Coxsackie and adenovirus receptor is a target and a mediator of estrogen action in breast cancer

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Abstract

The involvement of the coxsackie and adenovirus receptor (*CAR*), an adhesion molecule known to be the main determinant of adenovirus transduction of the cells, in cancer is currently under investigation. Recent reports suggest that *CAR* levels are elevated in breast cancer, and this may have an impact on its use as means of delivery for gene therapy. In this study, we show that estradiol (E_2) treatment of the estrogen receptor (ER)-positive breast cancer cell MCF-7 increases *CAR* levels and, in turn, enhances adenoviral transduction. Employing the transfection of *CAR* promoters in breast cancer cells, we show that this regulation of *CAR* expression occurs at the transcriptional level. In addition, and by chromatin immunoprecipitation, we have identified a crucial region of *CAR* promoter that controls E_2 responsiveness of *CAR* gene through the recruitment of ER. Moreover, utilizing *CAR* antibodies or *CAR* silencing by RNA interference repressed the estrogen-dependent growth of breast cancer cells, whereas the stable expression of *CAR* in MCF-7 or MDA-MB-231 cells led to an increased proliferation. Altogether, our data suggest that *CAR* is a novel estrogen-responsive gene, which is involved in the E_2-dependent proliferation of breast cancer cells.

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Introduction

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

So far, very little is known about *CAR* expression in breast cancer biopsy, and the situation appears to be significantly different from other cancers. A study showed that *CAR* levels are elevated in breast cancer and increase during progression and metastasis (Martin *et al*. 2005). In addition, *CAR* expression is correlated to estrogen receptor (ER) expression in breast cancer

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cells (Auer *et al*. 2009). Estrogens are potent mitogens in cancerous breast tissue (Henderson *et al*. 1988, Lazennec *et al*. 1999, Katzenellenbogen *et al*. 2000). Studies in breast cancer tissue, both *in vivo* and *in vitro*, have shown that estrogen dramatically escalates proliferative and metastatic activity in these tumor cells. The growth of ~70% of all human breast cancers is dependent upon the presence of an estradiol (E_2)–ER complex (Santen *et al*. 1990). The genomic action of estrogen is mediated by two ERs, namely ERα and ERβ (Katzenellenbogen *et al*. 2000). ERα is mainly involved in the mitogenic action of estrogens, whereas ERβ is rather anti-proliferative both in breast, prostate, and ovarian cancer (Lazennec *et al*. 2001, Bardin *et al*. 2004, Cheng *et al*. 2004, Lazennec 2006).

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

Materials and methods

Cell culture

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

RNA extraction and reverse transcriptase PCR

Total RNA was isolated with TRIzol reagent (Invitrogen) as described by the manufacturer. Reverse transcription was performed using random primers and Superscript II enzyme (Invitrogen). Real-time PCR quantification was then performed using a SYBR Green approach (Light Cycler; Roche), as described previously (Lucas *et al*. 2003). For each sample, *CAR* or *pS2/TFF1* mRNA levels were normalized with *RS9* or *Rplp0* mRNA levels (reference genes). The sequences of the oligonucleotides used were *CAR* (transmembrane CAR, exons 6/7, (Thoelen *et al*. 2001)) (left 5′-GCAGGAGCCATTATAGGAACTTTG-3′; right 5′-GGACCCCAGGGATGAATGAT-3′), pS2/TFF1 (left 5′-ACCATGGAGAACAAGGTGA-3′; right 5′-CCGAGCTCTGGGACTAATCA-3′), RS9 (left 5′-CAGGCGCAGACGGTGGAAGC-3′; right 5′-CGCGAGCGTGGTGGATGGAC-3′), Rplp0 (left 5′-AAYGTGGGCTCCAAGCAGATG-3′; right 5′-GAGATGTTCAGCATGTTCAGCAG-3′).

Recombinant adenovirus construction, propagation, and infection

The adenoviruses encoding the β-galactosidase (Ad-GAL) or ERα (Ad-ERα) used in this study have been previously described (Lazennec *et al*. 1999, 2001, Lucas *et al*. 2003). Briefly, the complete coding sequences of β-galactosidase, or human ERα cDNAs, were subcloned in a pACsk12CMV5 shuttle vector. To obtain recombinant viruses, permissive HEK-293 cells (human embryonic kidney cells) were cotransfected with pACsk12CMV5, or the recombinant pACsk12CMV5 plasmid, and with pJM17 that contains the remainder of the adenoviral genome. *In vivo* recombination of the plasmids generates infectious viral particles, non-recombinant adenovirus Ad5, or recombinant adenovirus Ad-GAL and Ad-ERα. MCF-7 or MDA-MB-231 cells were infected overnight at different multiplicities of infection (MOI) in DMEM/F12 10% CDFCS. The next day, the medium was changed and the cells were let to express β-GAL for 48 h before collecting the medium.

β-Galactosidase histochemical staining assay

After 48 h of expression, infected cells were washed twice with PBS and then fixed for 5 min at 4 °C in a fixing solution (2% formaldehyde, 0.2% glutaraldehyde, and 1×PBS). Cells were washed once and incubated for 4 h at 37 °C in histochemical staining solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 1×PBS, and 1 mg/ml X-GAL; Lucas *et al*. 2003). Cells were then washed twice with PBS and observed.

