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Adenosine A1 receptor, a target and regulator of ERα action, mediates the proliferative effects of estradiol in breast cancer

Zhihong Lin[*], **Ping Yin**[*], **Scott Reierstad**[*], **Meghan O'Halloran**[*], **John S. Coon V**[*], **Elizabeth K. Pearson**[*], **Gökhan M. Mutlu**[†], and **Serdar E. Bulun**[*,‡]

[*]Department of Obstetrics and Gynecology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611

[†]Department of Medicine, Division of Pulmonary and Critical Care Medicine, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611

Abstract

Estrogen receptor-α (ERα) and its ligand estradiol (E2) play critical roles in breast cancer growth and are key therapeutic targets. Here, we report a novel dual role of the adenosine A1 receptor (Adora1) as an E2/ERα target and a regulator of ERα transcriptional activity. In ERα-positive breast cancer cells, E2 up-regulated Adora1 mRNA and protein levels, an effect that was reversed by the E2 antagonist ICI 182,780. siRNA ablation of Adora1 in ERα-positive cells reduced basal and E2-dependent proliferation, whereas Adora1 over-expression in an ERα-negative cell line induced proliferation. The selective Adora1 antagonist, DPCPX, reduced proliferation, establishing Adora1 as a mediator of E2/ERα-dependent breast cancer growth. Intriguingly, Adora1 ablation decreased both mRNA and protein levels of ERα and, consequently, estrogen responsive element-dependent ERα transcriptional activity. Moreover, Adora1 ablation decreased binding activity of ERα to the promoter of its target gene TFF1 and led to reduced TFF1 promoter activity and mRNA levels, suggesting that Adora1 is required for full transcriptional activity of ERα upon E2 stimulation. Taken together, we demonstrated a short feed-forward loop involving E2, ERα, and Adora1 that favors breast cancer growth. These data suggest that Adora1 may represent an important target for therapeutic intervention in hormone-dependent breast cancer.

Keywords

Adora1; ERα; estradiol; breast cancer; G protein-coupled receptors; cell proliferation

Introduction

Breast cancer is one of the most common malignancies in women, and is the second leading cause of death for women in the United States (Landis *et al.*, 1999). The mechanisms of

[‡]To whom correspondence should be addressed: Department of Obstetrics and Gynecology, Feinberg School of Medicine, Northwestern University, Chicago, IL, 60611. s-bulun@northwestern.edu Tel: 312-503-0531. Fax: 312-503-0095.

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breast cancer pathogenesis have been intensively studied, and new treatments targeting this disease have emerged. Drugs such as tamoxifen (Shen *et al.*, 2008; Snoj *et al.*, 2008), which inhibit the ability of estrogen to activate the estrogen receptor, or aromatase inhibitors (Hayashi and Yamaguchi, 2005; Howell and Buzdar, 2005), which block aromatase enzyme activity necessary for estrogen production, are used to prevent and treat hormone-responsive breast cancer. Even with aggressive mammographic screening, adjuvant chemotherapy, and intensive therapy for existing cancer however, many of the women who develop breast cancer will die from it. Identification of additional factors that contribute to breast cancer cell proliferation may enhance our understanding of this disease and potentially facilitate the development of novel therapeutic agents.

Estrogen receptor-α (ERα) and its ligand estradiol (E2) play critical roles in breast cancer growth and are important therapeutic targets for this disease (DeNardo *et al.*, 2005; Kun *et al.*, 2003; Pettersson and Gustafsson, 2001). There is a significant interest in understanding the mechanisms by which ERα signaling is regulated in breast cancer and using this knowledge to develop interventions to that inhibit ERα signaling (Boulay *et al.*, 2005; Fan *et al.*, 1999; Namba *et al.*, 2005; Nonclercq *et al.*, 2007). We previously reported that the adenosine A1 receptor (Adora1) is a novel target of ERα (Lin *et al.*, 2007), the *in vivo* expression of which significantly correlates with the presence of ERα in breast cancer.

Adora1 is a member of the G protein-coupled receptor (GPCR) superfamily. Adora1 has been actively studied as a potential drug target for the treatment of fetal hypoxia, Pick's disease, and for the protection of brain from traumatic brain injury and heart from ischemia-reperfusion injury (Albasanz *et al.*, 2007; Kochanek *et al.*, 2006; Merighi *et al.*, 2003; Morrison *et al.*, 2006; Wendler *et al.*, 2007). Based on these varied roles of Adora1, it has also been suggested that the receptor may act as a potent regulator of normal and tumor cell growth by exerting antiapoptotic and prosurvival effects.

Recently, evidence has emerged that Adora1 is over-expressed in various breast cancer cell lines (Mirza *et al.*, 2005). We demonstrated previously that Adora1 is one of many target genes of ERα, and we hypothesized that Adora1 may serve as a mediator of estrogen action in breast cancer growth. Here, we determined whether Adora1 in turn regulates the transcriptional activity of ERα in breast cancer cells. Our findings suggest that Adora1 may play a dual role as a target and a regulator of ERα in breast cancer cells, and a positive feedback loop between ERα and Adora1 signaling may modulate cancer cell proliferation.

Results

Estrogen up-regulates Adora1 mRNA and protein in breast cancer MCF-7 cells

Adora1 was identified as a novel target of ERα in our previous study (Lin *et al.*, 2007). To investigate effect of estrogen on expression of Adora1 in MCF-7 cells, after overnight starvation, various concentrations (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-11} M) of 17β-estradiol (E2) were added to the medium for a period of 3 hours. Real-time PCR was performed to measure the expression of Adora1. E2 treatment significantly up-regulated Adora1 mRNA, with the largest inductions seen at 10^{-5}, 10^{-8}, 10^{-9} M E2, and relatively lower inductions seen at 10^{-6}, 10^{-7}, 10^{-10} and 10^{-11} M E2 (Figure 1A). To explore the

temporal response of Adora1 expression to E2 stimulation, MCF-7 cells were treated with 10^{-8} M concentration E2 for 5 days and the level of Adora1 expression was quantified over time by real-time PCR (Figure 1B). E2 stimulated Adora1 mRNA levels as early as 60 minutes. Adora1 expression reached 8-fold baseline levels at 4 hours, after which the magnitude of stimulation decreased gradually and disappeared by 48 hours. E2 also induced Adora1 protein levels in a time-dependent manner in MCF-7 cells (Figure 1C). A mild E2 induction of Adora1 protein was noted at 3h. A robust E2 induction of Adora1 protein was observed at 24 h, which reached a maximum level at 48 h. The E2 antagonist ICI 182,780 reversed E2-stimulated Adora1 expression (Figure 1D). These results indicated that E2 up-regulates Adora1 mRNA and protein levels in a time-and concentration-dependent manner in MCF-7 breast cancer cells.

