae* carcinoma cell line HeLa were used. MCF-7 and T47D cells were  
17 maintained in monolayer culture in Dulbecco's modified Eagle's/Ham's F-12 medium (1:1)  
18 (DMEM/F12; Gibco, USA) supplemented with 5 % calf serum (CS; Eurobio, F), 100 UI/ml penicillin  
19 (Eurobio, F), 100 ug/ml streptomycin (Eurobio, F) and 0.2 mM L-glutamine (Eurobio, F). HeLa cells  
20 were maintained in monolayer cultures in Minimal Essential Medium with Earle's salts (MEM; Gibco,  
21 USA) supplemented with 10% fetal bovine serum (Eurobio, F), 100 UI/ml penicillin, 100 ug/ml  
22 streptomycin and 0.2 mM L-glutamine. Cells were passaged weekly using trypsin-EDTA (Eurobio, F)  
23 and media were changed every two days. Both cell lines were cultured at 37°C in 5% CO<sub>2</sub> in a  
24 humidified atmosphere.

1 In all experiments, steroids and growth factors were deprived from cells growing them in  
2 serum- and phenol red-free DMEM/F12 (SFM; Gibco, USA) containing 0.5 % BSA and 2 mM L-  
3 glutamine for 24 hrs.

#### 4 ***Fluorescence-Activated Cell Sorting.***

5 Serum starved MCF-7 cells were treated with 10 nM E2 for 4, 12, and 24 h or for 24h with  
6 increasing concentration of E2 (1, 10, 100 nM). Cells were detached with EDTA solution (Versene),  
7 washed and incubated with anti-integrin  $\alpha 5$  antibody (H-104, Santa Cruz Biotechnology, USA) or anti-  
8 integrin  $\alpha 4$  antibody (7.2, Santa Cruz Biotechnology, USA) for 1 h at 22°C. Cells were then washed  
9 twice in PBS and incubated with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody  
10 (Santa Cruz Biotechnology, USA) for 1 h at 37°C. After two washes in PBS, cells were fixed with 70%  
11 ethanol and subjected to FACS analysis. At least  $1 \times 10^4$  cells were measured in a FACS flow cytometer  
12 (Becton Dickinson, USA). MCF-10 cells were used as control.

#### 13 ***Western blotting***

14 To evaluate the influence of E2 on  $\alpha 5$  protein expression, MCF-7 and T47D cells, serum-  
15 starved for 24 h, were incubated with 10 nM E2 (Sigma Aldrich, USA) for 16, 24, and 48 h. In some  
16 experiments the cells were treated with 1  $\mu$ M of the E2 antagonist ICI 182,780 (ICI; Zeneca, UK). At  
17 the end of each incubation time, cells were washed with ice-cold PBS and lysed for 1 min at 4 °C in  
18 Triton lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 1 % Triton X-  
19 100, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, 1 % PMSF, 1 % Aprotinin). Cell lysates were cleared by centrifugation (14000  
20 rpm for 10 min at 4 °C) and the protein content was determined using Bradford Dye Reagent (Bio Rad,  
21 USA) according to manufacturer's instructions. Cellular lysates (10 to 30  $\mu$ g of protein/lane) were  
22 resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with either anti- $\alpha 5$   
23 integrin polyclonal antibody (H-104, Santa Cruz Biotechnology, USA) or anti-GAPDH polyclonal  
24 antibody (FL-335, Santa Cruz Biotechnology, USA). The antigen-antibody complexes were detected

1 by incubation of the membranes with peroxidase-coupled anti-rabbit IgG and developed using the ECL  
2 Plus Western Blotting detection system (Amersham Pharmacia Biotech, UK).

### 3 ***RT-PCR***

4 MCF-7 and T47D cells were incubated for 24 h in SFM and then treated with E2 10 nM for 24  
5 h and/or 1  $\mu$ M ICI 182,780 (ICI; Zeneca, UK). At the end of the treatment total cellular RNA was  
6 extracted using RNeasy (Qiagen, USA) and reverse transcribed using Moloney Murine Leukemia Virus  
7 (M-MLV) reverse transcriptase (Promega, USA). Briefly, reverse transcription (RT) was performed  
8 using 1  $\mu$ g of total RNA in a final volume of 10  $\mu$ l by incubation at 37  $^{\circ}$ C for 30 min with 200 U of M-  
9 MLV reverse transcriptase, 0.4  $\mu$ g oligo dT, 0.5  $\mu$ M deoxy-nucleotidetriphosphate (dNTP) and 24U  
10 RNAsin (Promega, USA), followed by heat denaturation for 5 min at 95  $^{\circ}$ C. Subsequent PCR  
11 analysis was performed on 1  $\mu$ l of the RT product in a final volume of 50  $\mu$ l. The following pairs of  
12 primers were used to amplify the 188 bp of  $\alpha_5$  integrin: 5'-CCCCGCTCGTGCCGCTGCTGTT-3' ( $\alpha_5$   
13 forward) and 5'-CCCCACCCCC-ATCCCGTCTCC-3' ( $\alpha_5$  reverse). The PCR amplification was  
14 performed using 1.25U GoTaq DNA polymerase (Promega, usa), 1X PCR buffer (10mM Tris-HCl,  
15 50mM KCl), 2.5mM MgCl<sub>2</sub>, 0.2mM of each dNTP, and 1.5 pM of primers for  $\alpha_5$  or 36B4. PCR  
16 conditions were 30 seconds (sec) at 94  $^{\circ}$ C, 40 sec at 59  $^{\circ}$ C, and 60 sec at 72  $^{\circ}$ C for 30 cycles. PCR  
17 products (10  $\mu$ l) were separated on a 1.0 % agarose gel. Amplification of the 408 bp of ribosomal RNA  
18 36B4 was performed as control using the following primers: 5'-CTCAACATCTCCCCCTTCTC-3'  
19 (36B4 forward) and 5'-CAAATCCCA-TATCCTCGTCC-3' (36B4 reverse).

### 20 ***Plasmid constructs***

21 To create the Luciferase expression vector pGL3-p $\alpha_5$  encoding the 5'-flanking region of the  $\alpha_5$   
22 gene [22], genomic DNA was extracted from MCF-7 by TRIzol reagent following the manufacturer's  
23 instructions and amplified by PCR using the following primer pairs 5'-ACTCCTGGGTT-

1 TTCAATACTG-3' (forward) and 5'-CCCGCTCTTCCCTGTCC-3' (reverse). PCR amplification was  
2 performed using the same reagents reported above. PCR conditions were 5 min at 95 °C followed by 1  
3 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C for 30 cycles. The obtained fragment was inserted in  
4 the pCR 2.1 plasmid using the TA cloning kit (Invitrogen, USA), sequenced, and cut with Hind III and  
5 Xho I. The insert was then cloned into the pGL3 basic vector (Promega, USA). The deletion mutant in  
6 positions -424 to -370 of the  $\alpha_5$  gene 5'-flanking sequence corresponding to a Sp1/ERE half-site motif  
7 (Figure 4A) was created using PCR (pGL3-p $\alpha_5$ - $\Delta$ Sp1/ERE). The oligonucleotides used as PCR  
8 primers were as follows: 5'-ACTCCTGGGTTTTCAATACTG-3' (forward), 5'-CCTTAAGAG-  
9 GAAGCCTGTATATGTGTG-3' (reverse) and 5'-CGAATTCGATTCCTTTCCTCATTAGGA-3'  
10 (forward), 5'-CCCGCTCTTCCC-TGTCC-3' (reverse). The PCR products were digested with EcoRI  
11 (underlined in primers), ligated and inserted in the pCR 2.1 plasmid using the TA cloning kit. The  
12 obtained plasmid was digested with Hind III and Xho I. The insert was then cloned into the pGL3 basic  
13 vector. The pGL3-Sp1/ERE plasmid was obtained using two complementary oligonucleotides: 5'-  
14 ATATGAGCTCCATATACAGGCTTCCTCCGCCACCAGAGGTGATTCCTTTCCTCATTAGG-  
15 AAGCTTTATAT-3' and 5'-ATATAAAGCTTCCTAATGAGGAAAGGAATCACCTCTGGTGGGC-  
16 GGAGGAAGCCTGTATATGGAGCT-CATAT-3'. Self annealing was obtained by boiling and  
17 cooling down 10  $\mu$ g of each oligonucleotide for 5 min in water. The insert was digested with Sac I and  
18 Hind III (underlined in primers) and successively cloned in pGL3 basic vector.

19 Site mutation of the Sp1 site (-404 to -399) up-stream to the half-ERE binding motif was generated  
20 using PCR. The oligonucleotides used as PCR primers were as follows: 5'-AAGGTA-  
21 CCACTCCTGGGTTT TCAATACTG-3' (forward), 5'-CTCTGGTGGATGGAGGAAGCCTGTAT-3'  
22 (reverse) and 5'-ATACAGGCTTCCTCCatCCACCAGAG-3' (forward), 5'-AACTCGAGCCCG-  
23 CTCTTCCCTGTCC-3' (reverse). PCR products were inserted into the pCR 2.1 plasmid using the TA

1 cloning kit. The obtained plasmid was digested with Kpn I and Xho I (underlined in primers). The  
2 insert was then cloned into the pGL3 basic vector pGL3-p $\alpha$ 5 (-Sp1).

