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Coxsackie and Adenovirus Receptor is target and a mediator of estrogen action in breast cancer

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Short title: *CAR* is an estrogen receptor target

Keywords: estrogen receptor, breast cancer, Coxsackie and Adenovirus Receptor

Abbreviations: *CAR*: Coxsackie and Adenovirus Receptor, *ER*: estrogen receptor, E2: 17 β -
estradiol

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31 **ABSTRACT**

32 The involvement of the Coxsackie and Adenovirus Receptor (*CAR*), an adhesion molecule
33 known to be the main determinant of adenovirus transduction of the cells, in cancer is currently
34 under investigation. Recent reports suggest that *CAR* levels are elevated in breast cancer and this
35 may have an impact on its use as means of delivery for gene therapy. In the present study, we show
36 that estradiol treatment of the estrogen-receptor (*ER*) positive breast cancer cell MCF-7 increases
37 *CAR* levels, and in turn, enhances adenoviral transduction. Employing the transfection of *CAR*
38 promoters in breast cancer cells, we show that this regulation of *CAR* expression occurs at the
39 transcriptional level. Additionally, and by chromatin immunoprecipitation (ChIP), we have
40 identified a crucial region of *CAR* promoter that controls E2-responsiveness of *CAR* gene through
41 the recruitment of *ER*. Moreover, utilizing *CAR* antibodies or *CAR* silencing by RNA interference
42 repressed the estrogen-dependent growth of breast cancer cells, whereas the stable expression of
43 *CAR* in MCF-7 or MDA-MB-231 cells led to an increased proliferation. Altogether, our data
44 suggest that *CAR* is a novel estrogen-responsive gene, which is involved in the E2-dependent
45 proliferation of breast cancer cells.

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51 INTRODUCTION

52

53 The coxsackie and adenovirus receptor (*CAR*) is a transmembrane protein that was initially
54 characterized by its ability to allow adenovirus attachment through cells via the interaction with
55 the adenovirus fiber-knob protein (Bergelson et al., 1997; Glasgow et al., 2006; Lucas et al., 2003).
56 Splice variants of *CAR* have also been identified, which could generate soluble forms of the *CAR*
57 protein that lack a transmembrane domain (exon 6) (Thoelen et al., 2001). In normal cells, *CAR* is
58 associated with tight junctions protein complexes (Cohen et al., 2001). *CAR* has been recently
59 suspected to be involved in cancer development, but its role remains controversial. Several studies
60 have shown that *CAR* expression was reduced in cancer tissues compared to normal tissues in the
61 case of bladder, renal and prostate cancers (Haviv et al., 2002; Li et al., 1999; Matsumoto et al.,
62 2005; Okegawa et al., 2000; Okegawa et al., 2001; Sachs et al., 2002), whereas in the breast, *CAR*
63 expression is increased in tumors (Martin et al., 2005). The loss of *CAR* expression is also
64 associated with increased metastasis of bladder tumors (Matsumoto et al., 2005).

65 So far, very little is known about *CAR* expression in breast cancer biopsy, and the situation
66 appears to be significantly different from other cancers. One study showed that *CAR* levels are
67 elevated in breast cancer, and increase during progression and metastasis (Martin et al., 2005). In
68 addition, *CAR* expression is correlated to estrogen receptor expression in breast cancer cells (Auer
69 et al., 2009). Estrogens are potent mitogens in cancerous breast tissue (Henderson et al., 1988;
70 Katzenellenbogen et al., 2000; Lazennec et al., 1999). Studies in breast cancer tissue-both *in vivo*
71 and *in vitro*, have shown that estrogen dramatically escalates proliferative and metastatic activity
72 in these tumor cells. The growth of approximately 70% of all human breast cancers is dependent
73 upon the presence of an estradiol-estrogen receptor (*ER*) complex (Santen et al., 1990). The
74 genomic action of estrogen is mediated by two estrogen receptors, namely *ER* α and *ER* β
75 (*Katzenellenbogen et al., 2000*). *ER* α is mainly involved in the mitogenic action of estrogens,

76 whereas *ER* β is rather anti-proliferative both in breast, prostate and ovarian cancer (Bardin et al.,
77 2004; Cheng et al., 2004; Lazennec, 2006; Lazennec et al., 2001).

78 The aim of this study was to analyze the link between *CAR* and estrogen signaling in breast
79 cancer, and to determine the possible role of *CAR* in breast cancer and the mechanisms of
80 regulation of *CAR* by estrogens. We report that the treatment of *ER*-positive breast cancer cells
81 leads to an increased transduction by adenovirus, which is concomitant with an induction of *CAR*
82 mRNA and protein levels. The analysis of the *CAR* promoter revealed that *CAR* regulation by
83 estrogens occurs at the transcriptional level, through an Estrogen-responsive element (ERE). Very
84 interestingly, the blockage of *CAR* action was sufficient to reduce the estrogen-dependent
85 proliferation of breast cancer cells, whereas the ectopic expression of *CAR* could increase the
86 proliferation of breast cancer cells.

87 MATERIALS AND METHODS

88

89 **Cell Culture.** MCF-7 and MDA-MB-231 cells were maintained in DMEM-F12, and supplemented
90 with 10% fetal calf serum (FCS) and gentamycin as previously described (Lazennec et al., 1996).
91 For infection assays, cells were seeded on 6-well plates. Before any experiment, cells were weaned
92 off steroids by being cultured in phenol red-free DMEM/F12 that had been supplemented with
93 10% CDFCS (charcoal dextran-treated FCS) for 5 days.

94

95 **RNA Extraction and Reverse Transcriptase PCR.** Total RNA was isolated with TRIzol reagent
96 (Invitrogen) as described by the manufacturer. Reverse transcription was performed using random
97 primers and Superscript II enzyme (Invitrogen). Real-time PCR quantification was then performed
98 using a SYBR Green approach (Light Cycler; Roche), as previously described (Lucas et al., 2003).
99 For each sample, *CAR* or *pS2/TFF1* mRNA levels were normalized with *RS9* or *Rplp0* mRNA
100 levels (reference genes). The sequences of the oligonucleotides used were: *CAR* (transmembranar
101 *CAR*, exons 6/7, (Thoelen et al., 2001)) (Left 5'- GCAGGAGCCATTATAGGAACTTTG -3';
102 Right 5'- GGACCCCAGGGATGAATGAT -3'), *pS2/TFF1* (Left 5'-
103 ACCATGGAGAACAAGGTGA-3'; Right 5'-CCGAGCTCTGGGACTAATCA-3'), *RS9* (Left 5'-
104 CAGGCGCAGACGGTGGGAAGC- 3';; Right 5'- CGCGAGCGTGGTGGATGGAC-3'), *Rplp0*
105 (Left 5'-AAYGTGGGCTCCAAGCAGATG-3'; Right 5'-GAGATGTTCAGCATGTTCAGCAG-
106 3').

107

108 **Recombinant adenovirus construction, propagation and infection.** The adenoviruses encoding
109 the β -galactosidase (Ad-GAL) or ER α (Ad-ER α) used in this study have been previously
110 described (Lazennec et al., 1999; Lazennec et al., 2001; Lucas et al., 2003). Briefly, the complete
111 coding sequences of β -galactosidase, or human ER α cDNAs, were subcloned in a pACsk12CMV5

112 shuttle vector. To obtain recombinant viruses, permissive HEK-293 cells (human embryonic
113 kidney cells) were cotransfected with pACsk12CMV5, or the recombinant pACsk12CMV5
114 plasmid, and with pJM17 which contains the remainder of the adenoviral genome. *In vivo*
115 recombination of the plasmids generates infectious viral particles, non-recombinant adenovirus
116 Ad5, or recombinant adenovirus Ad-GAL and Ad-ER α . MCF-7 or MDA-MB-231 cells were
117 infected overnight at different multiplicities of infection (MOI) in DMEM/F12 10% CDFCS. The
118 next day, the medium was changed and the cells were let to express β -GAL for 48h before
119 collecting the medium.

120
121 **β -Galactosidase histochemical staining assay.** After 48h of expression, infected cells were
122 washed twice with PBS, and then fixed for 5 min at 4°C in a fixing solution (2% formaldehyde,
123 0.2% glutaraldehyde, 1X PBS). Cells were washed once and incubated for 4h at 37°C in
124 histochemical staining solution (5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂, 1X PBS,
125 1mg/ml X-GAL) (Lucas et al., 2003). Cells were then washed twice with PBS and observed.

126 127 **Western blot experiments**

128 Cells were harvested in Tris-glycerol buffer (Tris-HCl 50mM, EDTA 1.5mM, 10% glycerol)
129 supplemented with protease inhibitor cocktail (Roche), and were then sonicated. 30 μ g of protein
130 extracts were subjected to SDS-PAGE protein samples under non-reducing conditions. Samples
131 were heated normally at 95°C for 5 minutes in a sample loading buffer that lacked mercaptoethanol.
132 Western blot analyses were done using CAR antibody (sc-56892, Santa Cruz), and β -actin (Sigma-
133 Aldrich). Immunoreactivity was detected with Amersham ECL system. Actin was used as a loading
134 control.