Adora1 silencing results in significantly decreased endogenous ERα in MCF-7 cells

Up-regulation of Adora1 expression in MCF-7 cells upon E2 stimulation suggests that Adora1 may play a role in breast cancer progression. Since ERα activation is known to promote cell cycle progression (Aitken and Lippman, 1982; Altucci *et al.*, 1996; Osborne and Schiff, 2005), we examined whether Adora1 modulates ERα function in MCF-7. Specific targeted knockdown of Adora1 mRNA and protein levels was achieved with siRNA in MCF-7 cells (Figure 2A and 2B).

We monitored Adora1 knockdown efficiency by real-time PCR prior to downstream experiments. We repeated Adora1 knockdown 3 times using optimized experimental conditions. Compared with control siRNA-transfected MCF-7 cells, the real-time PCR CT values for Adora1 mRNA in cells transfected with Adora1 siRNA were 4.5- to 5-fold higher; real-time PCR delta/delta CT calculation indicated that Adora1 mRNA levels were significantly lower by 95.6% to 96.9% in cells transfected with Adora1-siRNA. The average knockdown efficiency was 96.2%±0.58 ($p<0.01$, t test).

In the presence of Adora1 siRNA, we found that ERα mRNA was down-regulated to approximately 60% compared with that in non-silenced cells (Figure 2A). Silencing of Adora1 also caused a significant decrease of ERα protein level (Figure 2B). These present results showed that depletion of Adora1 resulted in a marked reduction of both ERα mRNA and protein levels.

To further demonstrate that Adora1-dependent signaling regulates ERα levels, we incubated MCF-7 cells in the presence or absence of the Adora1-selective adenosine antagonist DPCPX (10^3 μM and 10^4 μM) for 12 h and measured ERα protein levels using western analysis. DPCPX is a xanthine derivative compound and highly selective Adora1 ligand (Factor *et al.*, 2007; Haleen *et al.*, 1987). DPCPX inhibits the effect of adenosine on Adora1 by specifically binding to this receptor. DPCPX treatment significantly abolished ERα protein (Figure 2C). This verifies the conclusion that Adora1 knockdown or inhibition of its signaling pathway ablates ERα.

Silencing of Adora1 is associated with a decrease in breast cancer cell proliferation

It has been shown that the Adora1 gene is located proximally to an ERα binding site and that its expression is induced by E_2 (Lin *et al.*, 2007). Here, we showed that depletion of Adora1 resulted in significantly decreased mRNA and protein level of ERα (Figure 2). Because ERα activation and its signaling are critical for cell proliferation (Chalbos *et al.*, 1982; Fu *et al.*, 2006; Laganiere *et al.*, 2005), we tested the possibility that Adora1 modulates ERα-mediated cell proliferation in breast cancer pathobiology. MCF-7 cells were cultured in steroid-deprived medium for 3 days, and siRNA against Adora1 or control siRNA was transiently transfected into the cells for 48 hours followed by 20 to 24-hour treatment with E2 or vehicle. We confirmed Adora1 knockdown by immunoblot (Figure 3A). As shown in Figure 3B, knockdown of Adora1 in MCF-7 cells treated with or without E_2 resulted in significantly decreased cell proliferation (24.9% and 14.8%, respectively) compared with cells transfected with control siRNA (14.0% and 9.5%, respectively). We confirmed this result by quantification of proliferating cell nuclear antigen (PCNA), a marker of cell proliferation and prognosis (23, 24), by immunoblot. Knockdown of Adora1 decreased cell proliferation as shown specifically by a decrease in PCNA immunostaining (Figure 3C). The lowest level of PCNA was observed in Adora1-depleted cells incubated with vehicle. In E2-treated cells, silencing of Adora1 resulted in remarkable decrease in PCNA level compared to that in the non-silenced MCF-7 cells. Data obtained from quantification of PCNA corroborated those from the MTT assay in Figure 3B. To further demonstrate a role of Adora1 in cell proliferation, we inhibited the effect of adenosine on this receptor using the Adora1-selective antagonist, DPCPX, which also attenuated in MCF-7 breast cancer cell proliferation (Figure 3D) and E2-induced cell proliferation by 2.46-fold (Figure 3E). These results indicate that depletion of Adora1 or inhibition of the downstream signaling pathway significantly decreased mRNA and protein levels of ERα, and inhibited E2-induced cell proliferation in MCF-7 cells.

Over-expression of Adora1 is associated with an increase in proliferation of ERα negative MDA-MB-231 breast cancer cells

The inhibitory effect of Adora1 depletion on cell proliferation in ER-positive MCF-7 cell prompted us to investigate whether over-expression of a full-length Adora1 cDNA in ER-negative MDA-MB-231 cells could stimulate cell proliferation. Over-expression of Adora1 increased cell proliferation as shown in Figure 3F specifically by an increase in viable cells measured by MTT assay and by PCNA immunostaining, compared with cells transfected with an empty pcDNA 3.1 vector (control). Transfection with the Adora1 expression vector significantly increased the viable cell numbers. These results further support our conclusion that Adora1 is a mediator of breast cancer cell growth.

Targeted knockdown of Adora1 inhibits ERα transcriptional activity

To determine whether Adora1 mediates ERα transcription, we examined the transcriptional activity of endogenous ERα on an ERE-dependent reporter construct in MCF-7 and T47D cells after siRNA silencing of Adora1. Specific targeted knockdown of Adora1 protein was achieved (Figure 4A in MCF-7 cell and Figure 4C in T47D). Breast cancer cells were cultured in charcoal-stripped serum for 3 days, followed by transfection of control siRNA or

siRNA to Adora1 for 24 hours, then co-transfection with an $(ERE)_2$-Luc reporter construct for 24 hours. Cells were stimulated overnight with E2 or vehicle. As shown in Figure 4, in both MCF-7 (Figure 4B) and T47D (Figure 4D) cell lines and regardless of the absence or presence of E2, ERE-mediated transcription was significantly decreased by the addition of siRNAs directed against Adora1 compared to that in non-silenced cells. In agreement with previous data, these results indicate that silencing of Adora1 significantly reduced ERα level leading to reduced transcriptional activity of ERα.