### 3 ***Plasmids, transfections and ERE-luciferase assay***

4 In addition to the plasmids described above, the following plasmids were used: the SV40  
5 promoter-based pSG5 vector encoding ER $\alpha$  (wild-type) pSG5-HeG0 (HeG0, Tora et al, 1989); pCMV-  
6 hE241G, an ER $\alpha$  construct deleted in the nuclear localization signal region (NLS) (aa. 250-303) and  
7 inserted in the pCMV expression plasmid (E241G; a gift from Song R) and the *Renilla reniformis*  
8 luciferase expression vector pRL-Tk (Promega, USA).

9 To monitor the transcriptional activation of  $\alpha$ 5 by E2, MCF-7 and HeLa cells ( $5 \times 10^4$  density)  
10 were plated on a 24-well plates, grown in DMEM/F12 to an approximate confluence of 70-80 % and  
11 then co-transfected with either pGL3-p $\alpha$ 5 (encoding for luciferase under the control of the human  $\alpha$ 5  
12 promoter) and pRL-Tk (MCF-7 and HeLa) or with pGL3-p $\alpha$ 5, HeG0, and pRL-Tk, or pGL3-p $\alpha$ 5,  
13 E241G, and pRL-Tk (HeLa). Specific deletion and mutations of the pGL3-p $\alpha$ 5 plasmid were generated  
14 as described above. The effects of each site mutation/deletion were examined in transfection assays in  
15 MCF-7 cells. Transfections were carried out using Fugene 6 (Roche, USA). Cells were transfected in  
16 growing medium and 6 h after transfection were washed twice with SFM and shifted to SFM for 24 h  
17 and then treated for an additional 24 h with 10 nM E<sub>2</sub> and/or 1  $\mu$ M ICI 182,780 (ICI; Zeneca, UK)  
18 and/or 100nM mithramycin A (Sigma, USA). Where indicated, cells were treated with mithramycin A  
19 for 1 h before stimulation with E2.

20 Firefly luciferase and *Renilla reniformis* luciferase activities were determined using the Dual-  
21 Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instructions.  
22 Firefly luciferase activity was normalized to *Renilla reniformis* luciferase activity and expressed as  
23 relative luciferase units.

1 To evaluate the effects of ER $\alpha$  activity on  $\alpha_5$  integrin expression HeLa cells were transfected,  
2 as above with increasing concentrations of pSG5-HeG0 (1 and 2  $\mu$ g), as control HeLa cells were  
3 transfected with an empty pSG5 vector. Following transfection, cells were serum starved for 24 h and  
4 then treated for 24 h with 10 nM E2.

### 5 ***Knock-down of $\alpha_5\beta_1$ integrin gene expression***

6 siRNA was used for effective depletion of  $\alpha_5\beta_1$  integrin gene (Santa Cruz Biotech, USA). A  
7 scrambled siRNA (Invitrogen, USA) that lacks identity with known gene targets was used as control for  
8 non sequence-specific effects. MCF-7 and T47D cells were trypsinized, transfected in suspension with  
9 Lipofectamine 2000 (Invitrogen, USA), as suggested by the manufacturer, and then plated in six-well  
10 plates ( $3.5 \times 10^5$  cells per well). Briefly, cells were transfected with 100 pmol siRNAs in 3 ml total  
11 growing medium. siRNAs were diluted in 500  $\mu$ l of SFM without antibiotic; after 5 min lipofectamine  
12 2000 was added to the mix and incubated at room temperature for 20 min. siRNA-Lipofectamine 2000  
13 complex was then added to the cells and incubated at 37°C for 6 h, switched to fresh growing medium  
14 and further incubated for additional 18 h. Then cells were switched to SFM for other 24 h and treated or  
15 not with 10 nM E2 for 24 h before motility analysis. Forty eight hours of incubation with the siRNAs  
16 was chosen based on a time course performed to evaluate the time needed to obtain a knock down of at  
17 least 60% of  $\alpha_5$  integrin content.

### 18 ***Chromatin immunoprecipitation***

19 MCF-7 cells were grown in 10 cm plates. Confluent cultures (90%) were shifted to SFM for 24  
20 h and then treated with 10 nM E2 or left untreated in PRF-CT for 2 h. Following treatment, CHIP  
21 methodology was performed as described previously (Morelli et al., 2004). The precleared chromatin  
22 was precipitated with anti-ER $\alpha$  mAb (Santa Cruz, USA) for ER $\alpha$ , anti-Sp1 pAb (Upstate, USA) for  
23 Sp1 and anti-Polymerase II pAb (Santa Cruz, USA) for Pol II. A 4  $\mu$ l volume of each sample was used  
24 as template for PCR with specific primers. The following pairs of primers were used to amplify 312 bp

1 of the  $\alpha 5$  promoter region containing the Sp1/half-ERE 5'-ACCCCAAAGTCTCCTCCCTCCCG-3'  
2 (forward); 5'-CCCGCGTCCCCCACCCCTAAG-3' (reverse). The PCR conditions were: 1 min at 94  
3 °C, 45 sec at 59 °C, and 45 sec at 72 °C. The amplification products obtained in 28 cycles were  
4 analyzed in a 2 % agarose gel and visualized by ethidium bromide staining.

### 5 ***Gel Mobility Shift Assay***

6 Nuclear protein extracts were prepared and collected as reported in DAPA. The probes were  
7 generated by annealing single-stranded oligonucleotides (reported below) labeled with [ $\gamma$ - $^{32}\text{P}$ ]ATP and  
8 T4 polynucleotide kinase and then purified using Sephadex G50 spin columns. The oligonucleotides  
9 used as probes and as cold competitors (synthesized by Sigma Genosys, UK) are listed below. The  
10 protein binding reactions were carried out in 20  $\mu\text{l}$  of buffer (20 mM HEPES, pH 8, 1 mM EDTA, 50  
11 mM KCl, 10 mM dithiothreitol, 10 % glycerol, 1 mg/ml BSA, 50  $\mu\text{g/ml}$  poly (dI-dC) with 50000 cpm  
12 of labeled probe, 20  $\mu\text{g}$  of MCF-7 nuclear protein or 15 ng of Sp1 and 0.18 pmol of purified ER $\alpha$   
13 recombinant proteins (both purchased from Invitrogen, USA), and 5  $\mu\text{g}$  of poly (dI-dC)). The above-  
14 mentioned mixture was incubated at room temperature for 20 min in the presence or absence of  
15 unlabeled competitor oligonucleotide or specific anti-ER $\alpha$  (anti ER $\alpha$  F-10) or Sp1 (anti Sp1 1C6)  
16 antibodies, both purchased from Santa Cruz Biotechnology, USA. The entire reaction mixture was  
17 electrophoresed through a 6 % polyacrylamide gel for 3 h at 150 V. Gel was dried and subjected to  
18 autoradiography at -70 °C.

19 Oligonucleotides used (nucleotide motifs of interest are underlined): Probe - 5'-TTCCTCCGCCC-  
20 ACCAGAGGTGATTCC-3'; Mutated probe - 5'-TTCCTCCGCCCACCAGAaaTtATTCC-3'.

### 21 ***DNA affinity precipitation assay***

22 DNA affinity precipitation assay was performed as described by Suzuki T. et al [23] with minor  
23 modifications. Nuclear extracts were prepared from MCF-7 cells. Sub-confluent cultures (70%) were  
24 shifted to SFM for 24 h and then treated with 10 nM E2 for 24 h, or left untreated in SFM. Following

1 treatment, cells were scraped in cold PBS, and resuspended in 400  $\mu$ l of cold buffer A (10 mM HEPES-  
2 KOH, pH 7.9, at 4 °C, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM  
3 leupeptin). The cells were allowed to swell on ice for 10 min, centrifuged, and the pellet was  
4 resuspended in 50  $\mu$ l of cold Buffer B (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 1.5 mM MgCl<sub>2</sub>,  
5 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) and incubated  
6 on ice for 20 min for high salt extraction. Cellular debris was removed by centrifugation for 2 min at 4  
7 °C, and the supernatant fraction (containing DNA-binding proteins) was stored at -70 °C. The yield  
8 was determined by Bradford method.

9 The 26 bp probe was prepared by annealing the biotin-treated sense oligonucleotide (5'-  
10 TTCCTCCGCCACCAGAGGTGATTC-3') and non-biotinated anti-sense oligonucleotide. A  
11 3 pmol sample of the probe was added to 100  $\mu$ g of the protein nuclear lysates and placed on ice for  
12 40 min. Then 20  $\mu$ l of streptavidin-coated beads (Promega) were added and the sample was incubated  
13 for a further 3 h at 4 °C with rotation. After separation of the beads from the supernatant by  
14 centrifugation, the beads were washed five times with binding buffer, suspended with 40  $\mu$ l of sample  
15 buffer, boiled and used for Western blotting as a DNA-bound fraction. The supernatant was mixed with  
16 an equal volume of 2 $\times$  sample buffer, boiled and used for Western blotting as an unbound fraction. For  
17 competition, a 10-fold excess of unlabelled probe was added to the nuclear lysate and incubated on ice  
18 for 30 min before addition of the labeled probe.