135

136 **Constructs and Transient transfection.** A *CAR* promoter corresponding to sequences -1213/-127
137 (pGL3-1087) has been previously described (Pong et al., 2003). Deleted constructs of the *CAR*
138 promoter (pGL3-522: -648/-127; pGL3-428: -554/-127) were cloned into pGL3-basic vectors
139 (Promega). Site directed mutagenesis of *CAR* ERE was performed with *CAR*-EREm primers
140 (TCGCATCCCGTGgGCATGtcGTCAGAGAACCTGCC) to generate pGL3-EREm construct.
141 The ERE-TK-LUC construct consists of two ERE in tandem upstream of TK promoters (Duong et
142 al., 2006). $3 \cdot 10^5$ of steroid-weaned cells were plated in 12-well plates in phenol red-free DMEM-
143 F12, and supplemented with 10% CDFCS 24 h before transfection. Transfections were performed
144 using lipofectamine according to the manufacturer's recommendations, using 2 μ g of the *CAR*
145 promoter pGL3-1087, luciferase reporter, or *CAR* promoted deleted constructs along 0.5 μ g of the
146 internal reference reporter plasmid (CMV-Gal) per well. After 6 h incubation, the medium was
147 removed and the cells were placed into a fresh medium supplemented with a control vehicle
148 (ethanol) or E2. Twenty-four hours later, cells were harvested and assayed for luciferase activity
149 using a Centro LB960 Berthold luminometer. β -galactosidase was determined as previously
150 described (Duong et al., 2006).

151

152 **siRNA experiments**

153 *CAR* protein was knocked down by transfection of MCF-7 cells with specific siRNA
154 (GGAAGUUCAUCACGAUUAUC, Eurofins) according to manufacturer's protocol. Non
155 targeting control siRNA (siGLO red) was purchased from Dharmacon. Protein levels were
156 determined by western blot.

157

158 ***CAR* stable transfectants**

159 The stably transfected MCF-7-*CAR* and MDA-MB-231-*CAR* cell lines were obtained after
160 transfection with the plasmid pcDNA3-h*CAR* (a kind gift of Dr S. Hemmi), encoding the human
161 *CAR* cDNA under the control of the CMV promoter. Transfected cells were then selected by G418

162 at a concentration of 2 mg/ml. After 2 weeks of selection, a pool of resistant cells was collected
163 and used for further experiments.

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166 Chromatin Immunoprecipitations (ChIPs).

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These assays were conducted with some modifications from Métivier et al. 2003 (Metivier et al., 2003), using 1.10^7 MCF-7 or MDA-MB-231 cells that were placed in DMEM/0.5% CDFCS for three days. Afterwards, some were treated with 10 nM E₂ four different times. Following cross-linking (1.5% formaldehyde for 10 min at room temperature), cells were collected in a 1 ml collection buffer (100 mM Tris-HCl (pH 9.4) and 100 mM DTT), incubated on ice for 10 min, and subsequently at 30°C for 10 min. Cells were then lysed in a 300 µl lysis buffer (10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 1% SDS, 0.5% Empigen BB (Sigma)). Extracts were then sonicated for 14 min using a BioRuptor apparatus (Diagenode), with 30 sec on/off cycles. Following 10 min centrifugation at 10,000 x g, 50 µl of the supernatants were used as inputs, and the remainder diluted 3-fold in IP buffer (2 mM EDTA, 100 mM NaCl, 20 mM Tris-HCl (pH 8.1), and 0.5% Triton X-100). Extracts were pre-cleared for 3 hr at 4°C with 150 µl of a 50% protein A-Sepharose bead (Amersham Pharmacia Biosciences) slurry containing 10 µg of yeast tRNA (Sigma). Following centrifugation at 800 x g for 1 min, a third of the extracts were taken for immunoprecipitation using 1 µg of antibodies directed against ER (HC20, Santa Cruz) or the epitope HA (HA-probe Y11, Santa Cruz), and diluted 3 times in a IP buffer. Complexes were recovered by a 3 hr incubation at 4°C with 50 µl of protein A-Sepharose lurry containing 10 µg of yeast tRNA. Precipitates were then serially washed, using 300 µl of Washing Buffers (WB) I (2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 0.1% SDS, 1% Triton X-100, 150 mM NaCl), WB II (2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 0.1% SDS, 1% Triton X-100, 500 mM NaCl), WB III (1 mM EDTA, 10 mM Tris-HCl (pH 8.1), 1% NP-40, 1% Deoxycholate, 0.25 M LiCl), and then twice with 1 mM EDTA, 10 mM Tris-HCl (pH 8.1). Precipitated complexes were removed from the beads through

188 three sequential incubations of 10 min with 50 μ l of 1% SDS, 0.1 M NaHCO₃. Cross-linking was
189 then reversed by an overnight incubation at 65°C. DNA was purified with Qiaquick columns
190 (Qiagen). Subsequent quantitative PCRs analysis used 1 μ l of input material and 5 μ l of ChIP
191 samples, and were performed on a BioRad MyiQ apparatus using BioRad iQ SYBR Green supermix.
192 The sequence of the oligonucleotides used, designed using Primer3 ([http://frodo.wi.mit.edu/cgi-](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)
193 [bin/primer3/primer3_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) are: *CAR* (Left 5'-CCTCTCGCGCTTTTATGTC-3'; Right 5'-
194 TCTCTGACCTCATGCTCACG-3'), *pS2/TFF1* (Left 5'-TCATCTTGGCTGAGGGATCT-3';
195 Right 5'-TTCCGGCCATCTCTCACTAT-3'), *Rplp0* (Left 5'-
196 ATCTAACTAGCACACGAACCTT-3'; Right 5'-CTGTATTCGTTTCAGCTTTGTCT-3'). The
197 PCR efficiency was calculated for each oligonucleotides set, using serial dilutions of inputs. Once
198 normalized to inputs following the Δ Ct method, the results were normalized again to the ChIP
199 performed using the control anti-HA antibody.

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201

202 **Proliferation assay.** MCF-7 cells which had been stably transfected with a CMV-LUC reporter
203 were grown in 24-well plates in the presence of 10⁻⁹ M estradiol for 4-days, and in the presence of
204 20 μ l of whole mouse serum or E1-1 anti-CAR hybridoma supernatant (Hemmi et al., 1998). Cells
205 were then collected and luciferase activity was measured. Concerning wild-type MCF-7, MCF-7-
206 *CAR*, MDA-MB-231 and MDA-MB-231-*CAR*, their proliferation was quantified by counting the
207 cells on a cell counter.

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210

211 **RESULTS**

212 **Estradiol increases the transduction of *ER*-positive breast cancer cells by adenovirus.**

213 The role and the regulation of *CAR* expression in breast cancer cells remain elusive. We
214 hypothesized that estrogens, which regulate the growth of *ER*-positive breast cancer cells, could
215 modify their sensitivity to adenoviral infection, through a regulation of *CAR* levels. To analyze
216 whether estrogens could modify the adenoviral transduction, we pre-treated estrogen-receptor
217 (*ER*)-positive breast cancer cells MCF-7 with estradiol (E2) for 24h before infecting them with an
218 adenovirus encoding β -galactosidase (Ad- β gal) at increasing MOIs. We observed that adenoviral
219 transduction was strongly enhanced in E2-treated cells as compared to control cells (Fig. 1A),
220 which suggests that E2 modifies the expression of a factor involved in adenoviral transduction.
221 Microscopic observation confirmed that the increased staining of the plates was the result of a
222 higher number of infected cells (Fig. 1B). On the other hand, *ER*-negative MDA-MB-231 cells
223 were similarly infected by the virus in the absence or the presence of E2, demonstrating that *ER*
224 was required for E2 regulation of adenoviral transduction (Fig. 1C).

226 ***CAR* levels are up-regulated by E2.**

227 As Coxsackie and Adenovirus Receptor (*CAR*) is the main determinant of adenovirus infection, we
228 hypothesized that its expression could be regulated by estrogens, which in turn, explains the effects
229 of E2 on adenoviral transduction. To determine if E2 was modulating *CAR* RNA levels, we treated
230 MCF-7 cells at different times with E2. We measured the levels of transmembranar *CAR* isoform
231 (see materials and methods), by real time PCR. We observed that *CAR* RNA expression was
232 induced at 8h of E2 treatment and returned to basal levels after 24h (Fig. 2A). To ensure that the
233 regulation of *CAR* RNA levels by E2 were mediated by the estrogen receptor, MCF-7 cells were
234 cotreated for 8 h with E2, and the pure anti-estrogen ICI_{182,780}, which could strongly reduce *CAR*
235 RNA induction by E2 (Fig. 2A). *CAR* levels were also induced in another ER-positive breast

236 cancer cell line, CAMA-1 (supplemental Fig. 2), suggesting that this was not specific of MCF-7
237 cells. In addition, *CAR* protein levels were also increased upon E2 treatment, as shown by Western
238 blot, but this induction by E2 returned to basal levels only at 48h (Fig. 2B). We performed the
239 same analysis in the *ER*-negative breast cancer cell line MDA-MB-231 (Fig. 2C), and observed
240 that *CAR* protein levels were not significantly affected by E2 in these cells. This further
241 demonstrates that *ER* is necessary for *CAR* induction by E2.