Adora1 silencing decreases binding of ERα to TFF1 promoter and E2-induced expression of the endogenous ERα target gene- TFF1

We examined whether Adora1 plays a functional role in transcriptional activation of a known ERα target gene, TFF1, by transfecting siRNAs directed against Adora1 in MCF-7 cells. As shown in Figure 5A, knockdown of Adora1 expression resulted in a marked reduction of E2-induced recruitment of ERα to the TFF1 promoter. This result suggested that Adora1 might be required for ERα transactivation of E2-responsive gene. As shown in Figure 5B, a TFF1-luciferase promoter construct was poorly induced by E2 in Adora1 silenced MCF-7 cells compared with Adora1 non-silenced MCF-7 cells. Thus, introduction of siRNAs directed against Adora1 considerably decreased the ability of ERα to stimulate transcription from the TFF1 promoter. Next we investigated whether the presence of Adora1 is required for binding of ERα to the TFF1 promoter. To determine more directly whether Adora1 is involved in regulating expression of ERα target, we examined the effect of inhibiting Adora1 expression on E2-induced expression of ERα target gene, TFF1. The presence of the siRNAs significantly reduced the ability of E2 to stimulate the expression of a selected ERα target, TFF1 (Figure 5C), but not the non-target siRNA. These results suggest that Adora1 is required for the full ERα transcription activity. It also, in agreement with the luciferase assay with ERE-dependent construct performed in MCF-7 cells, confirms the involvement of Adora1 in the regulation of ERα target gene expression. These results demonstrate that Adora1 plays an important role in ERα binding and transcriptional activity and the necessity of Adora1 for full ligand-dependent activity of ERα.

Discussion

In this study, we provide evidence for a novel dual role of the Adora1 as a target and a regulator of E2/ERα action in breast cancer. We show that E2 up-regulates Adora1 mRNA and protein level, and that the inhibition of Adora1 either by RNAi or its selective antagonist attenuated MCF-7 breast cancer cell proliferation by abolishing ERα and its E2-dependent transcriptional activity. These findings suggest the existence of a short feed-forward loop involving E2, ERα, and Adora1 that modulate breast cancer cell proliferation.

Studies have revealed that the ERα gene is regulated at the levels of activity of its transcription factor, such as AP2 (McPherson *et al.*, 1997), transcribed ERα mRNA stability (Kenealy *et al.*, 2000), and its protein degradation (Alao *et al.*, 2004). In present study, we found that ablation of Adora1 resulted in markedly decreased ERα mRNA and protein levels; this may represent a novel mechanism, by which Adora1 decrease the binding of transcription factor, such as AP2 to ERα promoter regions, which result in decreased ERα

mRNA transcribed and protein translated, and therefore the reduced the binding of ERα to its target genes. While our findings do not exclude the possibility that loss of Adora1 may shorten the half-life of ERα message or enhance ubiquitin degradation of ERα, others have suggested that suppression of ERα expression, rather than decreased stability of ERα mRNA and protein, is a more likely mechanism by which ERα activity is regulated (Huang *et al.*, 2006; Lu *et al.*, 2003). The ERα gene contains multiple promoters, some of which are as far as 150 kb upstream of the primary transcriptional start site (Reid *et al.*, 2002). Only a few transcription factors are known to regulate ERα expression (McPherson *et al.*, 2007), including AP2. Additional experiments are needed to elucidate the mechanism of ERα down-regulation by ablation of Adora1.

How Adora1 exerts of its effects on ERα function in the proliferation of human breast cancer MCF-7 cells is also unclear. It is possible that Adora1 cooperates with ERα in regulating E2-dependent cell proliferation. E2 treatment significantly increased expression of Adora1, indicating that Adora1 up-regulation, mediated by ligand-activated ERα, may be involved in breast cancer initiation and progression. It is possible that ERα-mediated E2 signaling might cross-talk with Adora1-mediated signaling through an unknown mechanism that regulates transcription and proliferation.

In this study, we identified Adora1 as being essential for ERα-stimulated TFF1 promoter activity and expression. TFF1 is a prototypic gene representing a subset of ERα target promoters, and has been shown to have a important role in breast cancer cell proliferation (Prest *et al.*, 2002). The results of the present study not only presents a new paradigm in the control of estrogen action but a mechanism by which Adora1 modulates ERα action to regulate specific genes and biological responses. Cooperation of the downstream effector is essential to both initiate and propagate the hormonal signal (Carroll *et al.*, 2005). The ablation of Adora1 resulted in decrease of ERα and E2-induced ERα transcriptional activity. As a consequence, ERα-mediated breast cancer cell proliferation was therefore reduced.

In the study, we demonstrated the inhibitory effect of DPCPX, an Adora1 selective adenosine antagonist, on ERα signaling. DPCPX inhibits the effect of adenosine on Adora1 by specifically binding to this receptor. DPCPX was used under *in vitro* and *in vivo* conditions to evaluate the role of Adora1 in the lungs (Factor *et al.*, 2007), brain (Ilie *et al.*, 2009), gut (Brunsden and Grundy, 1999), heart and kidney (Moosavi *et al.*, 2009). Preliminary experiments revealed that antagonism of Adora1 activity result in a reduction in ERK1/2 phosphorylation (data not shown), which is critical for E2-mediated cell growth (Keshamouni *et al.*, 2002). It has been shown that ERK phosphorylation is associated with increased ERE-mediated transcription in ovarian cancer (Bourguignon *et al.*, 2005) and breast cancer (Kuske *et al.*, 2006). In present study, we found that depletion of Adora1 caused a decrease in both ERα mRNA and protein levels in MCF-7 breast cancer cell line. *In vivo*, we previously demonstrated a significant positive correlation between ERα and Adora1 mRNA levels in ERα+ breast cancer tissues (Lin *et al.*, 2007). Our data may have clinical implications in that the ablation or antagonism of Adora1 appears to inhibit ERα-mediated tumor cell growth. Our data suggest that the blockade of signaling pathways downstream of Adora1 may have a protective effect against tumor development and that the

Adora1 pathway may represent an important target for therapeutic intervention in hormone-dependent breast cancer.