### 19 **Motility assay**

20 To evaluate the role of  $\alpha$ 5 $\beta$ 1 integrin on cell motility we performed a knock down of  $\alpha$ 5 $\beta$ 1  
21 integrin, as reported in *plasmids and transfections* section, in both MCF-7 and T47D cell lines, before  
22 motility assays. After transfection, cells were maintained in SFM for 24 h and then treated with E2 for  
23 additional 24h. At the end, cells were dispersed with versene (Eurobio, F), washed twice, re-suspended  
24 in SFM, counted using a haemocytometer and plated on 24-well modified Boyden chambers containing

1 porous (8 $\mu$ m) polycarbonate membranes (Costar, USA). For cell invasion experiments the chambers  
2 were coated on the internal surface, with 30  $\mu$ g/ml Fibronectin (Fn) by incubation at room temperature.  
3 The lower chambers were loaded with 500  $\mu$ l of either SFM for migration assays or SFM containing  
4 5% CS for invasion assays, while synchronized cells ( $1 \times 10^5$ ) suspended in 200  $\mu$ l of SFM with or  
5 without 10 nM E2 were plated into the upper chambers. After 8 hrs of incubation in 5% CO<sub>2</sub> at 37° C,  
6 the cells in the upper chamber were removed by a cotton swab, so that only cells that had migrated  
7 through the membrane remained. The membranes were then fixed and stained in Coomassie blue  
8 solution (0.25g Coomassie blue, 45ml water, 45ml methanol, 10ml glacial acetic acid) for 5 min, then  
9 each well was rinsed three times with distilled water. The migrated cells were counted using an  
10 inverted microscope.

### 11 *Statistical analysis*

12 All data were expressed as the mean $\pm$ SD (standard deviation) of at least three independent experiments.  
13 Statistical significances were tested using Student's t test.

14

## 15 **RESULTS**

### 16 *$\alpha_5$ integrin subunit expression in MCF-7 cells upon E2 exposure.*

17 The expression of the  $\alpha_5$  integrin subunit in response to E2 stimulation was evaluated in MCF-7  
18 cells. We focused our attention on the  $\alpha_5$  integrin subunit, which is strongly indicative of integrin  $\alpha_5\beta_1$   
19 expression, since only the  $\beta_1$  subunit protein has been demonstrated to form a heterodimer with the  $\alpha_5$   
20 subunit, so the detection of the  $\alpha_5$  subunit has been reported to mean that the  $\alpha_5\beta_1$  integrin was also  
21 detected herein.

22 Serum-starved MCF-7 cells were treated with 10 nM E2 for 4, 12 and 24 hours (h) before  
23 immunodetection of the  $\alpha_5$  integrin subunit by FACS analysis (see *Materials and Methods*). As shown

1 in figure 1A, the exposure to E2 induced an increase in  $\alpha_5$  integrin subunit expression that became  
2 significant starting from 12 h of treatment. The significant up-regulation of  $\alpha_5$  integrin subunit levels  
3 was also observed in serum starved MCF-7 cells treated for 24 h with increasing concentrations of E2  
4 (1, 10, 100 nM) (Figure 1B). The increment of  $\alpha_5$  integrin subunit expression in response to E2 was  
5 also observed in T47D cells (data not shown).

6 To further assess the specificity of E2-dependent  $\alpha_5$  integrin subunit expression, we evaluated  
7 whether E2 was also able to regulate  $\alpha_4$  integrin subunit levels, since its expression has been reported  
8 to be associated with a reduction of breast cancer cell invasiveness [24]. E2 administration induced a  
9 dose-dependent increase of  $\alpha_5$  expression, but did not affect  $\alpha_4$  integrin subunit levels (Figure 1C,i).

#### 10 ***E2 regulates the expression of $\alpha_5$ integrin subunit***

11 To further investigate the mechanistic basis for the estrogen-dependent regulation of  $\alpha_5$  integrin  
12 subunit expression, we carried out a time course analysis. MCF-7 cells were serum starved for 24 h and  
13 then treated with 10 nM E2 for 16, 24 and 48 h. The  $\alpha_5$  integrin subunit expression was detected on  
14 protein lysates. As shown in Figure 2A a marked decrease of  $\alpha_5$  integrin subunit was observed  
15 following a prolonged starvation (48 h), while E2 stimulation induced a significant increase of its  
16 expression. In addition, E2 induced a dose-dependent increase of  $\alpha_5$  integrin subunit expression (Figure  
17 2C). The up-regulation observed was mediated by ER activation since the addition of ICI 182,780  
18 abrogated it (Figure 2C).

19 The observed up-regulation was directly correlated to a significant increase in  $\alpha_5$  integrin  
20 mRNA transcription (Figure 2B and D).

21 To demonstrate that the increased expression of the  $\alpha_5$  integrin subunit was specifically mediated by  
22 ER $\alpha$ , we transfected HeLa cells with increased amounts of HeG0, treated with E2 (10 nM) for 24 h,  
23 and protein lysates were used to detect the  $\alpha_5$  integrin subunit. E2 stimulation increased  $\alpha_5$  integrin

1 expression in ER $\alpha$  content related manner (Figure 2C), supporting the conclusion that E2 regulates the  
2 expression of  $\alpha_5\beta_1$  integrin in ER $\alpha$ -positive cells. The increased expression of  $\alpha_5$  integrin subunit lead  
3 us to evaluate the role of E2 activated ER $\alpha$  on the regulation of  $\alpha_5$  integrin subunit expression.

#### 4 ***The 5'-flanking region of $\alpha_5$ gene is regulated by ER $\alpha$***

5 To investigate the mechanisms by which E2 regulates  $\alpha_5$  gene expression, we prepared a pGL3-  
6 p $\alpha_5$  plasmid containing a construct bearing the 5'-flanking region of the  $\alpha_5$  gene [22] obtained by  
7 PCR, as described in *Materials and Methods*, conjugated with a luciferase reporter gene. This region  
8 has been previously shown to be active as a promoter in the control of  $\alpha_5$  integrin gene expression [22].

9 MCF-7 cells were transfected with the pGL3-p $\alpha_5$  reporter plasmid and exposed to 10nM E2  
10 and/or 1 $\mu$ M ICI for 24h before luciferase assay. Figure 3A shows a significant increase in luciferase  
11 expression induced by E2 that is abolished by ICI. The same results were obtained in HeLa cells  
12 transfected with HeG0, pGL3-p $\alpha_5$ , and pRL-Tk (Figure 3C), while E2 failed to induce luciferase  
13 activity in HeLa cells transfected with the reporter plasmids alone (Figure 3B) and in the same cells  
14 transfected with pCMV-hE241G, a construct bearing ER $\alpha$  deleted in the nuclear localization signal  
15 (NLS), pGL3-p $\alpha_5$ , and pRL-Tk (Figure 3D). All together, these data indicate the  $\alpha_5$  integrin subunit as  
16 an E2/ER $\alpha$ -regulated gene.

#### 17 ***ER $\alpha$ and Sp1 bind to the 5'-flanking region of $\alpha_5$ integrin subunit***

18 The 5'-flanking region of the  $\alpha_5$  integrin subunit contains several elements located between  
19 positions -178 and -27 that are required for efficient basic transcription of the gene, such as AP1 sites,  
20 [22] and Sp1 and nuclear factor 1 (NF-1) sites which respond to cell adhesion on Fn [25]. The  
21 structural analysis of the  $\alpha_5$  promoter revealed the presence of an half ERE motif adjacent to a SP1 site  
22 which may potentially mediate the response to E2 treatment [26]. Since ER $\alpha$  can activate target  
23 promoters either in a classical way, by directly binding to specific ERE sequences, or indirectly,

1 through a functional interaction with other nuclear proteins such as Sp1 and subsequent binding to Sp1  
2 responsive sequences [27-32], we assessed the occupancy of the 5'-flanking region of the  $\alpha_5$  integrin  
3 subunit by ER $\alpha$  and Sp1 in MCF-7 cells using ChIP assay. As illustrated in Figure 4, unliganded ER $\alpha$   
4 is associated with the 5'-flanking region of the  $\alpha_5$  integrin gene containing the Sp1/half-ERE binding  
5 sites and its binding is strongly enhanced by the addition of E2. The binding of ER $\alpha$  to this promoter  
6 region is concomitant with the recruitment of Sp1. A small amount of Pol II was found to bind the  
7 promoter in untreated cells, reflecting a basal regulation of the  $\alpha_5$  integrin gene expression (Figure 4).  
8 E2 addition increased the occupancy of the 5'-flanking region of  $\alpha_5$  integrin gene by Pol II.

9 ***ER $\alpha$  regulates  $\alpha_5$  gene expression by binding to a Sp1/ERE half-site motif.***

10 On the basis of ChIP results, we hypothesized that both the half ERE motif and the SP1 site  
11 may be involved in E2 induced  $\alpha_5$  integrin subunit expression. To confirm our hypothesis two reporter  
12 plasmids were constructed as described in *Materials and Methods*, the pGL3-p $\alpha_5$ - $\Delta$ Sp1/ERE, which  
13 contains a specific deletion from positions -420 to -370 of the 5'-flanking sequence of  $\alpha_5$  gene  
14 corresponding to a Sp1/half ERE motifs (Figure 5A), and a pGL3-Sp1/ERE which contains the putative  
15 sequence responsible of E2 responsiveness (Figure 5A).

16 As reported in Figure 5B, the deletion of the Sp1/ERE half-site motif totally abolished the E2  
17 responsiveness of the promoter. As expected, an increase in luciferase expression was observed in cells  
18 transfected with the pGL3-Sp1/ERE construct in response to E2 that was abrogated by ICI (Figure 5C),  
19 showing that the SP1/half-ERE element is the target for the regulation of the  $\alpha_5$  integrin subunit  
20 expression in response to E2.