Western blot experiments

Cells were harvested in Tris–glycerol buffer (Tris–HCl 50 mM, EDTA 1.5 mM, and 10% glycerol) supplemented with protease inhibitor cocktail (Roche) and were then sonicated. Protein extracts (30 µg) were subjected to SDS-PAGE protein samples under

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non-reducing conditions. Samples were heated normally at 95 °C for 5 min in a sample loading buffer that lacked mercaptoethanol. Western blot analyses were done using *CAR* antibody (sc-56892, Santa Cruz, CA, USA) and β-actin (Sigma–Aldrich). Immunoreactivity was detected with Amersham ECL system. Actin was used as a loading control.

Constructs and transient transfection

A *CAR* promoter corresponding to sequences -1213/-127 (pGL3-1087) has been previously described (Pong *et al*. 2003). Deleted constructs of the *CAR* promoter (pGL3-522: -648/-127; pGL3-428: -554/-127) were cloned into pGL3 basic vectors (Promega). Site-directed mutagenesis of *CAR* ERE was performed with *CAR*-EREm primers (TCGCATCCCGTG**g**GCATG**tc**GTCAGA-GAACCTGCC) to generate pGL3-EREm construct. The ERE-TK-LUC construct consists of two ERE in tandem upstream of TK promoters (Duong *et al*. 2006). We plated 3.10^5 of steroid-weaned cells in 12-well plates in phenol red-free DMEM-F12 and supplemented with 10% CDFCS 24 h before transfection. Transfections were performed using lipofectamine according to the manufacturer's recommendations, using 2 μg of the *CAR* promoter pGL3-1087, luciferase reporter, or *CAR* promoted deleted constructs along 0.5 μg of the internal reference reporter plasmid (CMV-Gal) per well. After 6 h incubation, the medium was removed and the cells were placed into a fresh medium supplemented with a control vehicle (ethanol) or E_2. After 24 h, cells were harvested and assayed for luciferase activity using a Centro LB960 Berthold luminometer. β-galactosidase was determined as described previously (Duong *et al*. 2006).

siRNA experiments

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

CAR stable transfectants

The stably transfected MCF-7-*CAR* and MDA-MB-231-*CAR* cell lines were obtained after transfection with the plasmid pcDNA3-h*CAR* (a kind gift of Dr S Hemmi), encoding the human *CAR* cDNA under the control of the CMV promoter. Transfected cells were then selected by G418 at a concentration of 2 mg/ml. After 2 weeks of selection, a pool of resistant cells was collected and used for further experiments.

Chromatin immunoprecipitations

These assays were conducted with some modifications from Métivier *et al*. (2003), using 1.10^7 MCF-7 or MDA-MB-231 cells that were placed in DMEM/0.5% CDFCS for 3 days. Afterward, some were treated with 10 nM E_2 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 dithiothreitol), and 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, and 0.5% Empigen BB (Sigma)). Extracts were then sonicated for 14 min using a BioRuptor apparatus (Diagenode), with 30 s on/off cycles. Following 10 min centrifugation at 10 000 ***g***, 50 μl of the supernatants were used as inputs, and the remainder diluted threefold 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 h at 4 °C with 150 μl of a 50% protein A-Sepharose bead (Amersham Pharmacia Biosciences) slurry containing 10 μg yeast tRNA (Sigma). Following centrifugation at 800 ***g*** for 1 min, one-third of the extracts were taken for immunoprecipitation using 1μg of antibodies directed against ER (HC20, Santa Cruz) or the epitope HA (HA-probe Y11, Santa Cruz) and diluted three times in a IP buffer. Complexes were recovered by a 3 h incubation at 4 °C with 50 μl of protein A-Sepharose slurry containing 10 μg 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, and 150 mM NaCl), WB II (2 mM EDTA, 20 mM Tris–HCl (pH 8.1), 0.1% SDS, 1% Triton X-100, and 500 mM NaCl), WB III (1 mM EDTA, 10 mM Tris–HCl (pH 8.1), 1% NP-40, 1% deoxycholate, and 0.25 M LiCl), and then twice with 1 mM EDTA and 10 mM Tris–HCl (pH 8.1). Precipitated complexes were removed from the beads through three sequential incubations of 10 min with 50 μl of 1% SDS and 0.1 M $NaHCO_3$. Cross-linking was then reversed by an overnight incubation at 65 °C. DNA was purified with Qiaquick columns (Qiagen). Subsequent quantitative PCRs analysis used 1 μl of input material and 5 μl of chromatin immunoprecipitation (ChIP) samples and were performed on a Bio-Rad MyiQ apparatus using Bio-Rad iQ SYBR Green supermix. The sequences of the oligonucleotides used,

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designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), are: *CAR* (left 5′-CCTCTCGCGCTTTTTATGTC-3′; right 5′-TCTCTGACCTCATGCTCACG-3′), *pS2/TFF1* (left 5′-TCATCTTGGCTGAGGGATCT-3′; right 5′-TTCCGGCCATCTCTCACTAT-3′), *Rplp0* (left 5′-ATCTAACTAGCACACGAACCTT-3′; right 5′-CTGTATTCGTTCAGCTTTGTCT-3′). The PCR efficiency was calculated for each oligonucleotide set, using serial dilutions of inputs. Once normalized to inputs following the ΔCt method, the results were normalized again to the ChIP performed using the control anti-HA antibody.