Materials and methods

Cell lines

MCF-7, T47D and MDA-MB-231 cells (American Type Culture Collection, ATCC, Manassas, VA) were maintained in Minimum Essential Medium (MEM, Invitrogen, Carlsbad, CA) containing penicillin (25 U/ml), streptomycin (25 U/ml), insulin (0.01 mg/ml), and 10% fetal bovine serum (FBS). When indicated, the cells were cultured with charcoal-stripped serum for 3 days. After overnight starvation, cells were treated with E2 (10^{-8} M), ICI 182780, or vehicle for the indicated times. Cells were then collected and subjected to real-time PCR analysis, immunoblot, luciferase activity assay, and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Invitrogen, Carlsbad, CA).

Reagents

E2 and ICI 182780 (ICI) were obtained from the Sigma (St. Louis, MO). MTT assay kit was from Invitrogen. Final concentrations of 10^{-8} M and 10^{-6} of E2 and ICI were diluted into culture medium at the indicated times.

Real-time PCR

Total RNA was extracted from cells, treated with DNase I, and reverse transcribed using random hexamers and SuperScript III reverse transcriptase enzyme (Invitrogen). Real-Time PCR was done with SYBR Green or Taqman Real-Time Core Reagents (Applied Biosystems, ABI, Foster City, CA) on the ABI 7000 or 7900 HT Sequence Detection Systems. Primers and probes for quantification of Adora1 and ERα were purchased from ABI. Levels of expression were normalized to the GAPDH gene.

Knock-down of Adora1 by small interference RNA (siRNA)

RNA interference was carried out by using SMARTpool small interfering RNA (siRNA) designed against Adora1 and control (nontargeting) siRNA as a negative control (Dharmacon, Lafayette, CO). The target sequences of siRNA oligonucleotides against Adora1 were: 1) GGAGGAGCCUGGAGUGUAA; 2) GGUAGGUGCUGGCCUCAAA; 3) GGAGUCUGCUUGUCUUAGA; 4) CAAGAUCCCUCUCCGGUAC. To verify that these oligonucleotide sequences in the Adora1 siRNA pool specifically targeted Adora1 but not ERα mRNA, we aligned the four Adora1 siRNA oligonucleotide sequences with ERα mRNA (Locus number: NM_000125 from NCBI DNA database) by Blast alignment from National Center for Biotechnology Information (NCBI). Alignment of these sequences to ERα mRNA did not show any homology, indicating that these siRNA oligonucleotides do not target ERα mRNA. After 3 days of culture in MEM containing 10% charcoal-stripped calf serum, siRNA against Adora1 or control siRNA at a final concentration of 100 nmol/L was transfected into the MCF-7 cells for 48 hours. The cells were then stimulated with E2 (10 nM) or vehicle for 20–24 hours and harvested for analysis.

Plasmid constructs

To generate the estrogen-responsive luciferase reporter construct $(ERE)_2$-Luc for transient transfection assays in breast cancer cell lines, a synthesized oligo contained consensus ERE sites (GTACC*AGGTCACAGTGACCT*GATCAGCTAGTC*AAGGTCACAGTCCT*TCGTAC) was ligated into the blunted *HindIII* site of the pGL4.10 [luc 2] vector (Promega, Madison, WI). The promoter region of the TFF1 gene from nucleotides −428 to −332 (Nunez *et al.*, 1989) was amplified and then ligated into PGL4.10 [luc 2]. The plasmid constructs above were confirmed by sequencing.

To investigate inducible effect of Adora1 expression on cell proliferation, a full-length Adora1 cDNA was cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA). The cDNA has been amplified after reverse transcription of Adora1 mRNA from MCF-7 cells. We used the following primers for PCR amplification: forward primer, 5’ CAC CAT GCC GCC CTC CAT CT 3’ and reverse primer, 5’ GTC ATC AGG CCT CTC TTC TGG 3’. The PCR profile was 3 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 60°C, and 1min at 72°C, and a final extension of 7 min at 72°C. The amplified fragments were analyzed on a 1% agarose gel. The PCR fragments were directly cloned into the pcDNA3.1 expression vector (Invitrogen) as described in the manufacturer's protocol and sequenced to check its fidelity.

Luciferase assay

Hormone-depleted breast cancer cells were transfected with Adora1 or control siRNA (final concentration 100 nM, Dharmacon) using Fugene HD transfection reagent (Roche, Indianapolis, IN) for 24 hours. The cells were then co-transfected with $(ERE)_2$-Luc plasmid or empty vector for 24 hours followed by treatment with or without E2 (10^{-8} M) overnight. Cells were then harvested and assayed for luciferase and pCMVβ-gal (a constitutive β-galactosidase expression vector used to normalize transfection efficiency) activities (Promega).

Over-expression of Adora1 gene in MDA-MB-231 cells

Hormone-depleted MDA-MB-231 cells were transfected with Adora1 expression plasmid or pcDNA3.1 empty vector using Fugene HD transfection reagent for 48 hours. Adora1 expression vector or pcDNA3.1 empty plasmid (24 μg) was transfected to cells cultured in 10cm-dishes, as described in the manufacturer's protocol. Cells were then harvested at 48 hours after transfection and over-expression efficiency was checked by western. Cell proliferations were measured by MTT assay and by immunoblot of proliferating cell nuclear antigen (PCNA) as described below.

Chromatin immunoprecipitation (ChIP)

After knock-down of Adora1 with siRNA, cells were subjected to ChIP with ERα based on a protocol described previously (Lin *et al.*, 2007). The immunoprecipitated and input DNA samples were assayed for binding to the TFF1 promoter region. The primers were: Forward primer, 5’ GGCCATCTCTCACTATGAATCACTTCTGC 3’; Reverse primer, 5’ GGCAGGCTCTGTTTGCTTAAAGAGCG 3’. For PCR, 1 μl of purified DNA was used in

the following PCR profile: 3 minutes at 94 °C, followed by 35 cycles of 30 seconds at 94 °C, 30 seconds at 60 °C, and 30 seconds at 72 °C; and a final extension of 7 minutes at 72°C. The amplified PCR products were analyzed on a 1% agarose gel.