21 ***ER $\alpha$  enhances Sp1 binding to  $\alpha_5$  promoter.***

22 The results of our functional assays, which provide evidence for the involvement of the  
23 Sp1/half-ERE binding site in mediating the effects of E2 on  $\alpha_5$  integrin expression, fit well with our gel

1 mobility shift assay. Nuclear extracts of MCF-7 cells were incubated in the presence of a <sup>32</sup>P-labeled  
2 oligonucleotide reproducing the Sp1/half-ERE motif (Figure 6A, lane 6). The protein-DNA complexes  
3 formed revealed four slower migrating bands in the untreated nuclear extracts (Figure 6A, lane 1)  
4 which are depleted in the presence of an excess of unlabeled probe (Figure 6A, lane 2) and markedly  
5 increased in nuclear extracts obtained from E2 treated cells (Figure 6A, lane 3). The specificity of the  
6 binding was demonstrated by immunodepletion induced by either anti-ER $\alpha$  (Figure 6A, lane 4) or anti-  
7 Sp1 (Figure 6A, lane 5) antibodies. Addressing the binding of the bipartite ER-Sp1 complex to the  
8 selected Sp1/half-ERE responsive region.

9 To determine whether ER $\alpha$  “per se” is able to bind to its own reported half-site and to cooperate  
10 with Sp1 binding, gel mobility shift assays were performed using both ER $\alpha$  and Sp1 purified  
11 recombinant proteins with the same <sup>32</sup>P-labeled oligonucleotide in a cell free system (Figure 6B). A  
12 single gel-shifted band was visible using Sp1 purified protein (Figure 6B, lane 2), the specificity of this  
13 band was demonstrated using an excess of unlabeled probe that caused a dramatic reduction of the band  
14 (Figure 6B, lane 3), while the addition of a cold oligonucleotide mutated in the ERE half site slightly  
15 reduced the formation of the protein/DNA complex (Figure 6B, lanes 3 and 4, respectively).  
16 Unexpectedly, no band was observed incubating the labeled oligonucleotide with ER $\alpha$  purified  
17 recombinant protein (Figure 6B, lanes 5, 6, and 7). It is worth to note that the gel-shifted band observed  
18 incubating the probe with Sp1 native protein became newly detectable in the presence of increasing  
19 amounts of ER $\alpha$  (Figure 6B, lanes 8 to 10) that resulted drastically reduced using an excess of  
20 unlabeled probe (Figure 6B, lanes 11 to 13).

### 21 *Sp1 is required for E2 regulated expression of the $\alpha_5$ integrin subunit.*

22 To explore whether Sp1 contributes to the regulation of  $\alpha_5$  gene expression mediated by ER $\alpha$ ,  
23 we inhibited the SP1 binding to DNA using mithramycin A, a GC-specific DNA binding drug. As

1 shown in figure 7A, mithramycin A treatment caused a significant decrease in luciferase expression of  
2 the  $\alpha_5$  promoter construct in response to E2 stimulation in MCF-7 cells.

3 The requirement of Sp1 recruitment in the regulation of  $\alpha_5$  gene transcription in response to E2 was  
4 also confirmed by DAPA (Figure 7B). A mutation in the Sp1 site caused a dramatic reduction of ER $\alpha$   
5 binding to a biotinated oligonucleotide bearing the Sp1 consensus sequence and the half-ERE site,  
6 while Sp1 binding was completely abrogated (Figure 7B).

7 We confirmed these data using luciferase assays mutating the Sp1 site located at -404 to -399 of the 5'-  
8 flanking region of  $\alpha_5$  gene (Figure 7C). All together our data indicate that the half-ERE motif is not  
9 the target of the E2-dependent ER/Sp1 complex, suggesting that the regulation of  $\alpha_5$  gene transcription  
10 and expression occurs through a functional interaction of the complex with the Sp1 responsive  
11 sequences.

### 12 *$\alpha_5\beta_1$ integrin counteracts E2-induced cell motility in ER-positive breast cancer cells.*

13 Since we have previously demonstrated that ER $\alpha$  expression leads to a lower invasiveness and  
14 to a reduced metastatic potential of breast cancer cells through a genomic action [10], we investigated  
15 if  $\alpha_5\beta_1$  integrin expression was involved in ER $\alpha$  mediated reduction of breast cancer cells migration  
16 and invasion by knocking down  $\alpha_5\beta_1$  integrin (Figure 8). A knock down of about 60% of  $\alpha_5\beta_1$  integrin  
17 expression was obtained after 48 h (Figure 8A). In agreement with previous studies [8, 10], E2  
18 treatment produced a marked and significant reduction of both cell migration (Figure 8B) and invasion  
19 (Figure 8C) in MCF-7 cells as well as in T47D cells (data not shown). Interestingly, a dramatic  
20 increase of both cell migration (Figure 8B) and invasion (Figure 8C) was observed only in  $\alpha_5\beta_1$   
21 integrin silenced cells treated with E2, indicating that  $\alpha_5\beta_1$  integrin counteract E2-induced cell motility  
22 in ER-positive breast cancer cells.

23

## 1 **Discussion**

2 Previous findings have demonstrated that the concentration of the ECM protein Fn and  $\alpha_5\beta_1$   
3 integrin are developmentally and hormonally regulated by steroids in normal mouse mammary glands  
4 [9]. The expression of the Fn-specific integrin receptor  $\alpha_5\beta_1$ , was found to increase between puberty  
5 and sexual maturity and decrease in late pregnancy and lactation, indicating that Fn signaling may be  
6 required for mammary gland proliferation in response to ovarian steroids [9]. Breast tumours, which  
7 are regulated by ovarian steroids, are usually accompanied by striking changes in ECM components  
8 and expression of integrins. While the epithelial basal membrane is intact in normal mammary tissues,  
9 it is widely damaged in invasive carcinomas [33-34]. Particularly, in breast cancers exhibiting major  
10 aggressiveness and invasiveness, ER $\alpha$  is no longer expressed. For instance “*in vitro*” invasiveness and  
11 motility of ER $\alpha$  negative breast and ovarian cancer cells appear to be dramatically higher than ER $\alpha$   
12 positive cells [7-8, 35]. On the contrary, E2 has been found to enhance breast cancer cell migration and  
13 invasion through a rapid and dynamic cytoskeleton rearrangement [36-37] and a reversible  
14 epithelial/mesenchymal-like transition of ER-positive cells [38]. So, the role of E2 and ER $\alpha$  in breast  
15 cancer cell motility and invasion remains still controversial.

16 We report here for the first time that E2/ER $\alpha$  modulates the expression of  $\alpha_5\beta_1$  integrin in breast cancer  
17 cells, through the direct activation of the regulatory region of  $\alpha_5$  gene.

18 Previously we demonstrated how cell adhesion on Fn induces ER $\alpha$  translocation from the  
19 cytoplasm into the nucleus increasing the expression of estrogen responsive genes involved in the  
20 down-regulation of cell motility and invasion [10]. The increased expression of  $\alpha_5$  integrin in response  
21 to E2 stimulation reinforces the evidence that ER $\alpha$  may induce the expression of genes which enhance  
22 cell adhesion and reduce cell migration. Some target genes have been described previously, like

1 fibulin-1, a Fn-binding ECM protein [35] that is up-regulated by ER $\alpha$ , metalloproteinase 9 [12] and  
2 type IV collagenase [13] whose expression is negatively regulated by ER $\alpha$ .

3 On the basis of previous finding demonstrating that motile cells express high levels of integrin  
4  $\alpha_3\beta_1$  and  $\alpha_v\beta_1$  fibronectin receptor [39], while stationary cells prevalently express integrin  $\alpha_5\beta_1$  [40],  
5 the expression of the latter integrin seems to be a key indicator of the ability of cells to migrate. Indeed,  
6 overexpression of  $\alpha_5\beta_1$  on the surface of Chinese hamster ovary cells suppress their transformed  
7 phenotype, causing the loss of their ability to grow in soft agar and to form tumors in nude mice [41].  
8 On the contrary,  $\alpha_5\beta_1$  integrin has been shown to contribute to the progression of breast cancer. For  
9 instance, ligand binding to  $\alpha_5\beta_1$  integrin, in the absence of  $\alpha_4\beta_1$ , activates invasion-stimulatory signals  
10 in breast cancer cells [24, 42].

11 Virtually little is known about the molecular mechanisms controlling  $\alpha_5$  expression. We  
12 demonstrated that the 5'-flanking region of the  $\alpha_5$  gene, previously reported to act as a promoter [22],  
13 is a useful tool in the study of the molecular events controlling  $\alpha_5$  gene expression. The 5'-flanking  
14 region lies within a CpG island, lacks TATA and CCAAT boxes, and contains consensus binding sites  
15 for nuclear proteins such as the *ets* family of proto-oncogenes, Sp1, AP-2, and AP-1 [22]. We have  
16 identified a half-ERE site (-393 to -388) located down-stream and in close proximity of a Sp1 binding  
17 site (-404 to -399) in the 5'-flanking region of the  $\alpha_5$  gene. These adjacent binding sites are crucial  
18 effectors of E2 action inducing  $\alpha_5$  gene expression as it emerges from our reporter assay studies. The  
19 transcriptional regulation of the  $\alpha_5$  gene was further investigated in HeLa cells ectopically expressing a  
20 wild type ER $\alpha$  and a mutant of the receptor containing a deletion in the nuclear localization signal. The  
21 results obtained confirmed estrogen responsiveness of the  $\alpha_5$  gene promoter in HeLa cells expressing  
22 ER $\alpha$  but not the deleted mutant, demonstrating how the activation of the  $\alpha_5$  promoter by E2/ER $\alpha$   
23 underlines a general mechanism of regulation not tightly linked to the cell type.