242

243 ***CAR* gene promoter is regulated by estrogens.**

244 Next, we investigated the possible regulation of *CAR* gene promoters by estrogens in MCF-7 cells.
245 The transfection of *CAR* promoter reporter gene construct showed that it was induced 1.8-fold by
246 estradiol in MCF-7 cells (Fig. 3A). On the contrary, in the *ER*-negative breast cancer cell MDA-
247 MB-231, the *CAR* promoter was not induced by estradiol (Fig. 3B), demonstrating that *CAR* is
248 required for the regulation of *CAR* promoter by estrogens. We performed a computer analysis of
249 the *CAR* promoter, and we identified 4 half-ERE (estrogen responsive element, *ER* binding site),
250 and one putative ERE (-571/-557) based on consensus ERE sequence (Gruber et al., 2004; O'Lone
251 et al., 2004) (Fig. 4A). We used first deletion constructs of the *CAR* promoter (Fig. 4B). pGL3-522
252 (-648/-127) construct retained E2-inducibility, but the further deletion of 94 bp (pGL3-428, -554/-
253 127) by removing the putative ERE was sufficient to lose the responsiveness to E2 (Fig. 4B). By
254 using a point mutation construct of the ERE, we observed a strong reduction of *CAR* promoter
255 induction by E2 as well (Fig. 4B), reinforcing the central role of this ERE sequence in modulating
256 *CAR* promoter activity. To validate the functionality of *CAR* ERE, we performed Chromatin
257 ImmunoPrecipitation (ChIPs) experiments. These experiments were performed in MCF-7 cells,
258 which exhibit an estrogenic stimulation of *CAR* and *pS2/TFF1* genes (Fig. 4C). ChIPs showed an
259 early recruitment of ER α to *CAR* ERE sequence in 30 min (Fig. 4D). The kinetic of recruitment of
260 ER α to *CAR* promoter was more rapid than the one observed for the *pS2/TFF1* promoter, which is
261 also in agreement with the distinct kinetics of estrogen-regulation of *CAR* and *pS2/TFF1* mRNA

262 (Fig. 4C, D). Finally, the ability of *ER* α to bind to *CAR* ERE sequence was also confirmed by gel
263 shift assays (Supplementary Fig. 1).

264

265 ***CAR* is involved in breast cancer proliferation**

266 As *CAR* expression is regulated by estrogens, we next hypothesized that *CAR* could be involved in
267 the proliferation of *ER*-positive cells. MCF-7 cells were cultured in the presence of estrogen and
268 treated either with control serum or anti-*CAR* antibody. Results showed that *CAR* antibody reduced
269 half the proliferation of cancer cells in the presence of E2 (Fig. 5A), which suggests that up-
270 regulation of *CAR* by estrogen is an important step in the control of estrogen-dependent growth of
271 *ER*-positive breast cancer cells. In the absence of E2, the *CAR* antibody did not significantly alter
272 cell proliferation. We used a second approach based on siRNA to demonstrate the role of *CAR* in
273 MCF-7 cell proliferation. Transfection of a siRNA against *CAR* inhibited the induction of
274 proliferation of MCF-7 cells by E2 (Fig. 5B). To further confirm the potential role of *CAR* in
275 breast cancer cell proliferation, we generated a pool of stable transfectant MCF-7 cells for *CAR*
276 (Fig. 5C). When comparing the MCF-7-*CAR* cells to wild-type MCF-7 cells, we observed that
277 MCF-7-*CAR* cells displayed a higher proliferation rate compared to wild-type MCF-7 cells, in the
278 absence and the presence of estradiol (Fig. 5D). Of particular note, MCF-7-*CAR* cells grown in the
279 absence of E2 had a similar proliferation as wild-type MCF-7 in the presence of E2. This data
280 confirms that *CAR* can enhance the proliferation of breast cancer cells. To demonstrate that *CAR*
281 effects were not specific of a single cell line, we also generated a *CAR* stable transfectant in MDA-
282 MB-231 cells (Fig. 5E). MDA-MB-231-*CAR* also displayed an enhanced proliferation compared to
283 wild-type cells (Fig. 5F), reinforcing the fact that *CAR* stimulates breast cancer cell proliferation.

284

285

286 **DISCUSSION**

287 Recent studies have highlighted the possible role of *CAR* in carcinogenesis. However, it remains
288 unclear whether *CAR* levels are modified in breast cancer cells, and what the signals controlling its
289 expression in such cells are. In this report, we have addressed the role of *CAR* in breast cancer and
290 its regulation by estrogens.

291 Whereas most of the studies have shown that *CAR* levels were decreased in the aggressive
292 forms of several cancers, including glioma and bladder cancer (Kim et al., 2003; Okegawa et al.,
293 2001); the breast cancer situation seems to be different. Indeed, it was shown, that *CAR* levels were
294 elevated in breast cancer tissues (Martin et al., 2005). Bruning et al. also observed in a murine
295 model of breast cancer that *CAR* expression was up-regulated in carcinoma compared to pre-
296 neoplastic lesion (Bruning et al., 2005). To date, the role of *CAR* in breast cancer and the factors
297 regulating *CAR* expression are poorly understood. Previous studies have shown that treatment of
298 cancer cells with histone deacetylase inhibitors upregulates *CAR* expression (Hemminki et al.,
299 2003; Kitazono et al., 2001; Okegawa et al., 2007; Pong et al., 2003). Other signals such as the
300 MAPK pathway, the action of glucocorticoids, TNF α and TGF- β also modulate *CAR* expression
301 (Anders et al., 2003; Bruning & Runnebaum, 2003). Pong et al. have also reported an induction of
302 *CAR* expression by the phytoestrogen genistein in bladder cells, but it remains unclear whether this
303 occurs through estrogen receptor (Pong et al., 2006).

304 *CAR* levels are one of the primary determinants of adenoviral transduction. We show here
305 that estradiol increases *ER*-positive breast cancer cell transduction by type 5 adenoviruses. We also
306 observed that *CAR* levels were increased by estradiol in estrogen-receptor positive breast cancer
307 cells, which is in agreement with a previous study, using the *ER*-positive breast cancer cell line
308 T47-D (Auer et al., 2009). This regulation was dependent on Estrogen receptors, as E2 had no
309 effect on *CAR* levels and promoter activity in *ER α -negative MDA-MB-231 breast cancer cells.
310 *CAR* expression was induced both at the RNA and protein levels by E2. However, *CAR* RNA*

311 induction by E2 was transient, whereas *CAR* protein induction could last for more than 24h. This
312 suggests that *CAR* RNA and protein kinetics of regulation and stability are different. It is possible
313 that *CAR* protein stability is affected indirectly by estradiol through the synthesis of factors
314 controlling its turn-over, which could explain the delayed induction of *CAR* protein expression.
315 Based on the rapid increase of *CAR* RNA levels, we thus speculated that the regulation of *CAR*
316 gene by estrogens could be the result of a direct transcriptional regulation. Transfection
317 experiments showed that *CAR* promoter was directly regulated by estrogens, through an ERE
318 located in the first 600 bp of *CAR* gene promoter as shown by transfection, ChIPs and gel shift
319 experiments. The ERE sequence found in *CAR* promoter was a palindrome non-consensus
320 sequence with a 3-bp spacing, which is the most common situation found in genes regulated by
321 estrogen (Gruber et al., 2004; O'Lone et al., 2004).

322 Estrogens are known to be potent mitogen signals for *ER*-positive breast cancer cells (Prall
323 et al., 1998), which could also potentially involve adhesion molecules such as *CAR*. It was
324 previously shown that estrogen can modulate the expression of other adhesion molecule such as *N-*
325 *cadherin*, which is upregulated by E2 (MacCalman et al., 1995), or *E-cadherin* that is down-
326 regulated by E2 (Oesterreich et al., 2003). These molecules might directly or indirectly modulate
327 cell proliferation, notably through cell architecture reorganization.