Cell proliferation assay

Cell proliferation was measured by immunoblot of proliferating cell nuclear antigen (PCNA) or by utilizing the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Invitrogen). After transfection and treatment, the MTT reagent was applied for 4 hours and the resulting formazan crystals were dissolved overnight. The result was measured on the plate reader set to record absorbance at 570 nm.

Protein extraction and immunoblotting

Protein was extracted from treated cells and immunoblot was performed by using ERα (Millipore, Billerica, MA), PCNA, or β-actin (loading control) antibodies based on a standard protocol as follows. Aliquots of 20 μg of total protein were separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore). The membrane was blocked for 1 hour at room temperature with 5% milk in TBS followed by hybridization with primary antibodies at a dilution of 1:1000 directed against the following: A1 adenosine receptor (Adora1, rabbit polyclonal, EMD), ERα (rabbit monoclonal, Millipore); PCNA (mouse monoclonal, Millipore); β-actin (mouse monoclonal, 1:10,000 dilution, Sigma). After washing, the membrane was then incubated for 1 hour at room temperature with appropriate horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA) at a dilution of 1:4,000. Immunoreactive bands were detected by a chemiluminescence (Pierce, Rockford, IL) and visualized by autoradiography.

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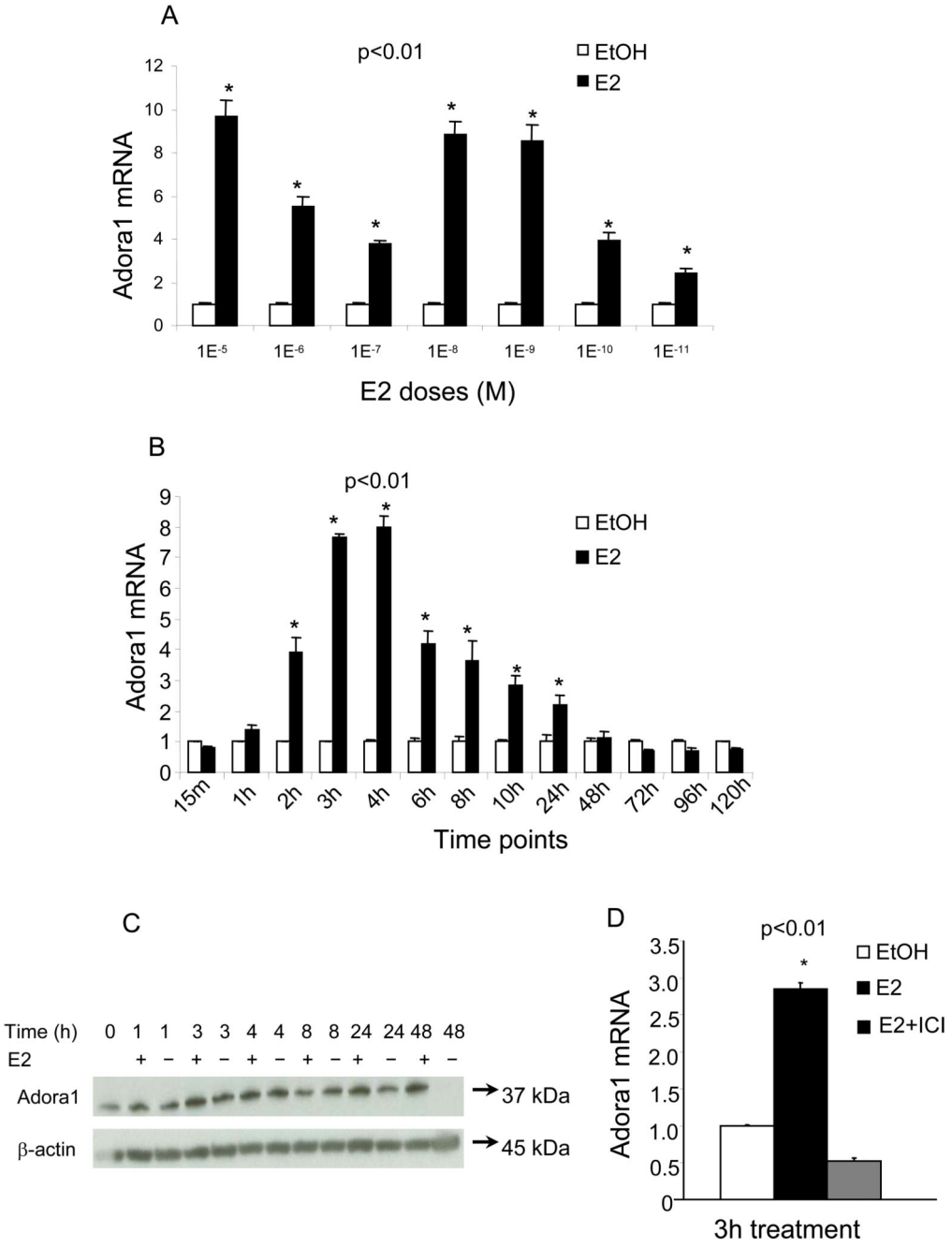

Figure 1.

Effect of E2 treatment on the expression of Adora1 in MCF-7 cells. Serum-starved MCF-7 cells were stimulated with (A) variable concentrations of E2 (ranging from 10^{-11} to 10^{-5} M) or vehicle (EtOH) for 3 hours; (B) E2 (10^{-8} M) or vehicle (EtOH) for 15 minutes or 1, 2, 3, 4, 6, 8, 10, 24, 48, 72, 96 or 120 hours; (C) E2 (10^{-8} M) or vehicle (EtOH) for 0, 1, 3, 4, 8, 24, 48 hours; or (D) E2 (10^{-8} M), ICI 182,780 (10^{-6} M) or vehicle for 3 hours. Cells were harvested, total RNA was isolated, and mRNA levels of Adora1 at each dose and time points were measured by real-time PCR and normalized by GAPDH. Data are the average of 3

replicates ± SD. *, $p<0.01$ determined by t-test. Adora1 protein levels were measured by immunoblot. β-actin was used as a loading control.