1           Theoretically, E2 regulation of the  $\alpha_5$  gene may be mediated by ER $\alpha$  binding to the 5'-  
2 regulatory half-ERE motif and/or ER $\alpha$  may interact with Sp1 to transactivate the  $\alpha_5$  gene by binding to  
3 the Sp1/ half-ERE binding site. Indeed, E2 has been found to activate the transcription of several ER $\alpha$ -  
4 regulated genes in a no classical way either through the binding of ER $\alpha$  and the recruitment of Sp1 to  
5 their own responsive element separated by 10-23 bases (Sp1(N)<sub>10-23</sub> ERE half-site) such as c-myc [43],  
6 cathepsin D [44], retinoic acid receptor  $\alpha_1$  [45], progesteron receptor A [26] and IRS-1 [46] or through  
7 its recruitment on E2-responsive GC-rich promoter motifs by interacting with Sp1 [27-32], as reported  
8 for epidermal growth factor receptor [47], progesteron receptor B [48], *cad* [32], and cyclin D1 [49].

9           EMSA studies, using oligonucleotides reproducing the Sp1/half-ERE site contained in the 5'-  
10 flanking region of the  $\alpha_5$  gene, revealed that  $\alpha_5$  gene transcription induced by E2-activated ER $\alpha$  is  
11 dependent on GC-rich SP1 binding sites at -404 to -399 in the 5'-flanking region of the  $\alpha_5$  gene.

12           Overall, our results suggest that the responsiveness of the  $\alpha_5$  integrin subunit to E2 is mediated  
13 by the enhanced binding of the Sp1/ER $\alpha$  complex to the GC-rich Sp1 region and not to the half ERE  
14 site.

15           The control of  $\alpha_5$  expression by E2 may play an important role in regulating the different  
16 biological processes that are dependent on  $\alpha_5\beta_1$  integrin-Fn interactions in ER $\alpha$  positive cells, such as  
17 the mechanisms that cells use to detach from and migrate out of the Fn-rich stroma in which they  
18 mature.

19           Generally,  $\alpha_5\beta_1$  integrin expression is also related to the maintenance of the differentiated and  
20 stationary status of the cells, so we may argue how the expression of  $\alpha_5$  integrin subunit could  
21 counteract E2/ER $\alpha$  capability to enhance “*per se*” breast cancer cell migration stimulating the  
22 reversible epithelial/mesenchymal-like transition [38]. Indeed the loss of  $\alpha_5\beta_1$  integrin expression is

1 concomitant with a dramatic increase of both breast cancer cell motility and invasion upon E2  
2 exposure.

3 Our data are apparently in contrast to recent data demonstrating how  $\alpha_4\beta_1$  integrin antagonizes  
4  $\alpha_5\beta_1$  integrin mediated cell invasion in both normal and cancerous mammary cells [24, 42]. The  
5 authors reported that activation of  $\alpha_5\beta_1$  integrin signals increases cell invasion on type I collagen  
6 matrix by up-regulating interstitial collagenase matrix metalloproteinase-1 expression. Since the  
7 substratum used in our cell invasion experiments is Fn, it is not surprising that the receptors involved in  
8 mediating cell invasion are distinct, and that the increased  $\alpha_5\beta_1$  integrin expression enhances cell  
9 adhesion on Fn coated membrane.

10 Here we demonstrate that in MCF-7 cells, not expressing  $\alpha_4\beta_1$  integrin, E2 exposure induces a  
11 strong increase of  $\alpha_5$  integrin expression concomitantly with a reduced cell migration as well as cell  
12 invasion on Fn. The increased expression of  $\alpha_5\beta_1$  integrin in response to E2 treatment appears to be  
13 consistent with previously reported observations demonstrating that an increased expression of  $\alpha_5\beta_1$   
14 integrin, such as in ERB-B2 positive breast cancer cells, is responsible of the long term survival of  
15 dormant micro-metastasis in bone marrow [50-51].

16 On the basis of all these observations, we may conclude that the up-regulatory effects induced  
17 by E2 on  $\alpha_5\beta_1$  integrin expression, may be crucially involved in the regulation of ER $\alpha$ -positive breast  
18 cancer cell motility and invasiveness.

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13 **Footnotes**

14 **Diego Sisci** and **Emilia Middea** contributed equally to this work.

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1 **Legends of figures**

2 **Figure 1 – Cell surface expression of  $\alpha_5$  integrin subunit in response to E2 treatment in MCF-7**

3 **cells.**  $\alpha_5$  integrin subunit expression was determined by Fluorescence activated cell sorting analysis  
4 and western blotting. A) MCF-7 cells were serum starved for 24 h, treated with 10 nM E2 for 4, 12, and  
5 24 h, and detached with EDTA solution. Cells were re-suspended in PBS and incubated with anti-  
6  $\alpha_5$  integrin antibody followed by fluorescein-conjugated anti-rabbit IgG. Cells were analyzed in a  
7 FACS flow cytometer. The results indicate the percentage of positive cells compared to cells labeled  
8 with an isotype matched control antibody; the results are representative of three independent  
9 experiments and reported as mean $\pm$ SD. Statistical analysis was performed by Student's t-test (\* $p$ <0.05  
10 *vs.* untreated).

11 B) MCF-7 cells serum-starved for 24 h were treated or left untreated with E2 (0, 1, 10, and 100 nM) for  
12 24 h. Cells lysates were subjected to western blotting with an anti- $\alpha_5$  integrin. GAPDH expression was  
13 evaluated as loading control.

14 The graph represents the mean $\pm$ SD of the optical density relative increase of  $\alpha_5$  integrin subunit levels  
15 with respect to untreated samples that were arbitrarily set at 1. The results are representative of three  
16 independent experiments. Statistical analysis was performed by Student's t-test (\* $p$ <0.05 *vs.* untreated).

17 C) Serum-starved MCF-7 cells (i) were incubated or not with E2 (0, 10, and 100 nM) for 24 h, and  
18 detached with EDTA solution. Cells were re-suspended in PBS and incubated with either an anti- $\alpha_5$   
19 (white bar) or an anti- $\alpha_4$  (grey bar) integrin antibody followed by fluorescein-conjugated anti-rabbit or  
20 anti-mouse IgG respectively. Cells were analyzed in a FACS flow cytometer. MCF-10 cells (ii) were  
21 used as control. The results indicate the percentage of positive cells compared to cells labeled with an  
22 isotype matched control antibody; the results are representative of three independent experiments and  
23 reported as mean $\pm$ SD. Statistical analysis was performed by Student's t-test (\* $p$ <0.05 *vs.* untreated).

1 **Figure 2 – 17 $\beta$ -estradiol increases the expression of the  $\alpha_5$  integrin subunit in MCF-7 cells.** MCF-  
2 7 cells serum-starved for 24 h were treated or left untreated with 10 nM E2 for 16, 24, and 48 h (A) and  
3 with 1, 10 and 100 nM of E2 and/or 1  $\mu$ M ICI 182,780 (C). Cells were lysed after incubation, and  
4 proteins were subjected to western blotting with an anti-integrin  $\alpha_5$  antibody or an anti-GAPDH as  
5 loading control. The results are representative of three independent experiments. The graph represents  
6 the mean $\pm$ SD of the optical density of  $\alpha_5$  integrin subunit normalized versus GAPDH and represented  
7 as relative increase with respect to untreated samples that were arbitrarily set at 1. Data were  
8 statistically analyzed by Student's t-test (\* p<0.05 vs untreated).

9 MCF-7 cells were treated for 24 and 48 h with 10 nM E2 (B) or with 1, 10 and 100 nM E2 and/or 1  
10 mM ICI 182,780 (D) and total RNA was extracted. The abundance of  $\alpha_5$  integrin subunit mRNA was  
11 detected by RT-PCR, as described in *Materials and Methods*. 36B4 mRNA levels were determined as  
12 control. The results are representative of three independent experiments. The graph represents the  
13 mean $\pm$ SD of the optical density of  $\alpha_5$  integrin subunit normalized versus 36B4 and reported as relative  
14 increase with respect to untreated samples that were arbitrarily set at 1. Data were statistically analyzed  
15 by Student's t-test (\* p<0.05 vs untreated).

16 E) HeLa cells were transfected with increasing amount of pSG5-HeG0 (ER $\alpha$ : 1  $\mu$ g and 2 $\mu$ g) or pSG5  
17 empty vector (Vector). Transfected cells were serum starved for 24 h and consecutively treated with E2  
18 (10 nM).  $\alpha_5$  integrin and ER $\alpha$  expression was detected by WB. The graph represents the mean $\pm$ SD of  
19 the optical density relative increase of  $\alpha_5$  integrin subunit levels with respect to untreated samples that  
20 were arbitrarily set at 1. The results are representative of three independent experiments. Data were  
21 statistically analyzed by Student's t-test (\* p<0.05 vs untreated).