328 The possible role of *CAR* in cancer cell proliferation remains controversial. Most studies
329 have shown in bladder, cervical, ovary cancers, and glioma that *CAR* was repressing cell
330 proliferation (Bruning & Runnebaum, 2004; Huang et al., 2005; Kim et al., 2003; Okegawa et al.,
331 2001; Wang et al., 2005; Zhang et al., 2007), whereas others have shown mitogenic properties for
332 *CAR* in lung cancer (Qin et al., 2004; Veena et al., 2009). The situation in breast cancer might be
333 unique compared to other cancers. Indeed, it was shown that *CAR* antagonizes the action of
334 apoptosis inducing agents, and in turn increases breast cancer cell survival (Bruning et al., 2005).
335 In a similar manner, as for lung cancer in which *CAR* silencing reduces tumor growth (Veena et
336 al., 2009), we show that *CAR* antibodies or *CAR* siRNA inhibit the estrogen-dependent growth of

337 MCF-7 cells. We did not observe any sign of mortality when cells were treated with the *CAR*
338 antibody, which suggests that *CAR* affects mainly cell proliferation than survival. In addition, we
339 report that stable expression of *CAR* in MCF-7 cells enhances both the basal and estrogen induced-
340 proliferation of these cells. This observation suggests that *CAR* could confer a hormone-
341 independent growth to *ER*-positive breast cancer cells. Moreover, *CAR* can also increase the
342 proliferation of *ER* α -negative MDA-MB-231 cells, suggesting that *CAR* could affect in a similar
343 manner different types of breast cancer cells. The mechanisms underlying *CAR* effects on
344 proliferation in breast cancer cells remain to be investigated. We believe that cell-specific factors
345 present in breast cancer cells, and not in cancer cells of other origins, might explain why *CAR*
346 stimulates the proliferation in breast cancer cells whereas it represses the proliferation in other
347 cancers.

348 The effects of *CAR* on proliferation, which is mainly described as an adhesion molecule,
349 might not be direct in breast cancer cells. *CAR* is a major component of tight junctions (TJs), as are
350 other molecules such as occludin, claudin, tricellulin and junction adhesion molecules (JAMs)
351 (Coyne & Bergelson, 2005). In polarized epithelial cells, *CAR* is detected at the apical pole of the
352 lateral membrane, where it colocalizes with the TJ protein zonula occludens-1 (ZO-1) (Cohen et al.,
353 2001). Recent reports suggest that TJs could regulate cell proliferation (Tsukita et al., 2008). TJs
354 contain aqueous pores or channels which regulate paracellular permeability, and serve to divide the
355 apical and basolateral membrane compartments. As a component of TJs, *CAR* could affect ionic or
356 pH conditions around epithelial cells, and in turn modulate cell proliferation (Tsukita et al., 2008).
357 Another hypothesis is that altering the features of cell adhesion by *CAR* could modify cell signaling
358 and interaction with other cofactors that directly control the proliferation or cell architecture.
359 Increasing evidence suggest that TJ's regulate gene expression (Balda & Matter, 2009). It has been
360 shown that the cytoplasmic plaque associated with TJs is formed by multiple adaptors, scaffold
361 proteins, and signaling components such as GTP-binding proteins, protein kinases and
362 phosphatases. These proteins can in turn modulate the expression or the activity of downstream

363 signaling molecules such as cyclins, Jun/fos or c-myc, which are known to regulate cell cycle
364 (Balda & Matter, 2009). In particular, it might be possible that *CAR* could indirectly affect
365 phosphorylation levels of *ER*, which is a known mechanism for hormone-independent proliferation
366 of *ER*-positive breast cancer cells. Cell context might explain the opposite effects of *CAR* in breast
367 cancer cells compared to other types of cancers. We cannot exclude the possibility that the presence
368 of *ER* in breast cancer cells could enable a different network of factors susceptible to act in
369 coordination with *CAR* to elicit cell proliferation.

370 These results, together with the up-regulation of *CAR* by estradiol, suggest that *CAR* could
371 be one of the early events leading to the induced proliferation of breast cancer cells. Our data could
372 open the road for novel strategies to target *ER*-positive breast cancers.

373

374 **Declarations of interest**

375 There is no conflict of interest that could be perceived as prejudicing the impartiality of the
376 research reported.

377

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383

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387

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- 501
502

503 **LEGENDS TO FIGURES**

504

505 **Fig. 1. Estradiol increases adenovirus transduction.** **A.** *ER*-positive breast cancer cells, MCF-7
506 cells, were treated for 24 h with ethanol vehicle (C) or estradiol (E2; 10^{-9} M) before infection with
507 Ad-GAL virus at increasing multiplicity of infection (MOI = 0, 1, 5, 25, 75, 375). The infected
508 cells were then grown for 48 h with ethanol vehicle or estradiol (10^{-9} M), and stained for β -gal
509 activity as described in "Materials and Methods". **B.** MCF-7 cells infected with adenovirus at MOI
510 = 1 and treated with or without E2. Quantification of β -gal positive cells after adenovirus infection.
511 β -gal-positive cells were counted from 2 non overlapping fields. Results represent the mean \pm SD
512 of 3 independent experiments, and are expressed as the percentage of control cells. **C.** *ER* α -
513 negative MDA-MB-231 cells were infected with adenovirus under the same conditions as in **B.**
514 Results represent the quantification of β -gal positive cells after adenovirus infection (n=3).

515

516 **Fig. 2. *CAR* expression is induced by E2.** **A.** MCF-7 cells were treated with control vehicle
517 ethanol, E2 (10^{-9} M) for 8h or 24h or with E2 + ICI_{182,780} (ICI; 10^{-6} M) for 8h. *CAR* RNA levels
518 were determined by real-time PCR. Results represent the mean of 3 independent experiments and
519 are expressed as the percentage of control cells. **B.** The levels of *CAR* protein were measured by
520 Western blot with *CAR* antibody using extracts from MCF-7 cells treated with E2 (10^{-9} M) for 4, 8,
521 or 24h. β -actin levels were detected to demonstrate equal loading. **C.** Same experiment as in B.,
522 but in MDA-MB-231 cells.

523

524 **Fig. 3. *CAR* promoter activity is enhanced by E2 in *ER*-positive breast cancer cells.** **A.** MCF-7
525 cells were transfected with pGL3, pGL3-1087, or ERE2-TK constructs, along with CMV-GAL as
526 a control for transfection efficiency. Cells were grown in the presence or the absence of E2 (10^{-9}
527 M). Results show relative luciferase activities (% of values of pGL3 vector without E2) after
528 normalization with β -gal activity (3 independent experiments). ** $p < 0.001$ using Student's t-test.

529 **B.** MDA-MB-231 cells were transfected with a pGL3-1087 construct, along with CMV-GAL, as a
530 control for transfection efficiency. Cells were grown in the presence or the absence of E2 (10^{-9} M).
531 Results show relative luciferase activities after normalization with β -gal activity (3 independent
532 experiments).

533

534 **Fig. 4. Identification of an ERE in CAR promoter.** **A.** Sequence of *CAR* promoters with the
535 presence of half-ERE sites and an ERE sequence (TGAGCatgAGGTCA). The sequences of *CAR*-
536 EREW^T and *CAR*-ERE^m (mutant) oligonucleotides used in gel shift experiments are indicated at
537 the bottom of the figure. **B.** MCF-7 cells were transfected with pGL3-1087, pGL3-522, PGL3-428
538 or PGL3-ERE^m constructs along with CMV-GAL as a control for transfection efficiency. Cells
539 were grown in the presence or the absence of E2 (10^{-9} M). Results show relative luciferase
540 activities (% of values of pGL3-basic vector without E2) after normalization with β -gal activity (3
541 independent experiments). **C.** MCF-7 cells were treated with control vehicle ethanol (C) or E2 (10^{-9}
542 M) for different times. *CAR* (left graph) and *pS2/TFF1* (right) mRNA levels were determined by
543 real-time PCR. Results represent the mean of 3 independent experiments and are expressed as % of
544 controls (C). **D.** Chromatin was prepared from MCF-7 cells treated as in RT-PCR experiments, or
545 from *ER*-negative MDA-MB-231 cells (MDA), which were used as a control for specific
546 immunoprecipitation. ChIP assays were then performed using antibodies directed against *ER* or the
547 epitope HA as an additional control. Specific enrichment of *CAR*, *pS2/TFF1* and control *Rplp0*
548 promoter regions in anti-*ER* Chip-ped material were then evaluated by quantitative PCR, with
549 values normalized to those obtained in samples immunoprecipitated using the control antibody (C).
550 The regions amplified are illustrated within the schemes above each graph. Results represent the
551 mean of 3 independent experiments.

552

553 **Fig. 5: CAR enhances breast cancer cell proliferation.** **A.** MCF-7 cells were grown in DMEM-
554 F12 supplemented with CD-FCS without (w/o E2), or with 10^{-9} M E2 (+E2), and incubated with
555 pre-immune serum (mock), or CAR antibody (CAR Ab). Cells stably expressing luciferase
556 reporters were lysed at days 2, 4 and 6 and subjected to luciferase activity. Results are expressed as
557 fold of control cells grown in the absence of estradiol at day 0, and in the absence of mouse pre-
558 immune serum and data represent the mean \pm SD from triplicates. **B.** MCF-7 cells were transfected
559 with non targeting siRNA (siC), or with CAR siRNA (siCAR), and treated with 10^{-9} M E2 (+E2).
560 Left panel: measure of CAR and protein levels in transfected cells by western blot at day 3. β -actin
561 levels were detected to demonstrate equal loading. Right panel: proliferation was measured at day
562 3 after transfection, and results are expressed as fold of control cells grown in the absence of
563 estradiol at day 0. Data represent the mean \pm SD from triplicates. **C.** Characterization of the pool
564 of MCF-7-CAR stable transfectants by western blot. β -actin levels were detected to demonstrate
565 equal loading. **D.** Wild-type MCF-7 cells or MCF-7-CAR cells (CAR) were grown in DMEM-F12
566 supplemented with CD-FCS without or with 10^{-9} M E2 (+E2). Proliferation rate is expressed as
567 fold of control cells grown in the absence of estradiol at day 0. The data represents the mean \pm SD
568 from triplicates. **E.** Characterization of the pool of MDA-MB-231-CAR stable transfectants by
569 western blot. β -actin levels were detected to demonstrate equal loading. **F.** Wild-type MDA-MB-
570 231 cells or MDA-MB-231-CAR cells (CAR) were grown in DMEM-F12 supplemented with FCS.
571 Proliferation is expressed as fold of control cells grown at day 0. Data represent the mean \pm SD
572 from triplicates.