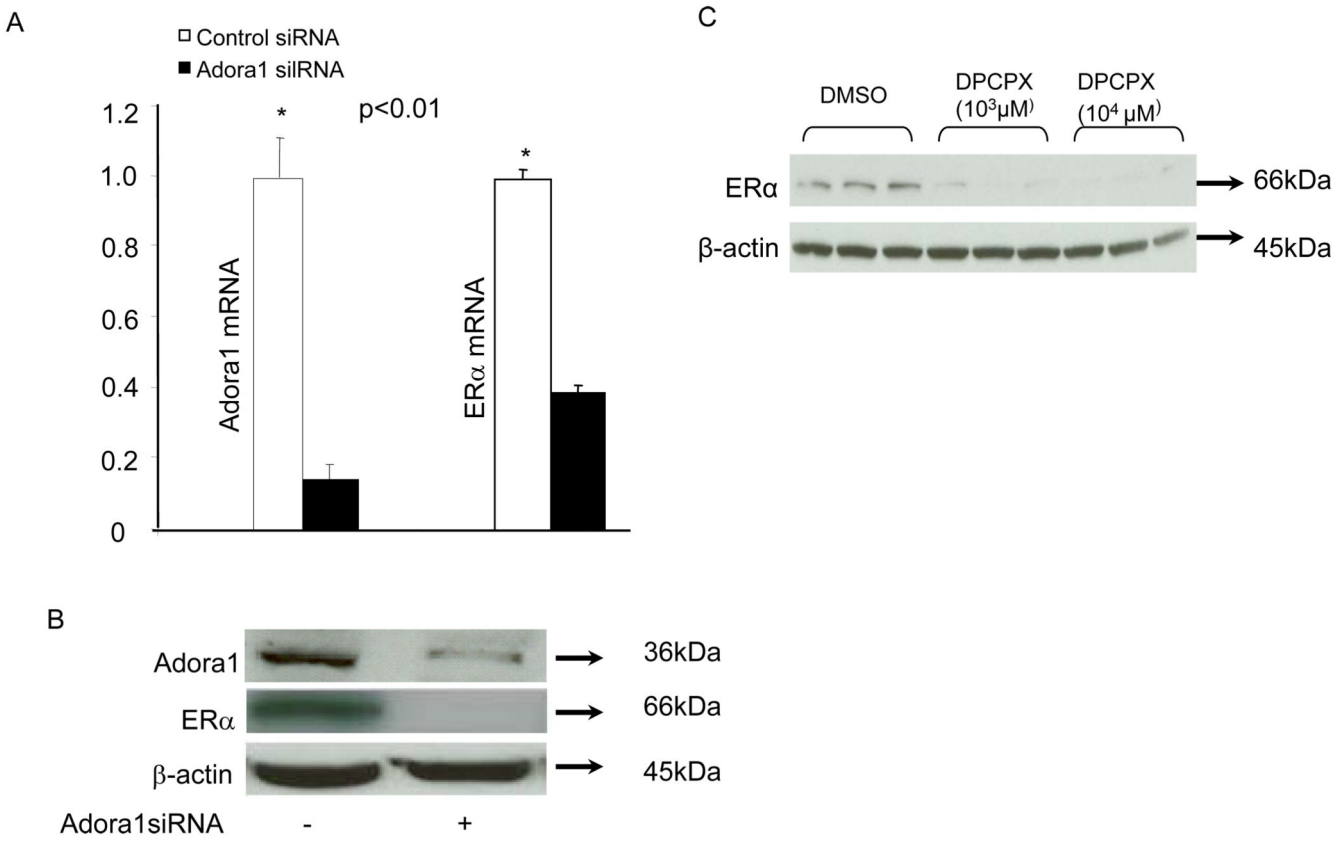

Figure 2.

Inhibition of Adora1 results in significantly decreased endogenous ERα in MCF-7 cells. MCF-7 cells were transiently transfected with an Adora1-targeted siRNA or a control siRNA construct; (A) mRNA levels of Adora1 and ERα were measured by real-time PCR; (B) protein levels of Adora1 and ERα were measured in immunoblot analyses using the indicated antibodies; and (C) MCF-7 cells were treated with vehicle or DPCPX at the concentration of 10^3 μM and 10^4 μM for 12 h, protein levels of ERα were measured in immunoblot analyses using ERα antibody. Data are the average of 3 replicates ± SD. *, $p<0.01$ determined by t-test. β-actin was used as a loading control.