Proliferation assay

MCF-7 cells that had been stably transfected with a CMV-LUC reporter were grown in 24-well plates in the presence of 10^{-9} M E_2 for 4-days, and in the presence of 20 µl of whole mouse serum or E1-1 anti-*CAR* hybridoma supernatant (Hemmi *et al.* 1998). Cells were then collected and luciferase activity was measured. Concerning wild-type MCF-7, MCF-7-*CAR*, MDA-MB-231, and MDA-MB-231-*CAR*, their proliferation was quantified by counting the cells on a cell counter.

Results

E_2 increases the transduction of ER-positive breast cancer cells by adenovirus

The role and the regulation of *CAR* expression in breast cancer cells remain elusive. We hypothesized that estrogens, which regulate the growth of ER-positive breast cancer cells, could modify their sensitivity to adenoviral infection, through a regulation of *CAR* levels. To analyze whether estrogens could modify the adenoviral transduction, we pre-treated ER-positive breast cancer cells MCF-7 with E_2 for 24 h before infecting them with an adenovirus encoding β-galactosidase (Ad-GAL) at increasing MOIs. We observed that adenoviral transduction was strongly enhanced in E_2-treated cells compared with control cells (Fig. 1A), which suggests that E_2 modifies the expression of a factor involved in adenoviral transduction. Microscopic observation confirmed that the increased staining of the plates was the result of a higher number of infected cells (Fig. 1B). On the other hand, ER-negative MDA-MB-231 cells were similarly infected by the virus in the absence or the presence of E_2, demonstrating that ER was required for E_2 regulation of adenoviral transduction (Fig. 1C).

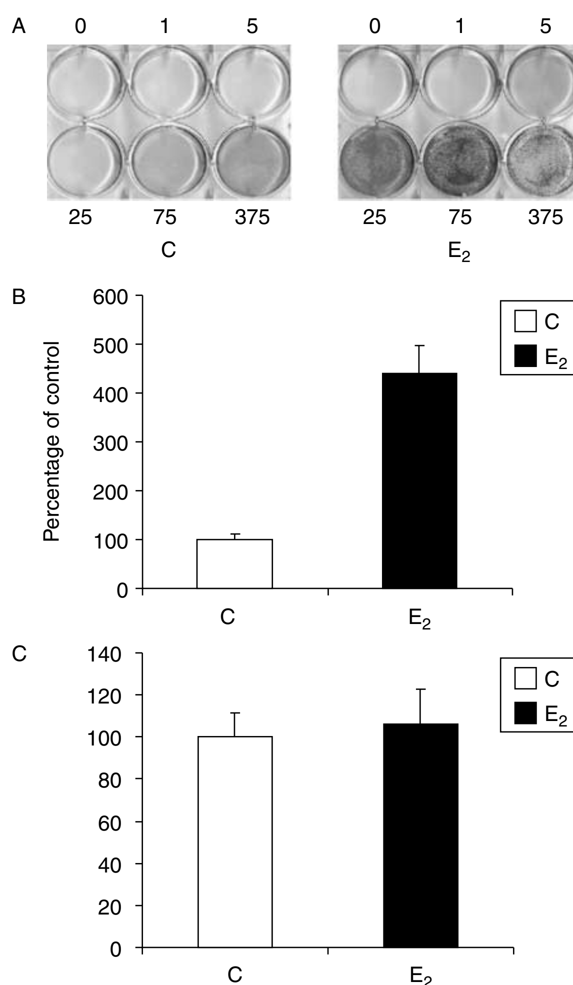

Figure 1 Estradiol increases adenovirus transduction. (A) ER-positive breast cancer cells, MCF-7 cells, were treated for 24 h with ethanol vehicle (C) or estradiol (E_2; 10^{-9} M) before infection with Ad-GAL virus at increasing multiplicity of infection (MOI=0, 1, 5, 25, 75, 375). The infected cells were then grown for 48 h with ethanol vehicle or estradiol (10^{-9} M) and stained for β-gal activity as described in ‘Materials and methods’. (B) MCF-7 cells infected with adenovirus at MOI=1 and treated with or without E_2. Quantification of β-gal-positive cells after adenovirus infection. β-gal-positive cells were counted from two non-overlapping fields. Results represent the mean±s.d. of three independent experiments and are expressed as the percentage of control cells. (C) ERα-negative MDA-MB-231 cells were infected with adenovirus under the same conditions as in B. Results represent the quantification of β-gal-positive cells after adenovirus infection (n=3).

CAR levels are up-regulated by E_2

As *CAR* is the main determinant of adenovirus infection, we hypothesized that its expression could be regulated by estrogens, which in turn explains the effects of E_2 on adenoviral transduction. To determine whether E_2 was modulating *CAR* RNA levels, we treated MCF-7 cells at different times with E_2.