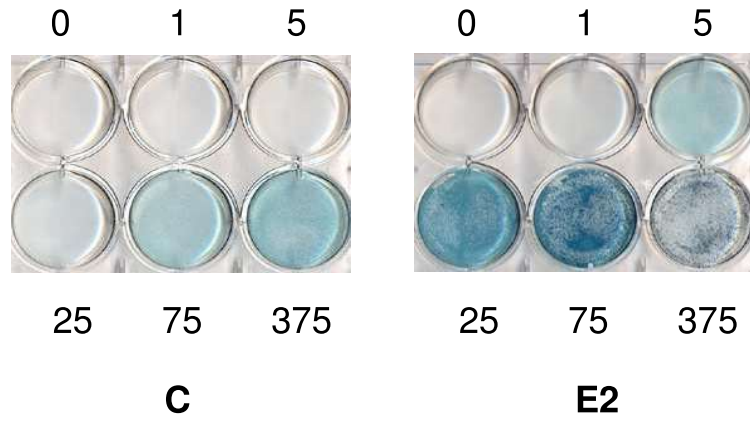
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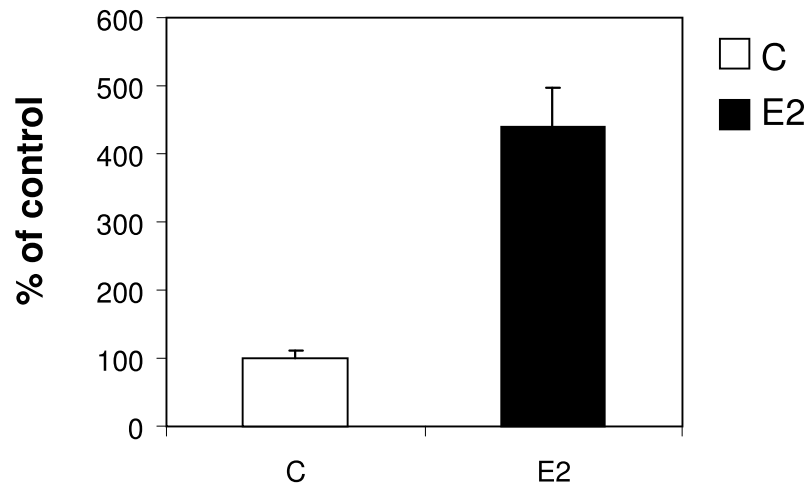
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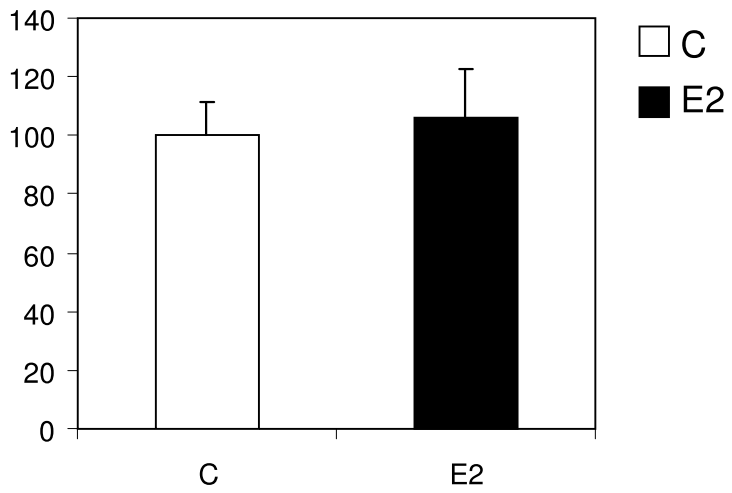
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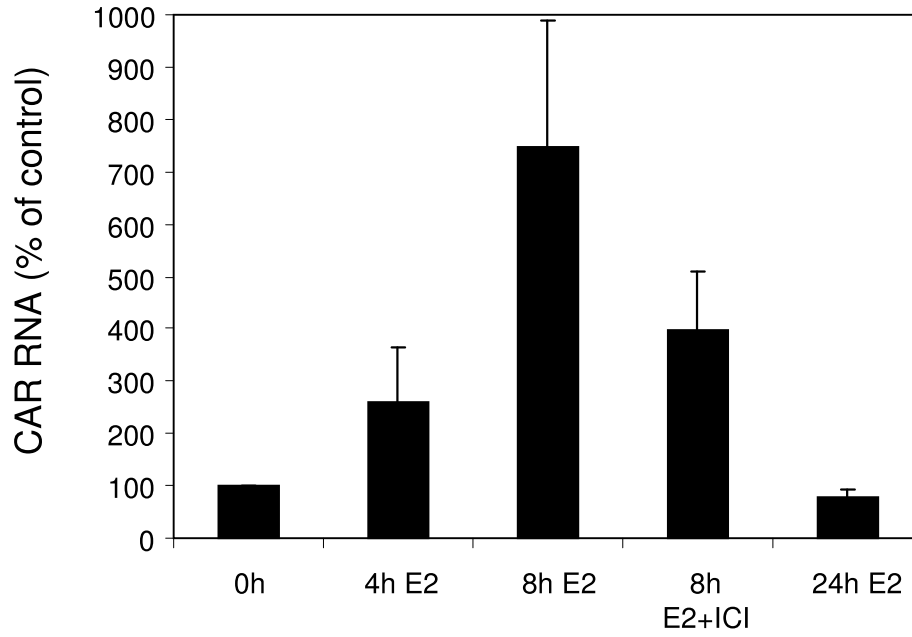
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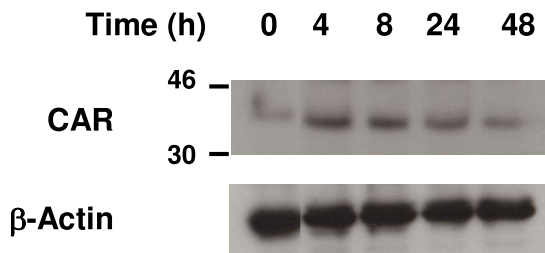
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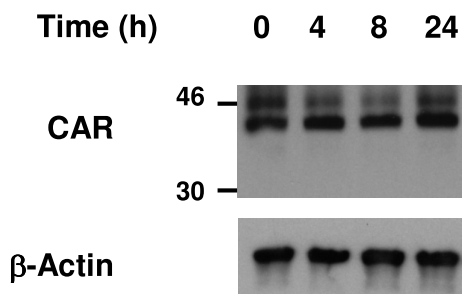