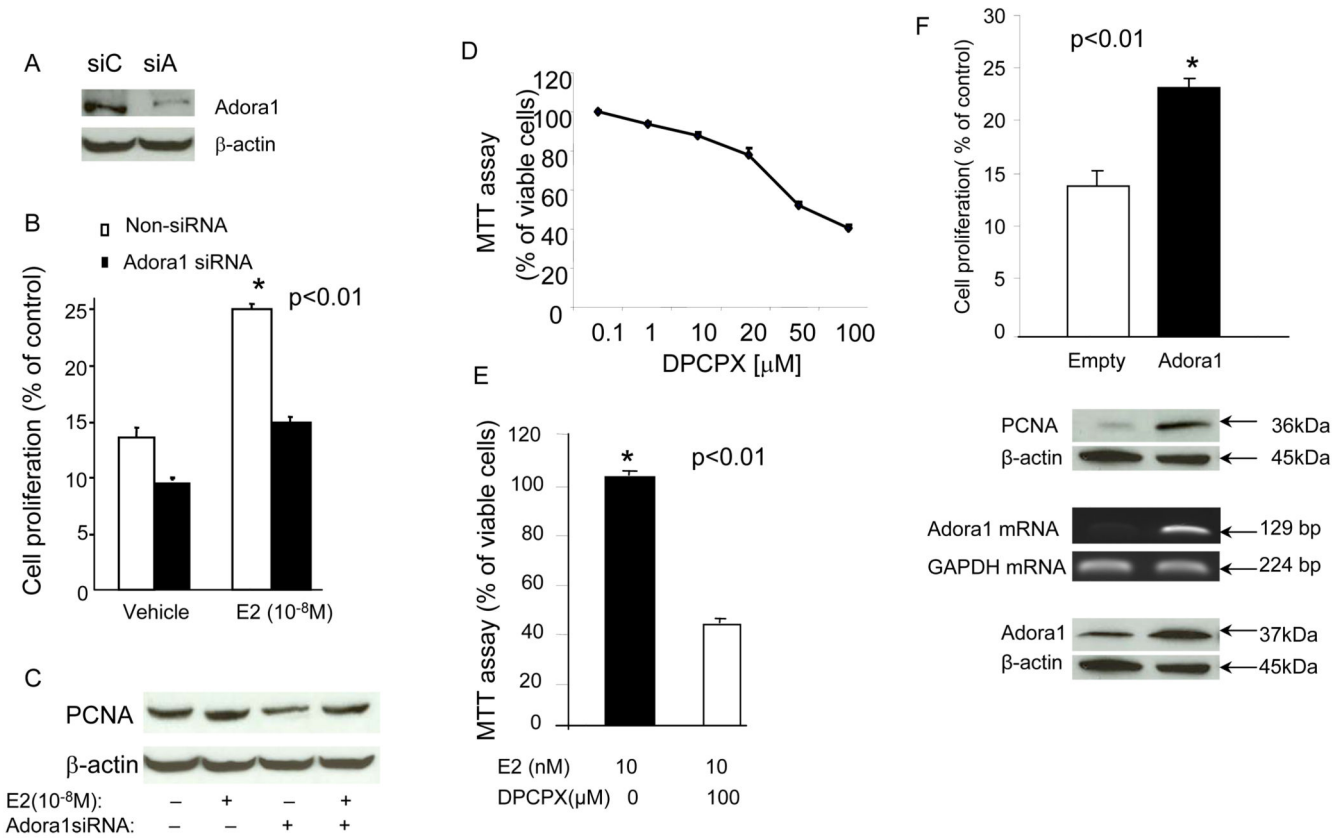

Figure 3.

Inhibition of Adora1 by siRNA in MCF-7 cells leads to reduced cell proliferation. (A) Adora1 protein expression in MCF-7 cells transfected with control (siC) or Adora1 (siA) siRNA. β-actin levels served as a control for specificity and gel loading. Cell proliferation was analyzed by MTT assay and PCNA immunoblot analysis. (B) MCF-7 cells were incubated in the presence of control or Adora1 siRNA in the medium supplemented with 5% charcoal-stripped FBS for 48 hours and stimulated with 10 nM E2 for an additional 24 hours. MCF-7 cells were harvested for determination of cell proliferation by MTT assay. Results represent the mean of at least 3 independent experiments ± SE. (C) Immunoblot of PCNA was performed with mouse anti-human monoclonal PCNA antibody. Control siRNA- or Adora1 siRNA-transfected MCF-7 cells were treated with or without E2 (10 nM) overnight. (D) Serum-starved MCF-7 cells were treated with variable concentrations of the Adora1 antagonist DPCPX or vehicle for 24 hours. The cells were harvested for determination of cell proliferation by MTT assay. (E) Serum-starved MCF-7 cells were treated with E2 (10nM) or E2 (10nM) plus 100μM DPCPX overnight. The cells were harvested for determination of cell proliferation by MTT assay. Data are the average of 3 replicates ± SD. *, $p<0.01$ determined by t-test. (F) Over-expression of Adora1 expression plasmid in serum-deprived MDA-MB-231 cells. Expression of the empty vector (*Empty*) was used as a reference standard. Expression plasmids and empty vector (24 μg) were separately transfected into ERα⁻ MDA-MB-231 cells in 10cm culture dishes. Cells were then harvested 48 h after transfection and over-expression efficiency was checked by Adora1 western. Cell proliferations were measured by MTT assay and by immunoblot of

proliferating cell nuclear antigen (PCNA). Blots were reprobed with β-actin antibody to control for loading *Columns*, mean of three independent experiments; *bars*, SE. *, $p < 0.01$, t test, statistically significant differences.

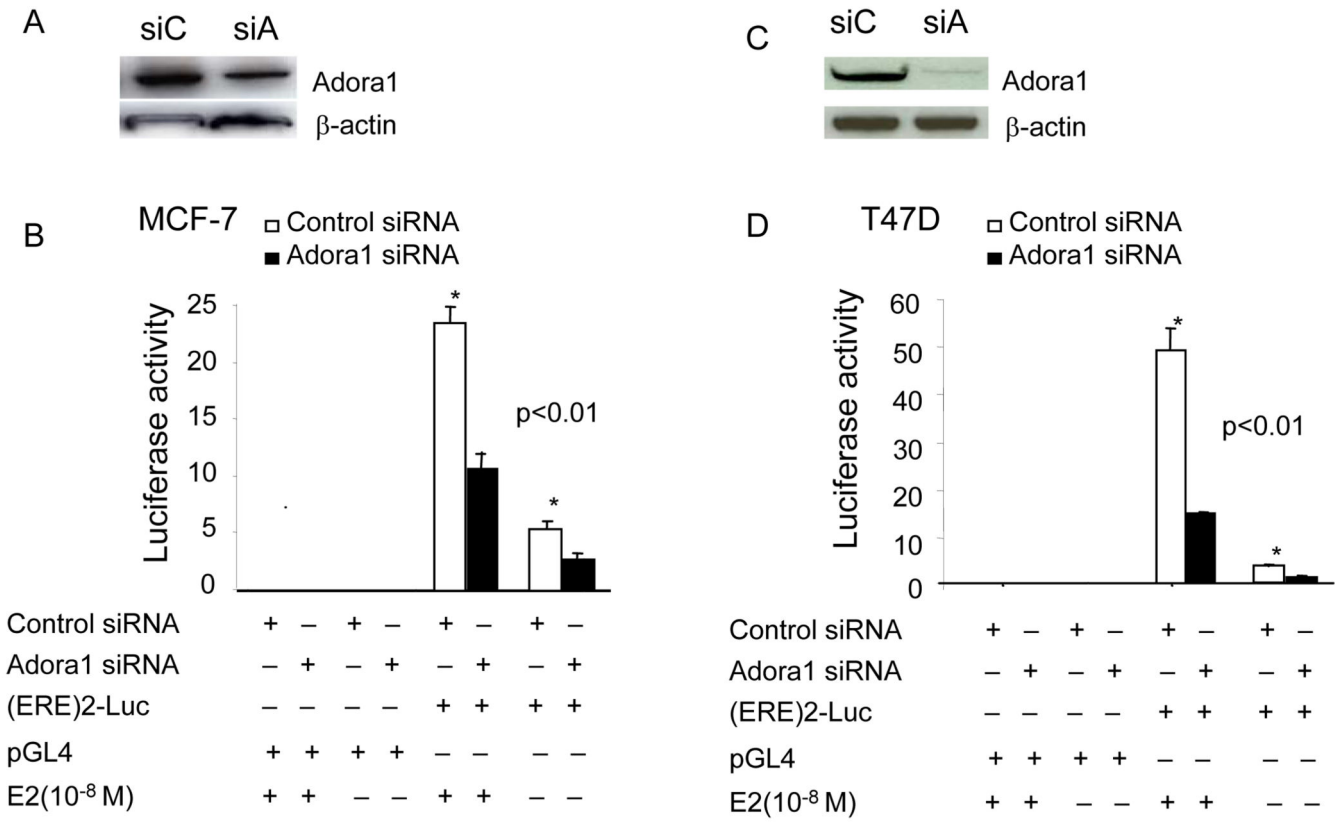

Figure 4.