22 **Figure 3 – The 5'-flanking region of  $\alpha_5$  gene acts as promoter and is activated by E2/ER $\alpha$ .** The  
23 transcriptional activity of ER $\alpha$  on the  $\alpha_5$  integrin subunit gene was evaluated using luciferase reporter

1 system, as described in *Materials and Methods*. MCF-7 cells (A), and HeLa cells (B) were transfected  
2 with a DNA mixtures containing 0,5 $\mu$ g pGL3-p $\alpha$ 5 reporter plasmid (detailed in *Materials and*  
3 *Methods*) and 50ng of pRL-Tk. Transfected cells were serum starved for 24 h and consecutively treated  
4 with E2 (10 nM) and/or ICI (1  $\mu$ M) for 24 h. C) HeLa cells transfected with 0,2 $\mu$ g pSG5-HeG0, 0,3 $\mu$ g  
5 pGL3-p $\alpha$ 5, and 50ng of pRL-Tk. D) HeLa cells were transfected with 0,2 $\mu$ g pCMV-hE241G, 0,3 $\mu$ g  
6 pGL3-p $\alpha$ 5, and 50ng of pRL-Tk. Firefly luciferase activity was internally normalized to Renilla  
7 luciferase activity and expressed as fold of increase with respect to untreated samples. Data were  
8 statistically analyzed by Student's t-test (\* p<0.05 vs control). The results are expressed as mean $\pm$ SD  
9 from four independent experiments

10 **Figure 4 – ER $\alpha$  is recruited to the 5'-flanking region of  $\alpha$ 5 gene.** Chromatin immunoprecipitations  
11 were carried out on serum-starved MCF-7 cells as described in *Materials and Methods*. Cells were  
12 treated or left untreated with E2 (10 nM) for 45 minutes, lysed and DNA-associated proteins were  
13 precipitated using either anti-ER $\alpha$  Ab, or anti-Sp1 Ab, or anti-Pol II Ab. The 5'-flanking sequence of  
14  $\alpha$ 5 gene containing both Sp1 and half-ERE sites was detected by PCR with specific primers listed in  
15 *Materials and Methods*. Inputs DNA were amplified as loading controls.

16 **Figure 5 – The Sp1/ERE half-sites contained in the 5'-flanking region of the  $\alpha$ 5 gene is**  
17 **responsible of E2/ER $\alpha$  activation.** A specific deletion (-420/-370) in the 5'-flanking region of the  $\alpha$ 5  
18 gene was obtained as described in *Materials and Methods* (A). MCF-7 cells were transfected with a  
19 DNA mixtures containing 0.5  $\mu$ g pGL3-p $\Delta$  $\alpha$ 5 reporter plasmid (B), 0,5 $\mu$ g pGL3-Sp1/ERE (C), and  
20 50ng of pRL-Tk. Transfected cells were serum starved for 24 h and then treated with E2 (10 nM)  
21 and/or ICI (1  $\mu$ M) for 24 h. Firefly luciferase activity was internally normalized to Renilla luciferase  
22 activity and expressed as fold of increase with respect to untreated samples. Results represent the

1 mean±SD from several experiments. Data were statistically analyzed by Student's t-test (\* p<0.05 vs  
2 control).

3 **Figure 6 - E2 enhances the binding of Sp1 to the 5'-flanking region of the  $\alpha_5$  gene containing the**  
4 **Sp1/half-ERE binding sites.**

5 A) Nuclear extracts from MCF-7 cells treated for 24 h with E2 10 nM (lanes 3 to 5) or untreated (lane 1  
6 and 2) were incubated with [ $\gamma$ -<sup>32</sup>]P-labeled oligonucleotides containing the Sp1 and the half-ERE  
7 binding sites (reported in *Materials and Methods*) and subjected to electrophoresis in a 6%  
8 polyacrylamide gel. The specificity of the binding was tested by adding to the reaction mixture either  
9 anti-ER $\alpha$  (lane 4) or anti-Sp1 antibodies (lane 5). Competition experiments were performed by adding  
10 a 100-fold molar excess of unlabeled probe (lane 2). Lane 6 contains the probe.

11 B), The [ $\gamma$ -<sup>32</sup>]P-labeled oligonucleotide (lane 1), described above, was incubated with either purified  
12 Sp1 protein (15 ng) (lane 2 to 4) or purified ER $\alpha$  protein (0,18 pmol) (lane 5 to 7). The presence of  
13 shifted complexes was also evaluated by incubating Sp1 (10 ng) with different amounts of the  
14 recombinant ER $\alpha$  (0; 0,18; 0,36 and 0,54 pmol). Competition experiments were performed by adding a  
15 100-fold molar excess of either unlabeled probe (lane 3, 6, and 11 to 13) or the probe mutated in the  
16 half-ERE site (reported in *Materials and Methods*) (lane 4, 7).

17 **Figure 7 - Sp1 is required for the expression of  $\alpha_5$  gene in response to E2.** MCF-7 cells (A) were  
18 transfected with DNA mixtures containing 0,5  $\mu$ g pGL3-p $\alpha_5$  reporter plasmid and 50 ng of pRL-Tk.  
19 Transfected cells were serum starved for 24 h and consecutively treated with E2 (10 nM) for 24 h  
20 and/or pre-treated with Mithramycin A (100  $\mu$ M). Firefly luciferase activity was internally normalized  
21 to Renilla luciferase activity and expressed as fold of increase with respect to untreated samples. Data  
22 were statistically analyzed by Student's t-test (\* p<0.05). The results are expressed as mean±SD. from  
23 several independent experiments.

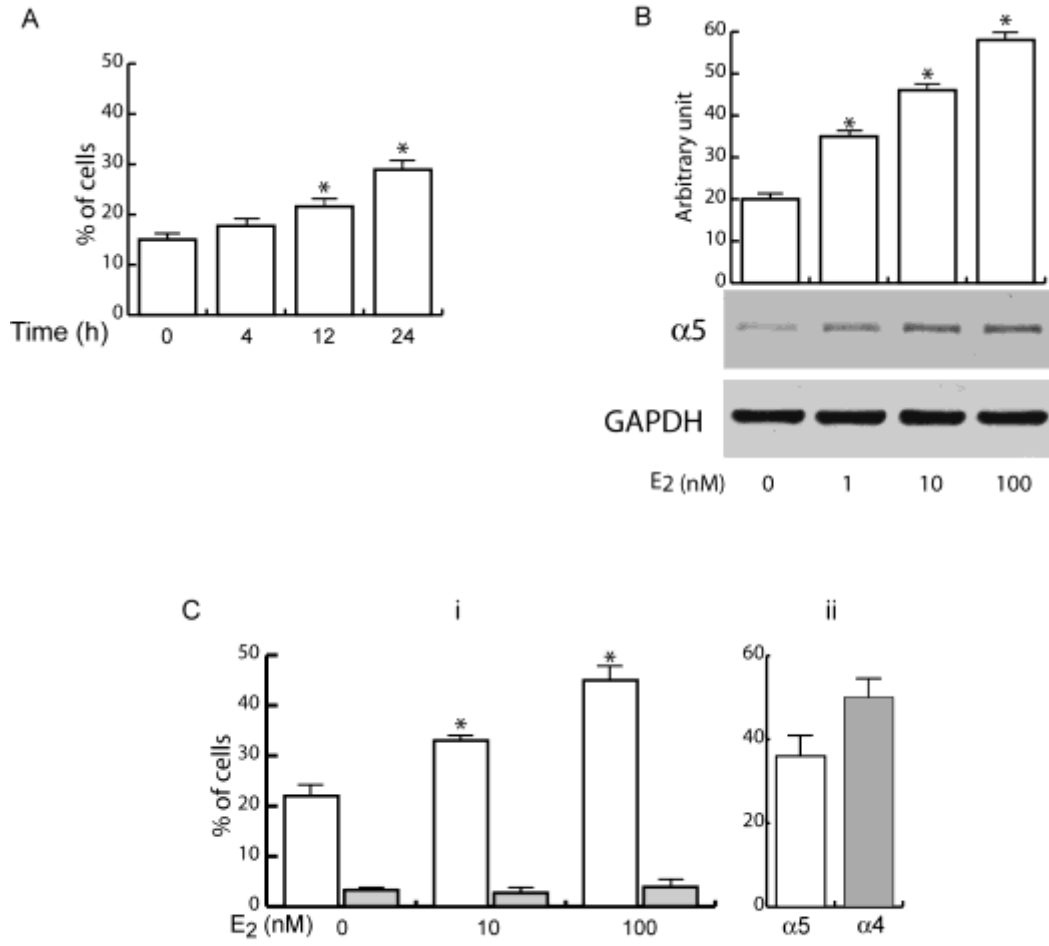
1 B) Nuclear extracts from E2-treated and untreated MCF-7 cells for 24 h were incubated with either a  
2 biotinated oligonucleotide containing the Sp1 and the half-ERE binding sites or a biotinated  
3 oligonucleotide mutated in the Sp1 consensus site and subjected to DNA affinity precipitation assay as  
4 described in Materials and Methods. The specificity of the binding was tested by loading the unbound  
5 fraction (C-). E2 treated MCF-7 cells lysate was used as positive control (C+). The results are  
6 representative of three independent experiments.

7 C) MCF-7 cells were transfected with DNA mixtures containing 0,5  $\mu$ g pGL3-p $\alpha$ 5 reporter plasmid, or  
8 the same plasmid mutated in the Sp1 site located at -404/-399 (detailed in *Materials and Methods*) and  
9 50ng of pRL-Tk. Transfected cells were serum starved for 24 h and consecutively treated with E2 (10  
10 nM) for 24 h. Firefly luciferase activity was internally normalized to Renilla luciferase activity and  
11 expressed as fold of increase with respect to untreated samples. Data were statistically analyzed by  
12 Student's t-test (\*  $p < 0.05$  vs control). The results are expressed as mean $\pm$ SD. from several independent  
13 experiments.