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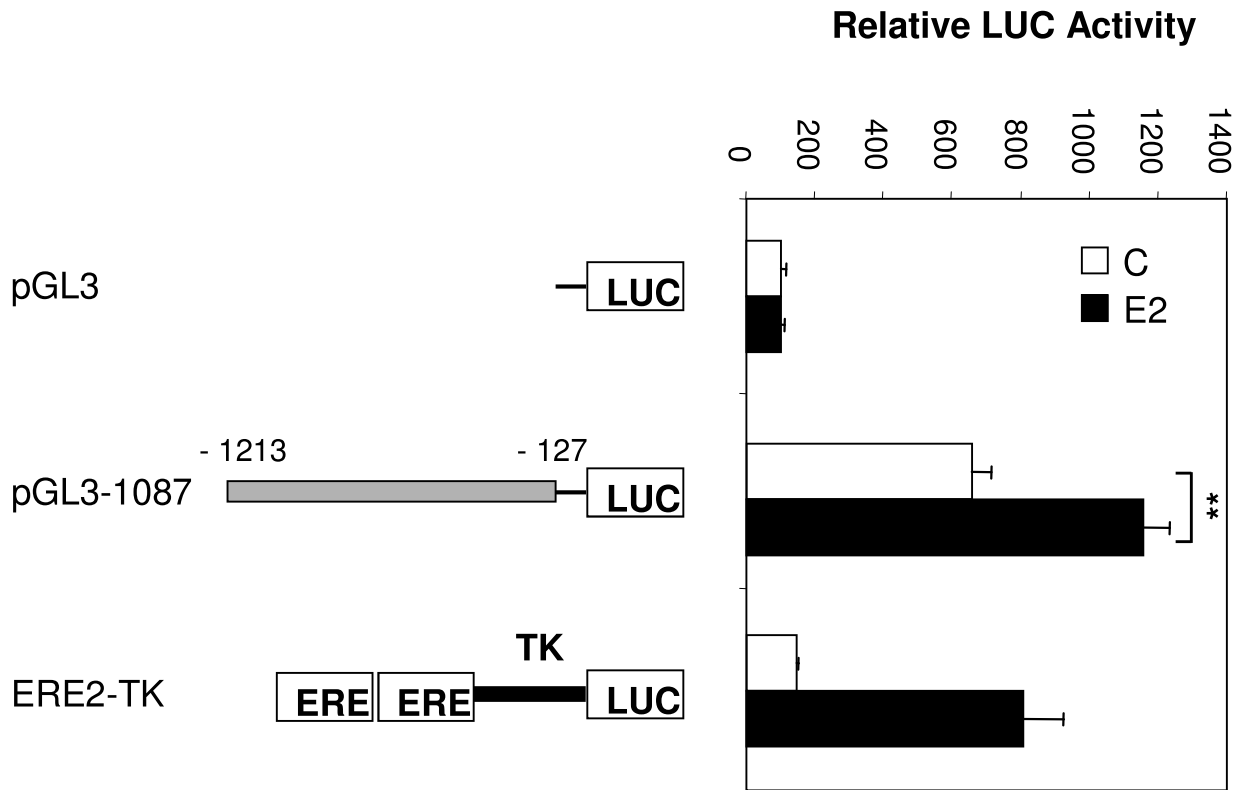
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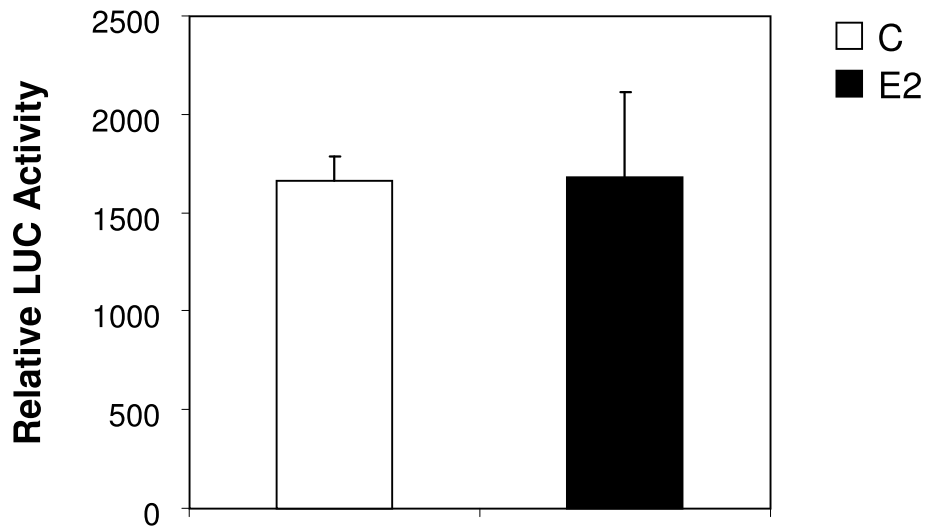
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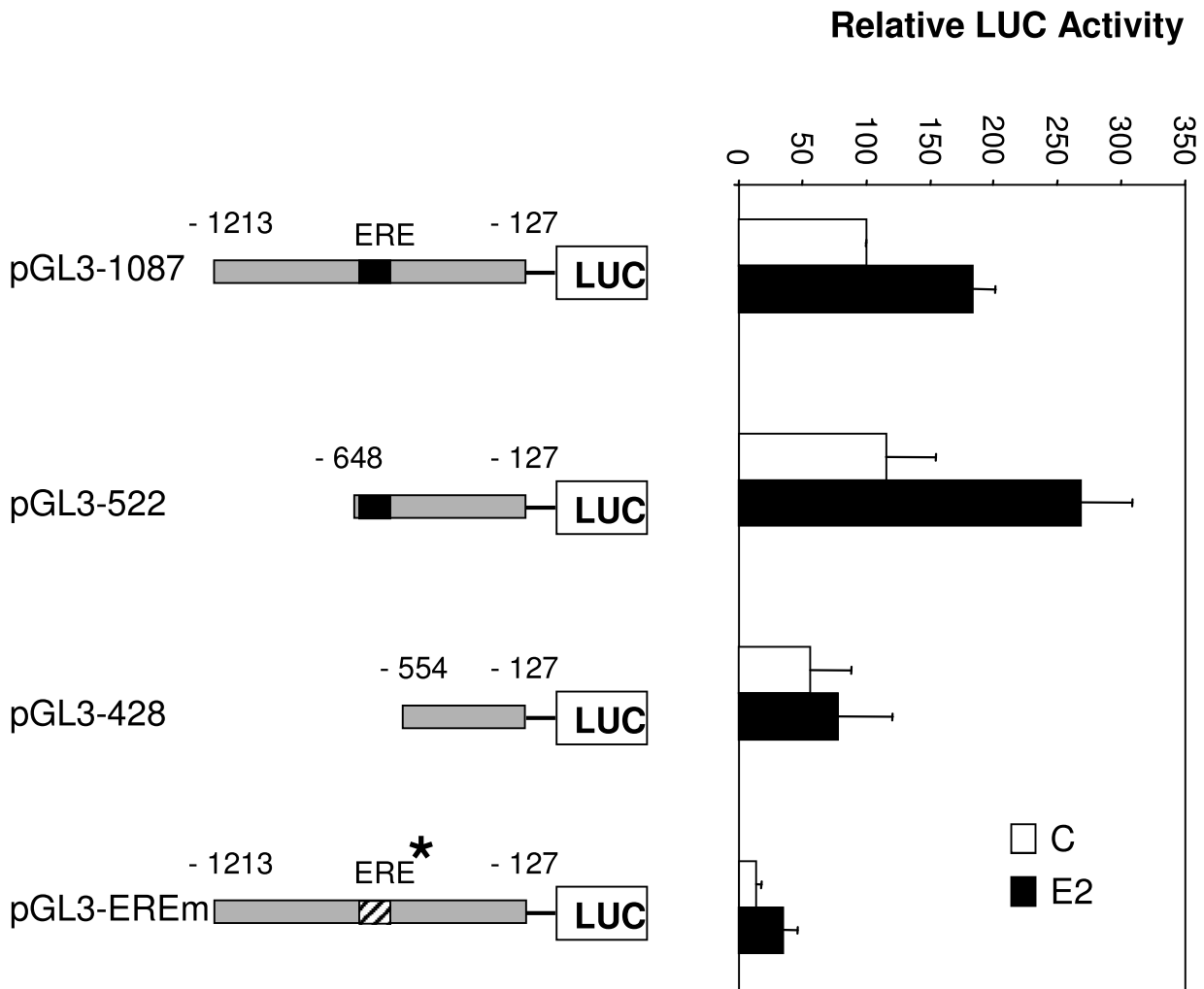
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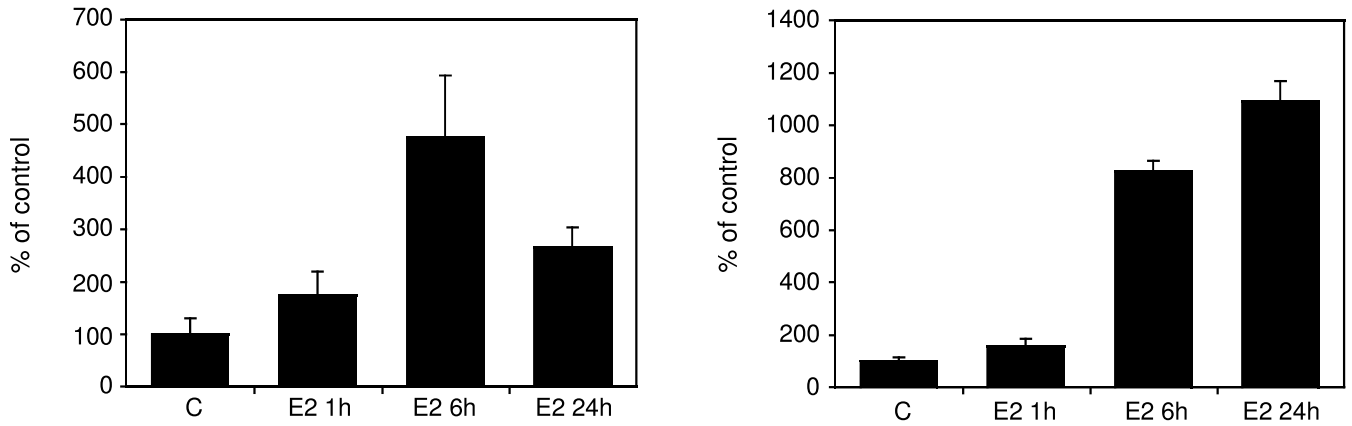
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CAR-EREm TCGCATCCCGgGCATGtCGTCAGAGAACCTGCC