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We measured the levels of transmembrane *CAR* isoform (see Materials and methods) by real-time PCR. We observed that *CAR* RNA expression was induced at 8 h of E_2 treatment and returned to basal levels after 24 h (Fig. 2A). To ensure that the regulation of *CAR* RNA levels by E_2 were mediated by the ER, MCF-7 cells were cotreated for 8 h with E_2 and the pure anti-estrogen $ICI_{182\ 780}$, which could strongly reduce *CAR* RNA induction by E_2 (Fig. 2A). *CAR* levels were also induced in another ER-positive breast cancer cell line, CAMA-1, suggesting that this was not specific of MCF-7 cells (data not shown). In addition, *CAR* protein levels were also increased upon E_2 treatment, as shown by western blot, but this induction by E_2 returned to basal levels only at 48 h (Fig. 2B). We performed the same analysis in the ER-negative breast cancer cell line MDA-MB-231 (Fig. 2C) and observed

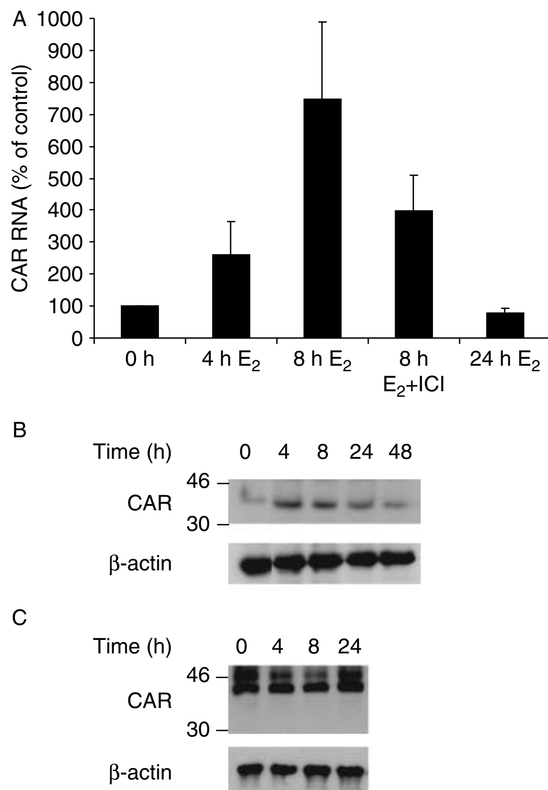

Figure 2 *CAR* expression is induced by E_2. (A) MCF-7 cells were treated with control vehicle ethanol, E_2 (10^{-9} M) for 8 or 24 h or with $E_2+ICI_{182\ 780}$ (ICI; 10^{-6} M) for 8 h. *CAR* RNA levels were determined by real-time PCR. Results represent the mean of three independent experiments and are expressed as the percentage of control cells. (B) The levels of *CAR* protein were measured by western blot with *CAR* antibody using extracts from MCF-7 cells treated with E_2 (10^{-9} M) for 4, 8, or 24 h. β-actin levels were detected to demonstrate equal loading. (C) Same experiment as in B, but in MDA-MB-231 cells.

that *CAR* protein levels were not significantly affected by E_2 in these cells. This further demonstrates that ER is necessary for *CAR* induction by E_2.

CAR gene promoter is regulated by estrogens

Next, we investigated the possible regulation of *CAR* gene promoters by estrogens in MCF-7 cells. The transfection of *CAR* promoter reporter gene construct showed that it was induced 1.8-fold by E_2 in MCF-7 cells (Fig. 3A). On the contrary, in the ER-negative breast cancer cell MDA-MB-231, the *CAR* promoter was not induced by E_2 (Fig. 3B), demonstrating that *CAR* is required for the regulation of *CAR* promoter by estrogens. We performed a computer analysis of the *CAR* promoter, and we identified four half-ERE (ER binding site) and one putative ERE (-571/-557) based on the consensus ERE sequence (Gruber *et al.* 2004, O'Lone *et al.* 2004; Fig. 4A). We used first deletion constructs of the *CAR* promoter (Fig. 4B). pGL3-522 (-648/-127) construct retained E_2 inducibility, but the further deletion of 94 bp (pGL3-428, -554/-127) by removing the putative ERE was sufficient to lose the responsiveness to E_2 (Fig. 4B). Using a point mutation construct of the ERE, we observed a strong reduction of *CAR* promoter induction by E_2 as well (Fig. 4B), reinforcing the central role of this ERE sequence in modulating *CAR* promoter activity. To validate the functionality of *CAR* ERE, we performed ChIPs experiments. These experiments were performed in MCF-7 cells, which exhibit an estrogenic stimulation of *CAR* and pS2/TFF1 genes (Fig. 4C). ChIPs showed an early recruitment of ERα to *CAR* ERE sequence in 30 min (Fig. 4D). The kinetic of recruitment of ERα to *CAR* promoter was more rapid than the one observed for the *pS2/TFF1* promoter, which is also in agreement with the distinct kinetics of estrogen regulation of *CAR* and *pS2/TFF1* mRNA (Fig. 4C, D). Finally, the ability of ERα to bind to *CAR* ERE sequence was also confirmed by gel shift assays (data not shown).