Adora1 silencing decreases ERα transcriptional activity in breast cancer cells. MCF-7 cells were cultured with charcoal-stripped serum for 3 days. (A) Immunoblot analysis of Adora1 expression in MCF-7 cells and (C) T47D cells transfected with control (siC) or Adora1 (siA) siRNA. β-actin levels were detected to control for specificity and gel loading. (B) MCF-7 cells and (D) T47D cells were co-transfected with control siRNA or Adora1 siRNAs (100 nM), $(ERE)_2$-Luc reporter or pGL4 vector (200 ng) and pCMVβGal (80 ng), in the presence or absence of E2 (10^{-8} M) overnight. Cells were then lysed and assayed for luciferase and β-gal activities. Reported normalized luciferase activities are the means ± SD from 3 independent experiments (*, $p<0.01$ determined by t-test).

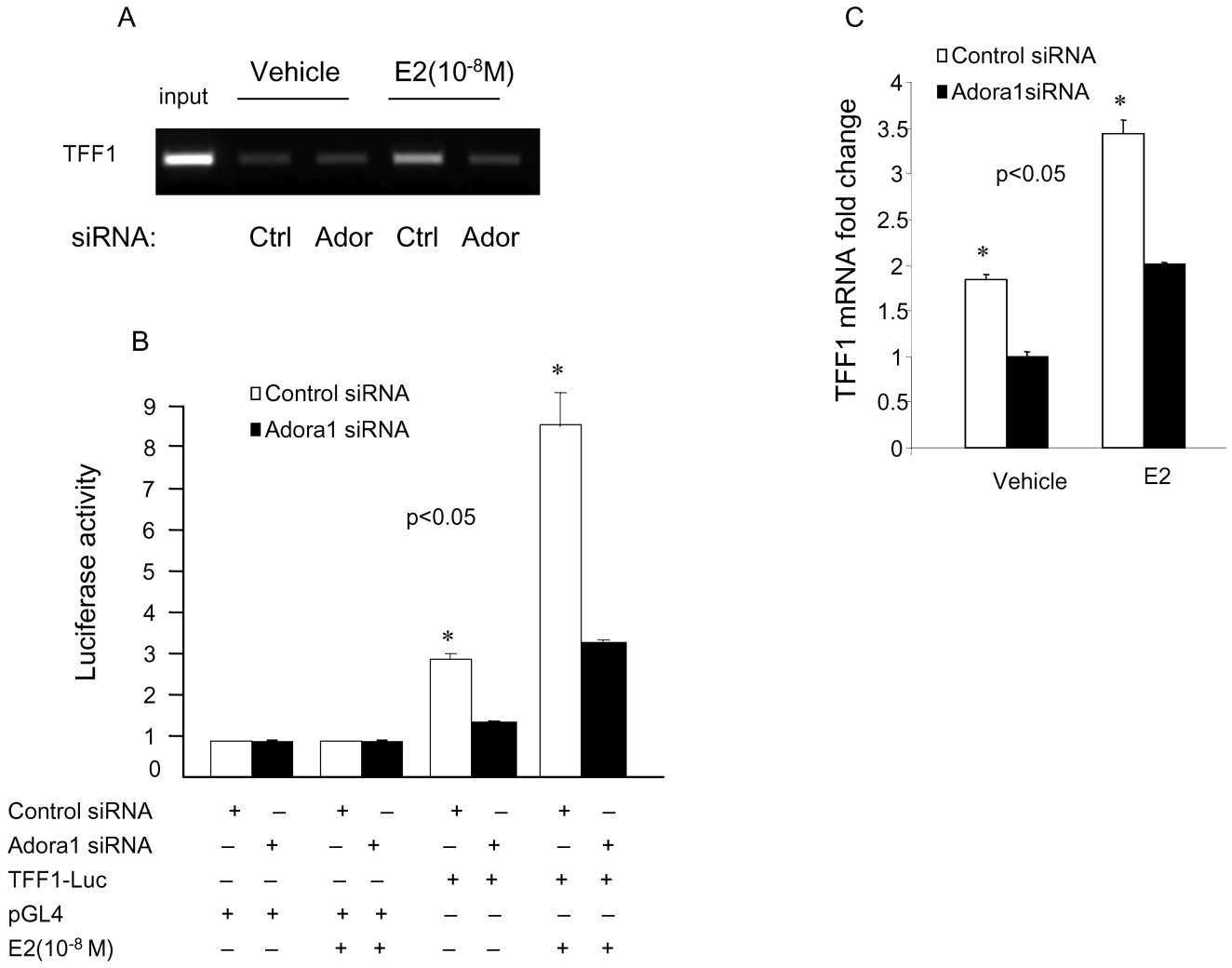

Figure 5.

Silencing of Adora1 in MCF-7 cells leads to reduced binding of ERα to TFF1 promoter, TFF1 promoter driven luferase activity, and mRNA expression of TFF1. (A) Knock-down of Adora1 results in decreased binding of ERa to the TFF1 promoter. B) siRNA knock-down of Adora1 expression decreases ERα-stimulated transcriptional activation of the TFF1 promoter. MCF-7 cells were co-transfected with a TFF1-Luc reporter or pGL4 vector (200ng), pCMVβGal (80ng), and either control or Adora1 siRNA (100 nM) in the presence or absence of E2 (10^{-8}M) overnight. C) Adora1 silencing results in significantly decreased TFF1 expression. MCF-7 cells were transfected either with control or Adora1 siRNA (100 nM) and treated with vehicle or E2 (10^{-8} M) overnight. Cells were then lysed, total RNA was extracted, and mRNA levels of Adora1 were measured by real-time PCR and normalized to GAPDH. Data represent the average of 3 replicates ± SD (*, p<0.05 determined by t-test).