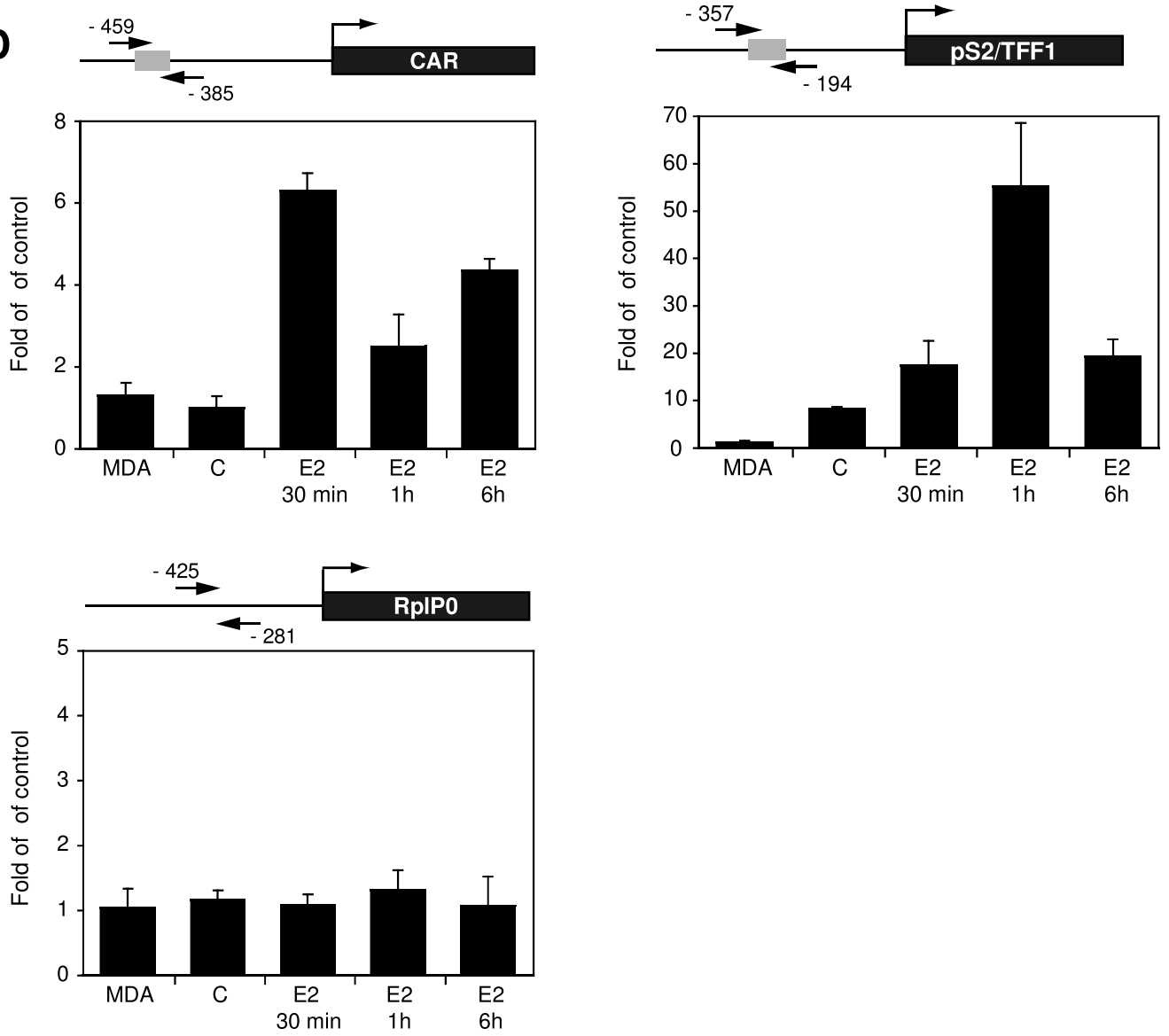
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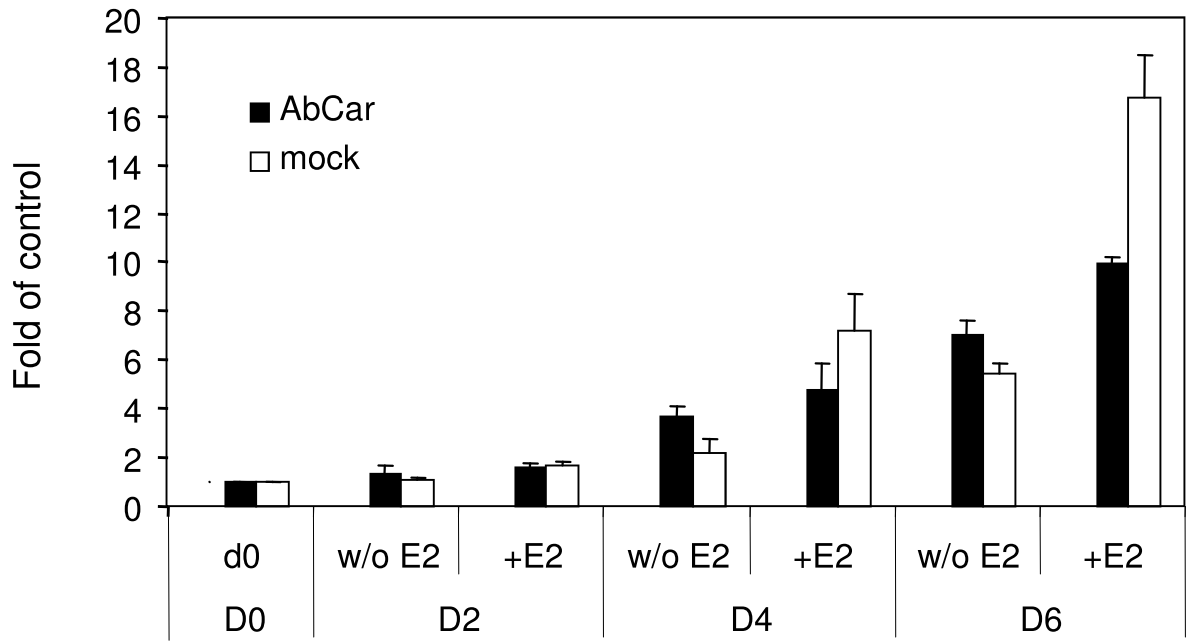
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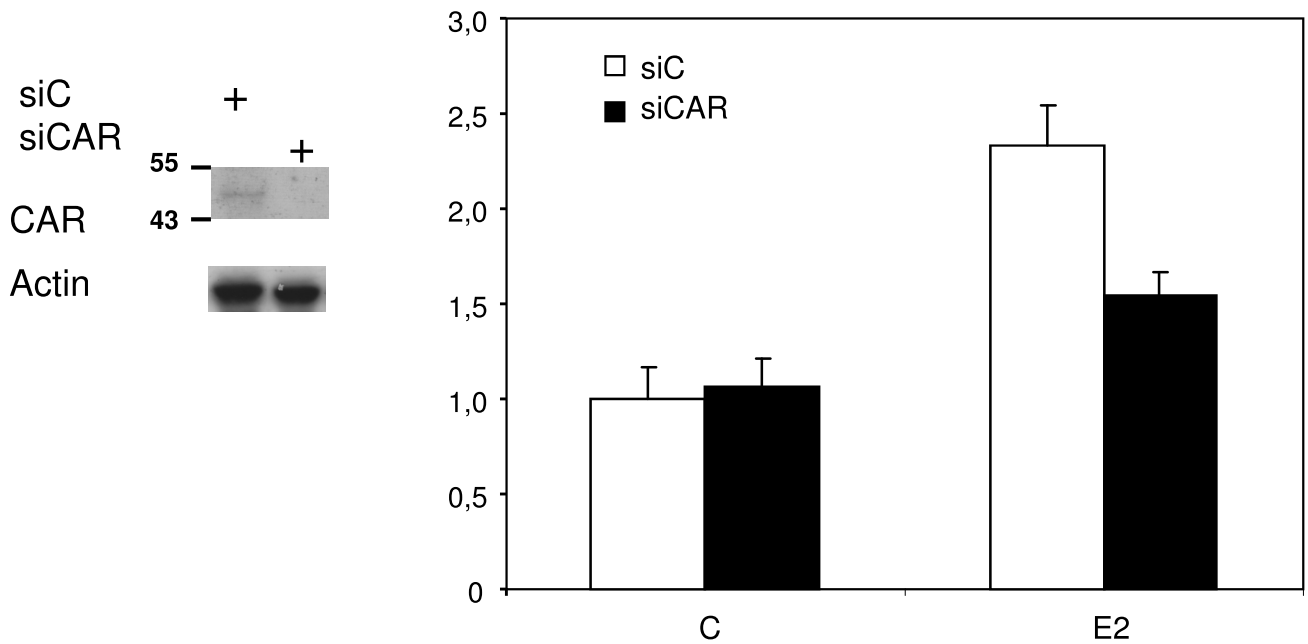
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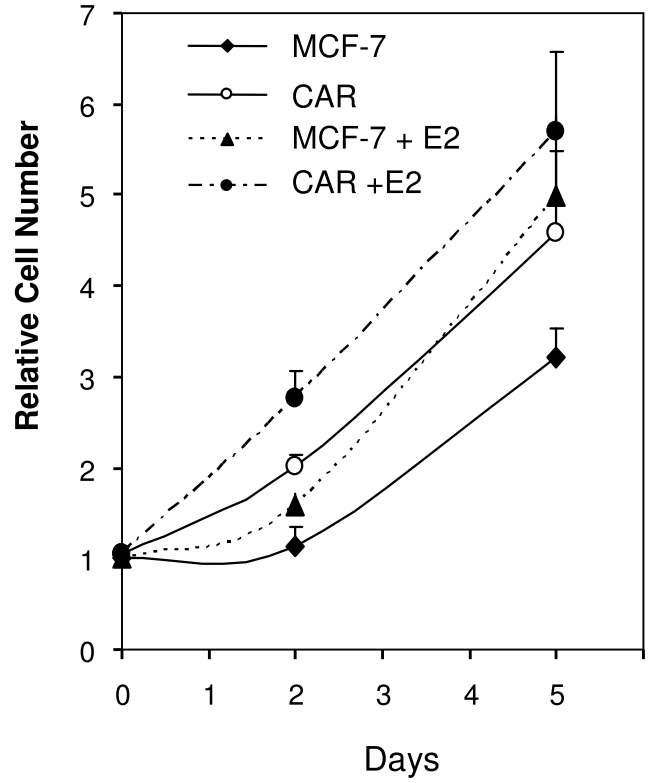
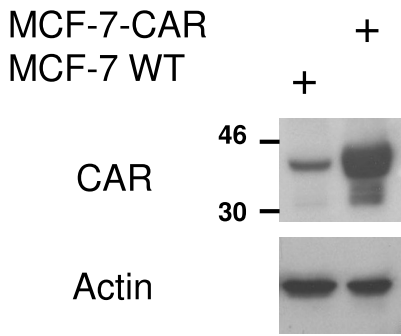
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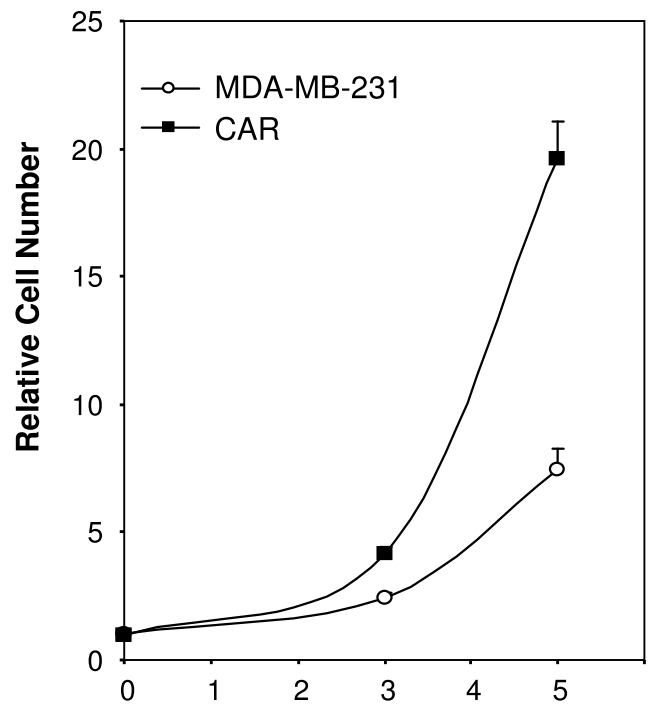
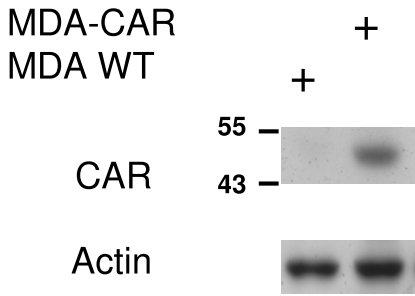
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Supplementary Materials and Methods