CAR is involved in breast cancer proliferation

As *CAR* expression is regulated by estrogens, we next hypothesized that *CAR* could be involved in the proliferation of ER-positive cells. MCF-7 cells were cultured in the presence of estrogen and treated either with control serum or anti-*CAR* antibody. Results showed that *CAR* antibody reduced half the proliferation of cancer cells in the presence of E_2 (Fig. 5A), which suggests that upregulation of *CAR* by estrogen is an important step in the control of estrogen-dependent growth of ER-positive breast cancer cells. In the absence of E_2, the *CAR* antibody did not significantly

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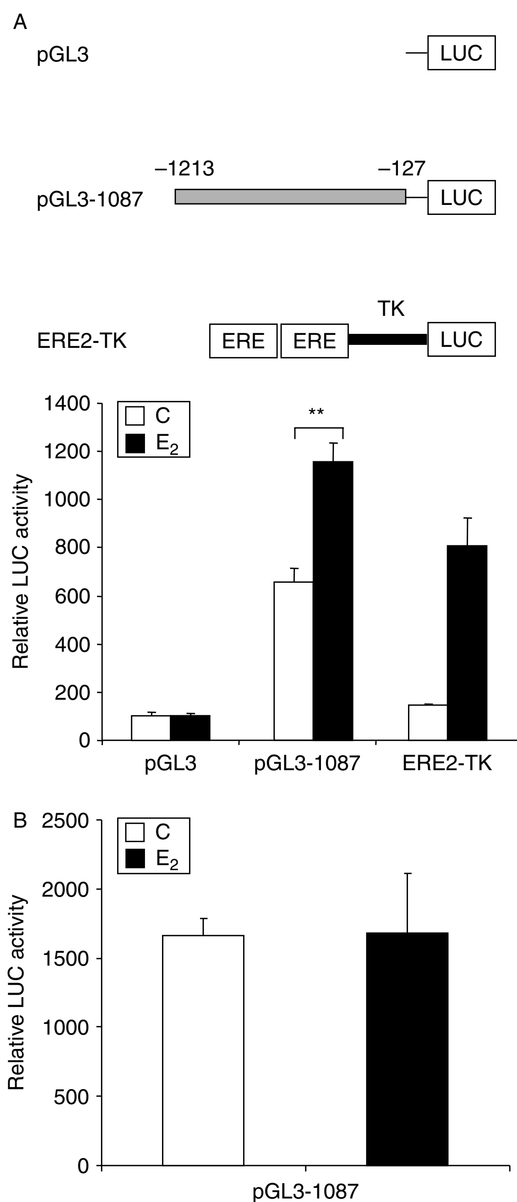

Figure 3 *CAR* promoter activity is enhanced by E_2 in ER-positive breast cancer cells. (A) MCF-7 cells were transfected with pGL3, pGL3-1087, or ERE2-TK constructs, along with CMV-GAL as a control for transfection efficiency. Cells were grown in the presence or the absence of E_2 (10^{-9} M). Results show relative luciferase activities (% of values of pGL3 vector without E_2) after normalization with β-gal activity (three independent experiments). **$P<0.001$ using Student's *t*-test. (B) MDA-MB-231 cells were transfected with a pGL3-1087 construct, along with CMV-GAL, as a control for transfection efficiency. Cells were grown in the presence or the absence of E_2 (10^{-9} M). Results show relative luciferase activities after normalization with β-gal activity (three independent experiments).

alter cell proliferation. We used a second approach based on siRNA to demonstrate the role of *CAR* in MCF-7 cell proliferation. Transfection of a siRNA against *CAR* inhibited the induction of proliferation of MCF-7 cells by E_2 (Fig. 5B). To further confirm the potential role of *CAR* in breast cancer cell proliferation, we generated a pool of stable transfectant MCF-7 cells for *CAR* (Fig. 5C). When comparing the MCF-7-*CAR* cells to wild-type MCF-7 cells, we observed that MCF-7-*CAR* cells displayed a higher proliferation rate compared with wild-type MCF-7 cells, in the absence and the presence of E_2 (Fig. 5D). Of particular note, MCF-7-*CAR* cells grown in the absence of E_2 had a similar proliferation as wild-type MCF-7 in the presence of E_2. This data confirms that *CAR* can enhance the proliferation of breast cancer cells. To demonstrate that *CAR* effects were not specific to a single cell line, we also generated a *CAR* stable transfectant in MDA-MB-231 cells (Fig. 5E). MDA-MB-231-*CAR* also displayed an enhanced proliferation compared with wild-type cells (Fig. 5F), reinforcing the fact that *CAR* stimulates breast cancer cell proliferation.