14 **Figure 8 –  $\alpha_5\beta_1$  integrin expression inhibits E2-mediated cell migration and invasion.** MCF-7 cells  
15 were transfected in suspension with 100 pmol siRNAs/well (si $\alpha_5\beta_1$  integrin or a scrambled siRNA for  
16 control samples), plated, starved for 24 h and treated with E2 (10 nM) for additional 24 h. Cytoplasmic  
17 protein extracts (A) (25  $\mu$ g/lane) were analyzed by WB to evaluate  $\alpha_5\beta_1$  integrin content. GAPDH was  
18 evaluated as loading control. The graph represents the mean $\pm$ SD of the optical density of  $\alpha_5$  integrin  
19 subunit normalized versus GAPDH. MCF-7 cells, transfected and treated as before, were detached,  
20 plated and allowed to migrate for 16 hrs on porous membranes coated (C) or not (B) with 30  $\mu$ g/ml  
21 Fibronectin (Fn) to evaluate the effects of Estradiol (10 nM) exposure on cell motility (B) and invasion  
22 (C). Cells were then fixed, stained and the cells migrated to the lower surfaces of the membranes were  
23 counted. The results represent the mean $\pm$ S.D. of three independent experiments. Data were statistically  
24 analyzed by Student's t test (\*  $p < 0.05$  vs untreated; ■  $p < 0.05$  vs the corresponding treatment in

1 scrambled).