Whole cell extracts and gel shift experiments.

MDA-MB-231 cells were lysed in 10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, and 10% glycerol containing protease inhibitors (5 µg/ml aprotinin, leupeptin and pepstatin A, and 0.1 mM phenylmethylsulfonylfluoride). Then, cells were sonicated and the cellular debris was pelleted by centrifugation at 13,000 X g for 20 min in Microfuge tubes.

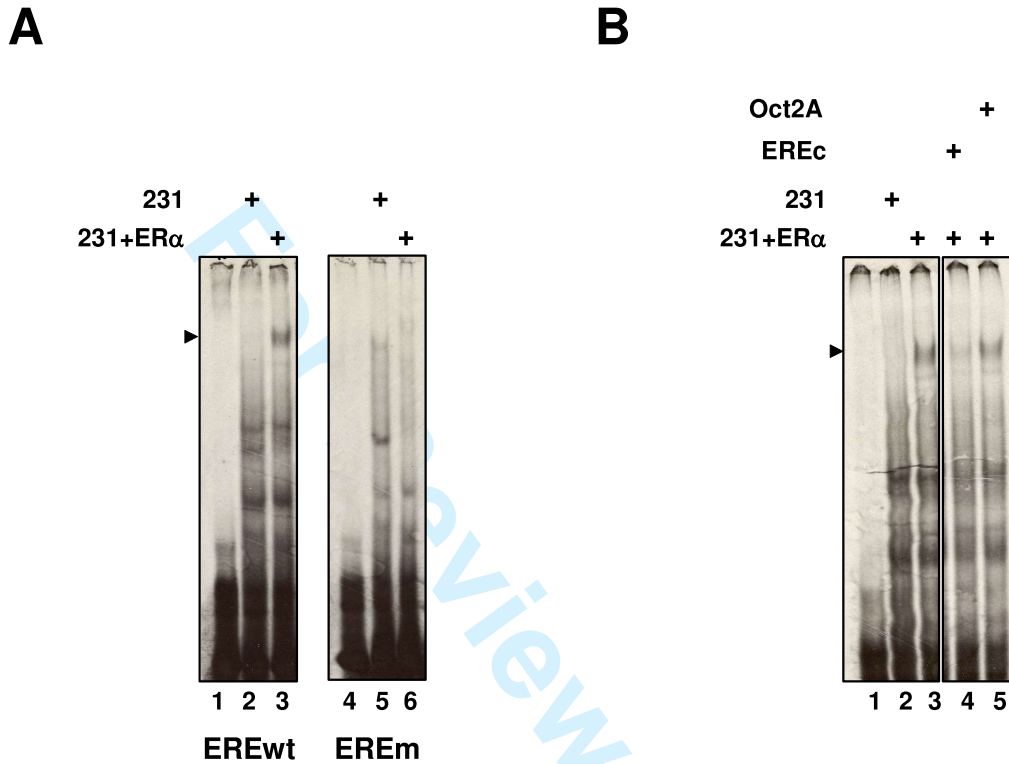
Gel shift experiments were performed as previously described (32). Briefly, 30,000 cpm of the [³³P]-labeled CAR-ERE (TCGCATCCCGTGAGCATGAGGTCAGAGAACCTGCC) or CAR-EREm (TCGCATCCCGTGgGCATGtcGTCAGAGAACCTGCC) double-strand oligonucleotides were combined with 1 µg poly (dI-dC) and 5 µg of MDA-MB-231 whole-cell extract. The reaction buffer contained 20 mM HEPES, pH 7.9; 1 mM DTT; 50 mM KCl; 10% glycerol; and 2.5 mM MgCl₂. Protein-DNA complexes were separated from the free probe by non-denaturing gel electrophoresis with 4% polyacrylamide gels in 0.5X Tris/borate/ EDTA. Competitions were performed with cold double-stranded oligonucleotides corresponding to consensus ERE sequence (AGCTCTTTGATCAGGTCACTGTGACCTGACTTT) or Oct2A binding site (5'-GTACGGAGTATCCAGCTCCGTAGCATGCAAATCCTCTGG-3').

Supplementary Figure 1 Legend

A. Whole cell extract was prepared from MDA-MB-231 infected mock Ad5 adenovirus or ER α encoding adenovirus and used in gel shift experiments. **A.** CAR-EREWT (left panel) or CAR-EREm (right panel) probes were incubated without protein extract (lanes 1 and 4), with 5 µg of mock infected WCE from MDA-MB-231 (lanes 2 and 5) or with ER α infected WCE from MDA-MB-231 cells (lanes 3 and 6). The arrow indicates the position of ER α complex.

B. Gel shift were performed in the same conditions as in A with CAR-EREWT probe.

Competition with a 50X excess of cold consensus ERE (EREc) or cold Oct2A oligonucleotides were performed in lanes 4 and 5, respectively. The picture corresponds to the assembly of lanes of the same gel.



Supplementary Figure 2 Legend

CAMA-1 cells were treated with control vehicle ethanol, E2 (10^{-9} M) for different times. CAR RNA levels were determined by real-time PCR. Results represent the mean of 3 independent experiments and are expressed as the percentage of control cells.