Discussion

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

Although most of the studies have shown that *CAR* levels were decreased in the aggressive forms of several cancers, including glioma and bladder cancer (Okegawa *et al.* 2001, Kim *et al.* 2003), the breast cancer situation seems to be different. Indeed, it was shown that *CAR* levels were elevated in breast cancer tissues (Martin *et al.* 2005). Bruning *et al.* 2005 also observed in a murine model of breast cancer that *CAR* expression was upregulated in carcinoma compared with pre-neoplastic lesion. To date, the role of *CAR* in breast cancer and the factors regulating *CAR* expression are poorly understood. Previous studies have shown that treatment of cancer cells with histone deacetylase inhibitors upregulates *CAR* expression (Kitazono *et al.* 2001, Hemminki *et al.* 2003, Pong *et al.* 2003, Okegawa *et al.* 2007). Other signals such as the MAPK pathway, the action of glucocorticoids, tumour necrosis factor α, and transforming growth factor-β also modulate *CAR* expression (Anders *et al.* 2003, Bruning & Runnebaum 2003). Pong *et al.* 2006 have also reported an induction of *CAR* expression by the phytoestrogen genistein in bladder cells, but it remains unclear whether this occurs through ER.

CAR levels are one of the primary determinants of adenoviral transduction. In this study, we show that E_2

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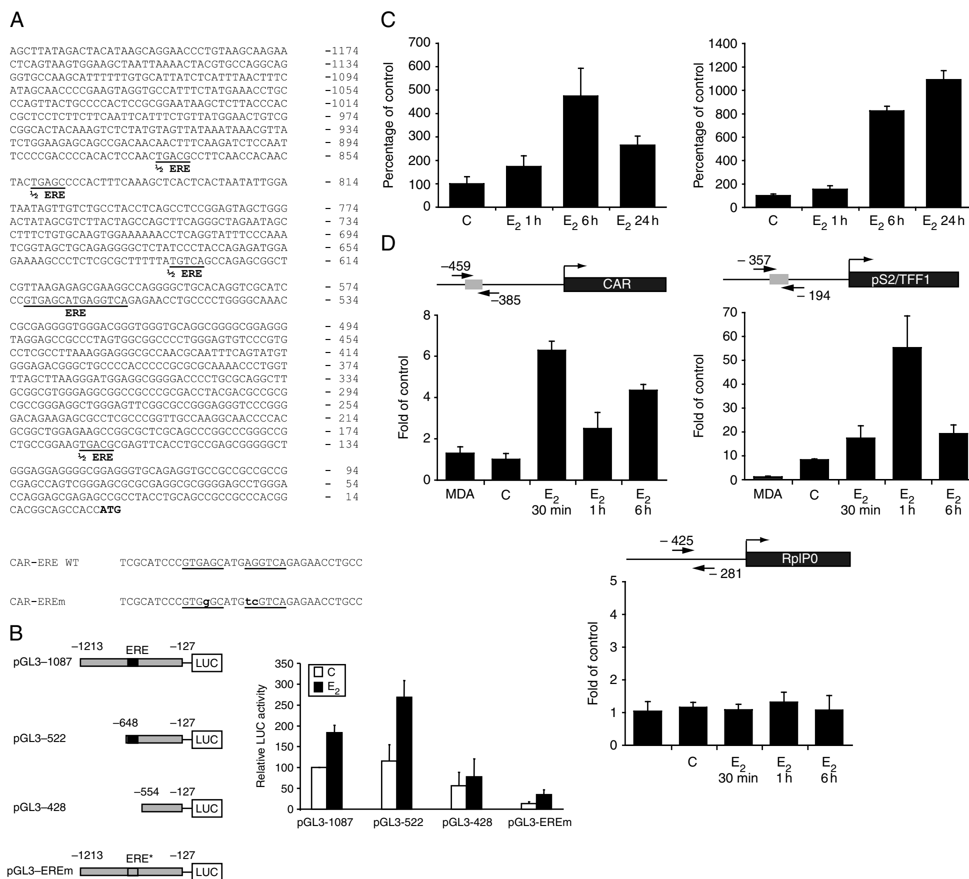

Figure 4 Identification of an ERE in *CAR* promoter. (A) Sequence of *CAR* promoters with the presence of half-ERE sites and an ERE sequence (TGAGCatgAGGTCA). The sequences of *CAR*-EREWT and *CAR*-EREm (mutant) oligonucleotides used in gel shift experiments are indicated at the bottom of the figure. (B) MCF-7 cells were transfected with pGL3-1087, pGL3-522, pGL3-428, or pGL3-EREm constructs along with CMV-GAL as a control for transfection efficiency. Cells were grown in the presence or the absence of E_2 (10^{-9} M). Results show relative luciferase activities (% of values of pGL3 basic vector without E_2) after normalization with β-gal activity (three independent experiments). (C) MCF-7 cells were treated with control vehicle ethanol (C) or E_2 (10^{-9} M) for different times. *CAR* (left graph) and *pS2/TFF1* (right) mRNA levels were determined by real-time PCR. Results represent the mean of three independent experiments and are expressed as % of controls (C). (D) Chromatin was prepared from MCF-7 cells treated as in RT-PCR experiments, or from ER-negative MDA-MB-231 cells (MDA), which were used as a control for specific immunoprecipitation. ChIP assays were then performed using antibodies directed against ER or the epitope HA as an additional control. Specific enrichment of *CAR*, *pS2/TFF1*, and control *Rplp0* promoter regions in anti-ER Chip-ped material was then evaluated by quantitative PCR, with values normalized to those obtained in samples immunoprecipitated using the control antibody (C). The regions amplified are illustrated within the schemes above each graph. Results represent the mean of three independent experiments.