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Figure 1

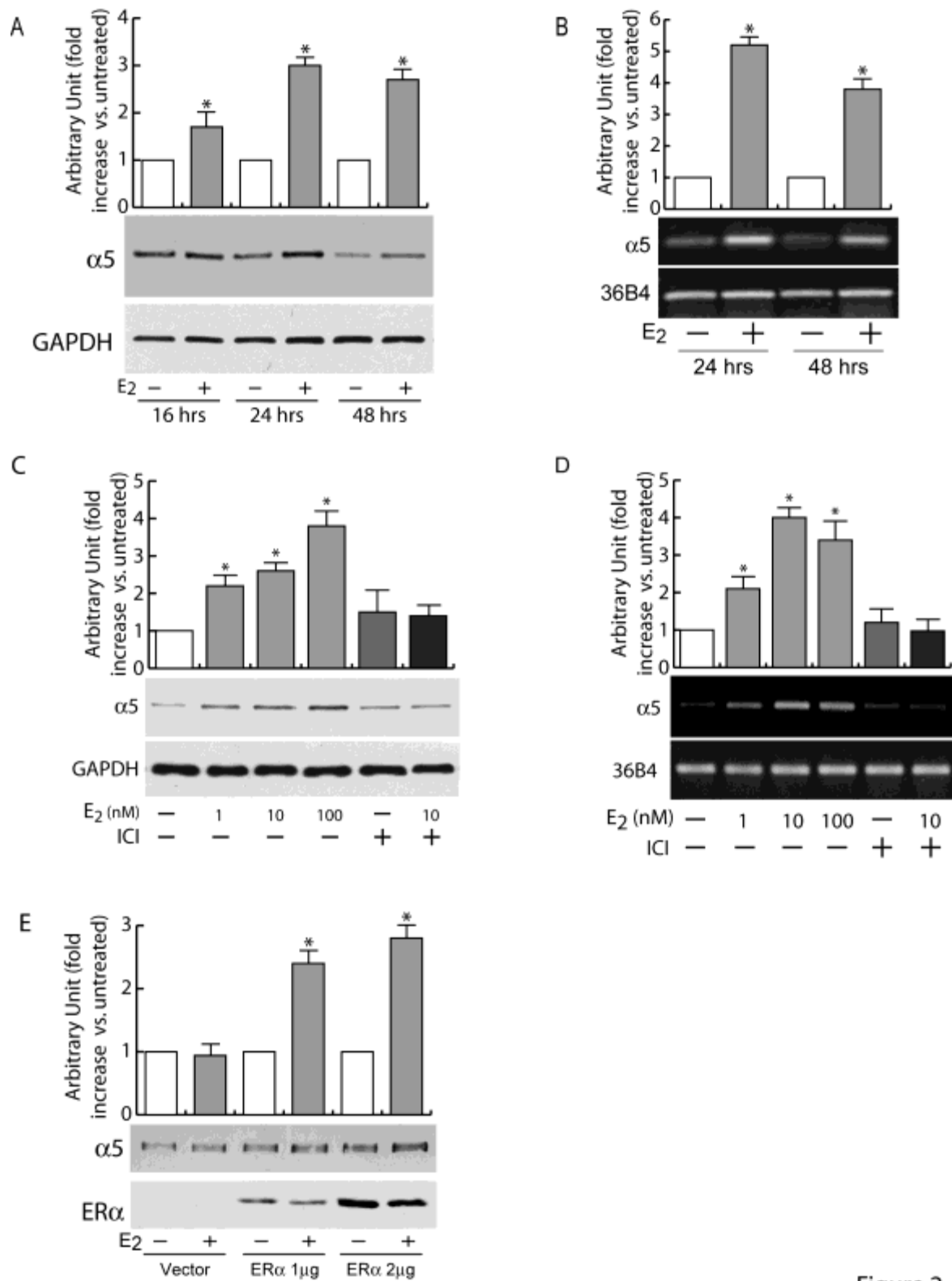


Figure 2

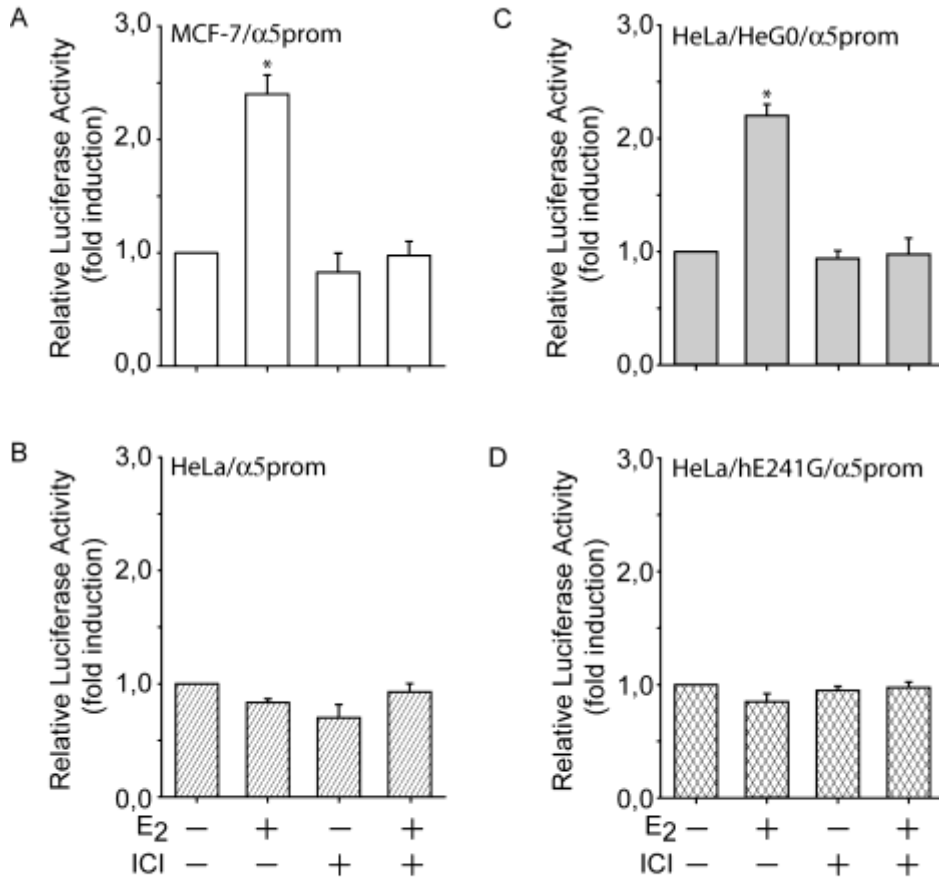


Figure 3

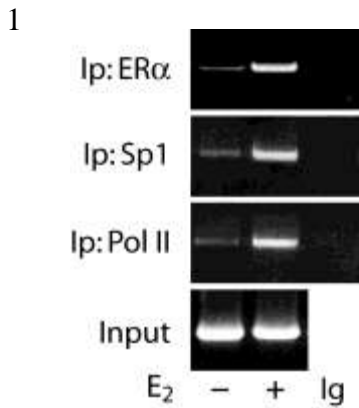


Figure 4

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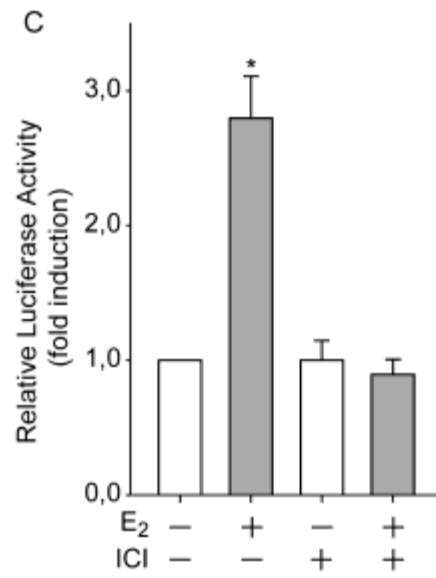
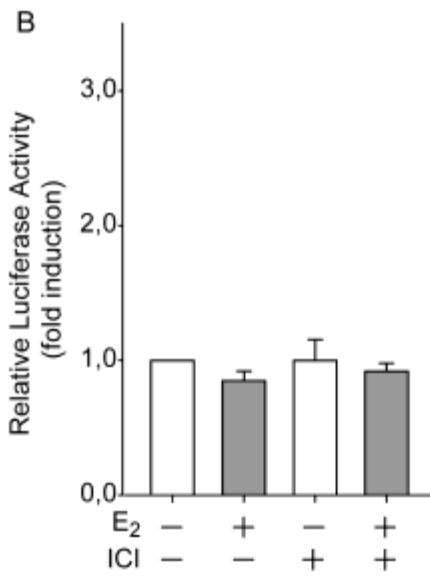
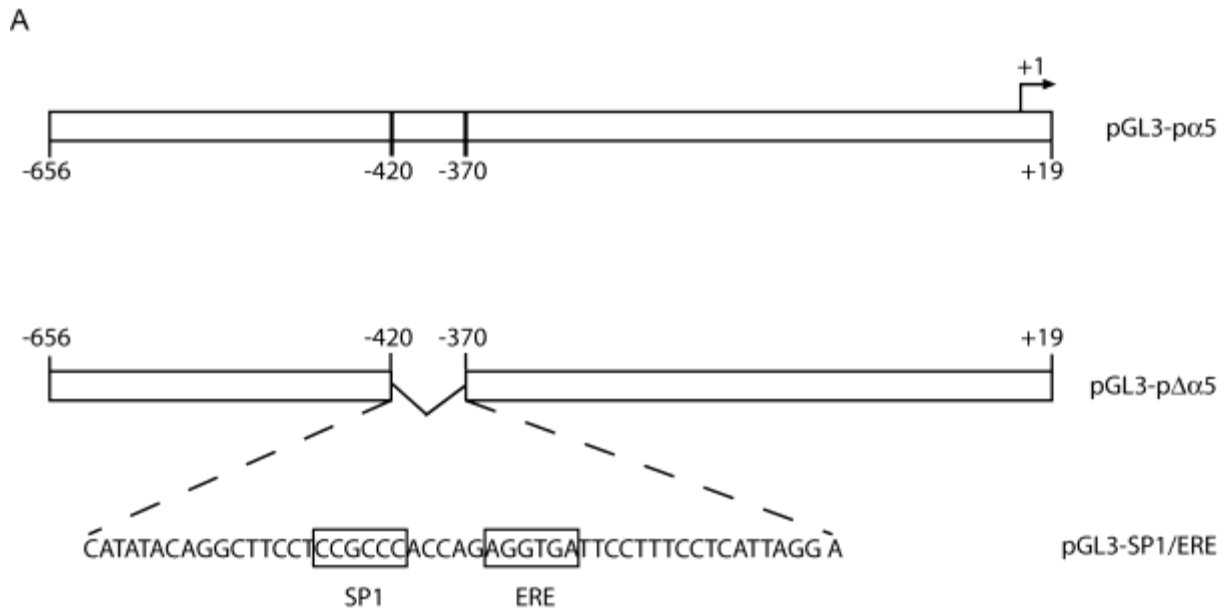
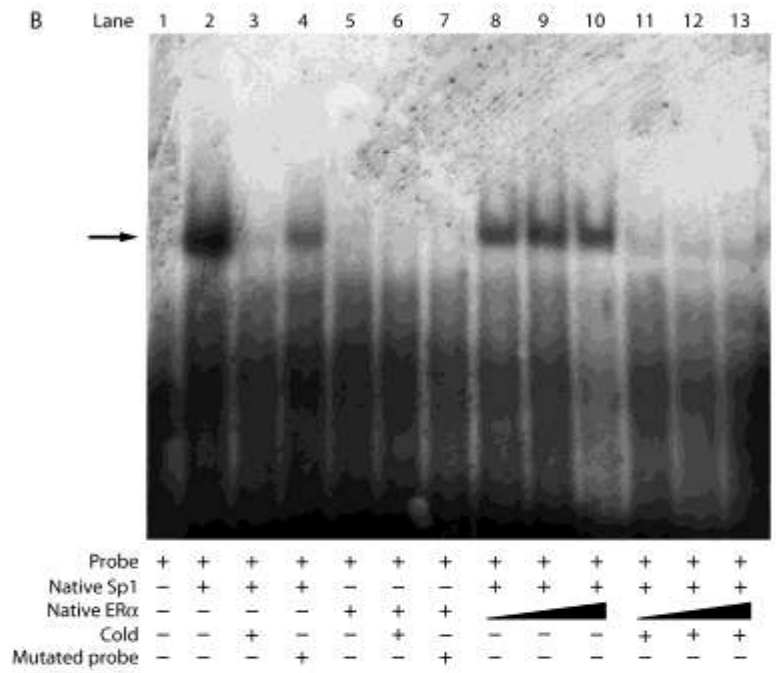
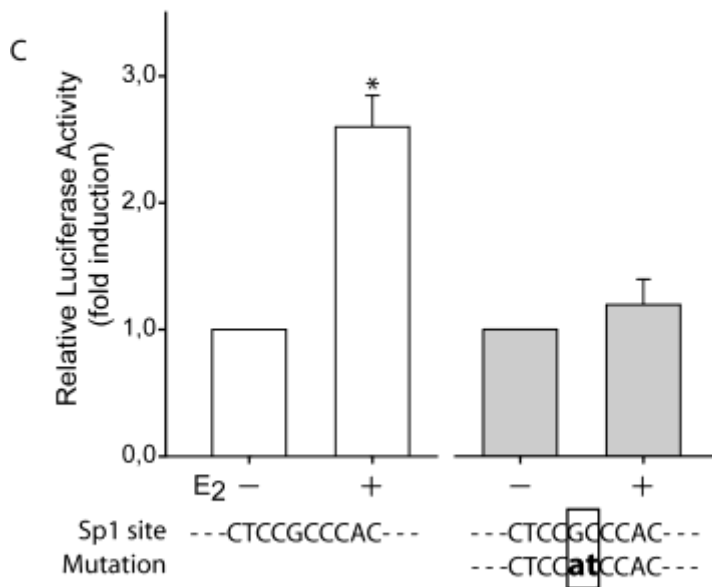
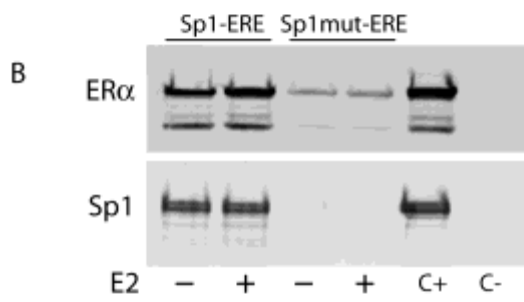
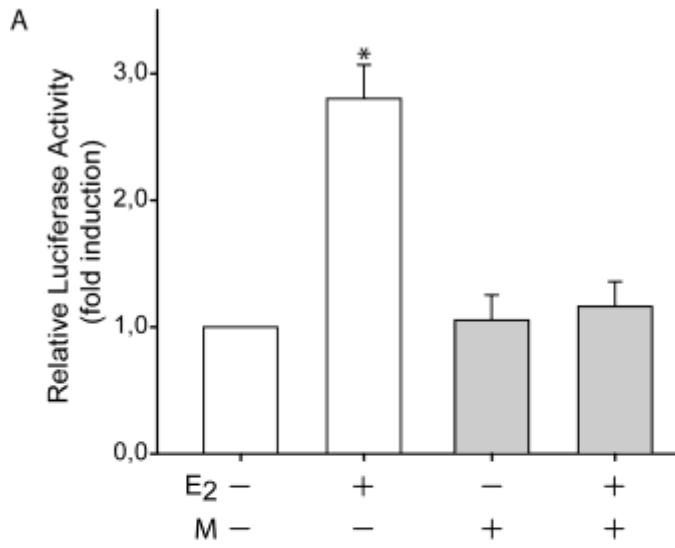


Figure 5



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Figure 6



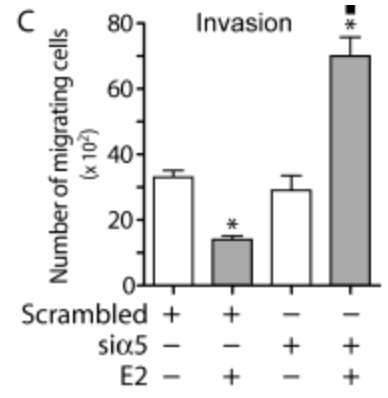
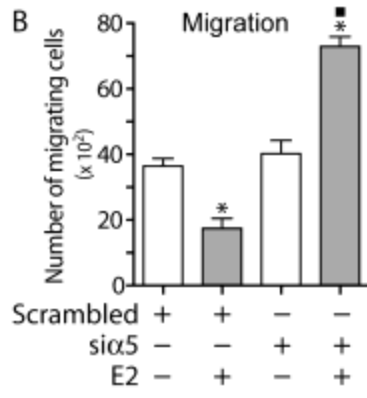
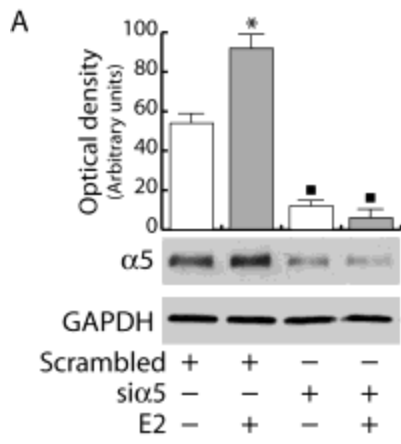


Figure 8