increases ER-positive breast cancer cell transduction by type 5 adenoviruses. We also observed that *CAR* levels were increased by E_2 in ER-positive breast cancer cells, which is in agreement with a previous study, using the ER-positive breast cancer cell line T47-D (Auer *et al.* 2009). This regulation was dependent on ERs, as E_2 had no effect on *CAR* levels and promoter activity in ERα-negative MDA-MB-231 breast cancer cells. *CAR* expression was induced both at the RNA and protein levels by E_2. However, *CAR* RNA induction by E_2 was transient, whereas *CAR* protein induction could last for more than 24 h. This suggests that *CAR* RNA and protein kinetics of regulation and stability are different. It is possible that *CAR* protein stability is affected indirectly by E_2 through the synthesis of factors controlling its turnover, which could explain the delayed induction of *CAR* protein expression.

Based on the rapid increase of *CAR* RNA levels, we thus speculated that the regulation of *CAR* gene by estrogens could be the result of a direct transcriptional regulation. Transfection experiments showed that *CAR* promoter was directly regulated by estrogens, through an ERE located in the first 600 bp of *CAR* gene promoter as shown by transfection, ChIPs, and gel shift experiments. The ERE sequence found in *CAR*

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promoter was a palindrome non-consensus sequence with a 3 bp spacing, which is the most common situation found in genes regulated by estrogen (Gruber *et al*. 2004, O'Lone *et al*. 2004).

Estrogens are known to be potent mitogen signals for ER-positive breast cancer cells (Prall *et al*. 1998), which could also potentially involve adhesion molecules such as *CAR*. It was previously shown that estrogen can modulate the expression of other adhesion molecule such as *N-cadherin*, which is upregulated by E_2 (MacCalman *et al*. 1995), or *E-cadherin* that is downregulated by E_2 (Oesterreich *et al*. 2003). These molecules might directly or indirectly modulate cell proliferation, notably through cell architecture reorganization.

The possible role of *CAR* in cancer cell proliferation remains controversial. Most studies have shown in bladder, cervical, ovary cancers, and glioma that *CAR* was repressing cell proliferation (Okegawa *et al*. 2001, Kim *et al*. 2003, Bruning & Runnebaum 2004, Huang *et al*. 2005, Wang *et al*. 2005, Zhang *et al*. 2007), whereas others have shown mitogenic properties for *CAR* in lung cancer (Qin *et al*. 2004, Veena *et al*. 2009). The situation in breast cancer might be unique compared with other cancers. Indeed, it was shown that *CAR* antagonizes the action of apoptosis inducing agents and in turn increases breast cancer cell survival (Bruning *et al*. 2005). In a similar manner, as for lung cancer in which *CAR* silencing reduces tumor growth (Veena *et al*. 2009), we show that *CAR* antibodies or *CAR* siRNA inhibit the estrogen-dependent growth of MCF-7 cells. We did not observe any sign of mortality when cells were treated with the *CAR* antibody, which suggests that *CAR* affects mainly cell proliferation than survival. In addition, we report that stable expression of *CAR* in MCF-7 cells

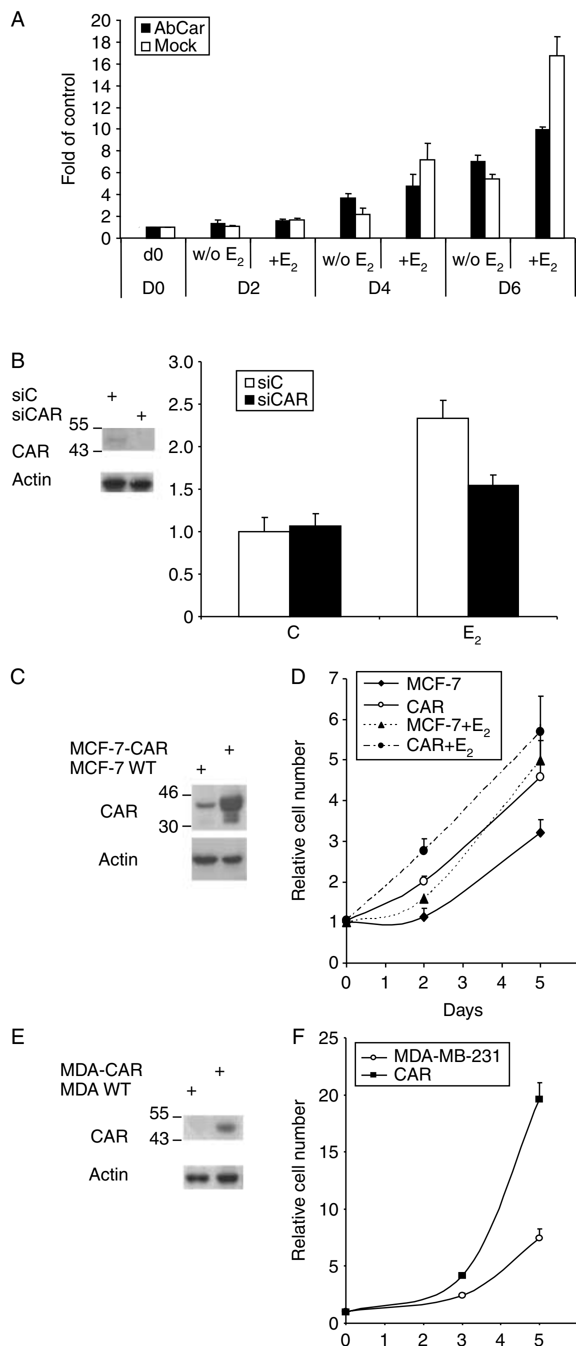

Figure 5 *CAR* enhances breast cancer cell proliferation. (A) MCF-7 cells were grown in DMEM-F12 supplemented with CD-FCS without (w/o E_2), or with 10^{-9} M E_2 (+E_2), and incubated with pre-immune serum (mock), or *CAR* antibody (*CAR* Ab). Cells stably expressing luciferase reporters were lysed at days 2, 4, and 6 and subjected to luciferase activity. Results are expressed as fold of control cells grown in the absence of estradiol at day 0, and in the absence of mouse pre-immune serum and data represent the mean ± s.d. from triplicates. (B) MCF-7 cells were transfected with non-targeting siRNA (siC), or with *CAR* siRNA (siCAR), and treated with 10^{-9} M E_2 (+E_2). Left panel: measure of *CAR* and protein levels in transfected cells by western blot at day 3. β-Actin levels were detected to demonstrate equal loading. Right panel: proliferation was measured at day 3 after transfection, and results are expressed as fold of control cells grown in the absence of estradiol at day 0. Data represent the mean ± s.d. from triplicates. (C) Characterization of the pool of MCF-7-*CAR* stable transfectants by western blot. β-actin levels were detected to demonstrate equal loading. (D) Wild-type MCF-7 cells or MCF-7-*CAR* cells (*CAR*) were grown in DMEM-F12 supplemented with CD-FCS without or with 10^{-9} M E_2 (+E_2). Proliferation rate is expressed as fold of control cells grown in the absence of estradiol at day 0. The data represents the mean ± s.d. from triplicates. (E) Characterization of the pool of MDA-MB-231-*CAR* stable transfectants by western blot. β-actin levels were detected to demonstrate equal loading. (F) Wild-type MDA-MB-231 cells or MDA-MB-231-*CAR* cells (*CAR*) were grown in DMEM-F12 supplemented with FCS. Proliferation is expressed as fold of control cells grown at day 0. Data represent the mean ± s.d. from triplicates.

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enhances both the basal and the estrogen-induced proliferation of these cells. This observation suggests that *CAR* could confer a hormone-independent growth to ER-positive breast cancer cells. Moreover, *CAR* can also increase the proliferation of ERα-negative MDA-MB-231 cells, suggesting that *CAR* could affect different types of breast cancer cells in a similar manner. The mechanisms underlying *CAR* effects on proliferation in breast cancer cells remain to be investigated. We believe that cell-specific factors present in breast cancer cells, and not in cancer cells of other origins, might explain why *CAR* stimulates the proliferation in breast cancer cells, whereas it represses the proliferation in other cancers.

The effects of *CAR* on proliferation, which is mainly described as an adhesion molecule, might not be direct in breast cancer cells. *CAR* is a major component of TJs, as are other molecules such as occludin, claudin, tricellulin, and junction adhesion molecules (JAMs; Coyne & Bergelson 2005). In polarized epithelial cells, *CAR* is detected at the apical pole of the lateral membrane, where it colocalizes with the TJ protein zonula occludens-1 (ZO-1; Cohen *et al*. 2001). Recent reports suggest that TJs could regulate cell proliferation (Tsukita *et al*. 2008). TJs contain aqueous pores or channels that regulate paracellular permeability and serve to divide the apical and basolateral membrane compartments. As a component of TJs, *CAR* could affect ionic or pH conditions around epithelial cells and in turn modulates cell proliferation (Tsukita *et al*. 2008). Another hypothesis is that altering the features of cell adhesion by *CAR* could modify cell signaling and interaction with other cofactors that directly control the proliferation or cell architecture. Increasing evidence suggest that TJs regulate gene expression (Balda & Matter 2009). It has been shown that the cytoplasmic plaque associated with TJs is formed by multiple adaptors, scaffold proteins, and signaling components such as GTP-binding proteins, protein kinases, and phosphatases. These proteins can in turn modulate the expression or the activity of downstream signaling molecules such as cyclins, Jun/fos, or c-myc, which are known to regulate cell cycle (Balda & Matter 2009). In particular, it might be possible that *CAR* could indirectly affect phosphorylation levels of ER, which is a known mechanism for hormone-independent proliferation of ER-positive breast cancer cells. Cell context might explain the opposite effects of *CAR* in breast cancer cells compared with other types of cancers. We cannot exclude the possibility that the presence of ER in breast cancer cells could enable a different network of factors susceptible to act in coordination with *CAR* to elicit cell proliferation.

These results, together with the up-regulation of *CAR* by E_2, suggest that *CAR* could be one of the early events leading to the induced proliferation of breast cancer cells. Our data could open the road for novel strategies to target ER-positive breast cancers.

Declaration of interest

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

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