Targeting Estrogen Receptor-α Reduces Adrenocortical Cancer (ACC) Cell Growth *in Vitro* and *in Vivo*: Potential Therapeutic Role of Selective Estrogen Receptor Modulators (SERMs) for ACC Treatment

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Context: Adrenocortical carcinoma (ACC) is a rare tumor with a very poor prognosis and no effective treatment. ACC is characterized by an increased production of IGF-II and by estrogen receptor (ER)-α up-regulation.

Objective: The objective of this study was to define the role played by ERα in 17β-estradiol (E2)- and IGF-II-dependent ACC growth and evaluate whether selective estrogen receptor modulators are effective in controlling ACC growth *in vivo.*

Experimental Design: The human adrenocortical cell line H295R was used as an *in vitro* model and to generate xenograft tumors in athymic nude mice.

Results: In H295R cells IGF-II controlled expression of steroidogenic factor-1 that, in turn, increased aromatase transcription and, consequently, estrogen production, inducing cell proliferation. ERα silencing significantly blocked E2- and IGF-II-dependent cell proliferation. This effect was dependent on the regulation of cyclin D1 expression by ERα, activated in response to both E2 and IGF-II. In fact, IGF-II induced ERα activation by phosphorylating serine 118 and 167. Furthermore, we demonstrated that ERα mediated E2-induced nongenomic signaling that stimulated IGF-I receptor (IGF1R), ERK1/2, and AKT phosphorylation, resulting in a ligand-independent activation of the IGF1R-induced pathway. In addition, E2 potentiated this pathway by up-regulating IGF1R expression as a consequence of increased cAMP-responsive element binding protein activation and binding to IGF1R promoter. The estrogen antagonist, hydroxytamoxifen, the active metabolite of tamoxifen, reduced IGF1R protein levels and both E2- and IGF-II-induced cell proliferation. Moreover, H295R xenograft growth was strongly reduced by tamoxifen.

Conclusion: These findings establish a critical role for ERα in E2- and IGF-II-dependent ACC proliferation and provide a rationale for targeting ERα to control the proliferation of ACC. **(*J Clin Endocrinol Metab* 97: E2238–E2250, 2012)**

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Abbreviations: ACC, Adrenocortical carcinoma; AG, AG1024; AP-1, activator protein-1; CCND1, cyclin D1; ChIP, chromatin immunoprecipitation; CREB protein, cAMP-responsive element binding protein; DHEAS, dehydroepiandrosterone sulfate; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; E2, 17β-estradiol; ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFX, GF109203X; IGF1R, IGF-I receptor; LY, LY294002; MTT, 3-[4,5-Dimethylthiaoly]-2,5-diphenyltetrazolium bromide; OHT, hydroxytamoxifen; pAKT, phosphorylated form of AKT; pCREB, antiphosphorylated cAMP-responsive element binding protein; PD, PD98059; pERK1/2, phosphorylated levels of ERK1/2; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PPT, 4,4′,4′-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol; SERM, selective estrogen receptor modulator; SF-1, steroidogenic factor-1; siRNA, small interfering RNA; TAM, tamoxifen.

Adrenocortical carcinoma (ACC) is a rare tumor with a very poor prognosis. Currently, in localized ACC, only surgery provides a chance for long-term cure. Additionally, recurrence rates as high as 60–80%, after radical resection, have been reported (1, 2), indicating a need for adjuvant treatment. To date, treatment with mitotane remains the common therapy mainly because of its ability to impair adrenal steroidogenesis (3). The lack of specific treatment comes from limited knowledge of molecular mechanisms underlying ACC development. Studies in the past 10 yr suggest that genetic mutations in the adrenal gland lead to the initiation of a malignant tumor (4, 5). The most consistent and dominant genetic change in ACC is the perturbation of the IGF-II locus (11p15) that is imprinted (6). IGF-II is overexpressed in 90% of ACCs together with IGF-I receptor (IGF1R). The direct involvement of the IGF-II/IGF1R system in adrenocortical tumor cell proliferation has also been shown *in vitro* using the adrenal cancer cell line H295R (7). It has been shown in several tissues that, upon ligand binding, the intrinsic tyrosine kinase of the IGF1R caused the activation of phosphatidylinositol 3-kinase (PI3K)/AKT (8) and the Raf-1/MAPK kinase/ERK pathways stimulating cellular proliferation. In addition, receptors for growth factors, like IGF-II, are able to also activate protein kinase C (PKC).

Transgenic mice, overexpressing IGF-II postnatally, were generated and were demonstrated to have adrenocortical hyperplasia, although frank malignancy was not observed (9). This observation suggested that IGF-II is important for the abnormal proliferation of adrenal cells but that additional steps are required for transformation to neoplasia. In addition, the anti-IGF1R monoclonal antibody, figitimumab, has been used in phase I clinical trials for the treatment of refractory adrenocortical carcinoma. However, no objective responses were seen in the refractory ACC patients (10).

CYP19 is the gene encoding for the enzyme (aromatase) responsible for estrogen synthesis using androgens as substrate (11). Although *CYP19* is not usually considered a member of the adrenocortical cytochrome P450 family, we have shown that this enzyme is overexpressed in human ACCs (12). Aromatase has been detected in the H295R adrenocortical cancer cell line by mRNA analyses (13) as well as enzyme activity (14, 15). We have previously shown that H295R cells exhibit estrogen-sensitive proliferation, which can be inhibited by exposure to antiestrogens ICI182,780 and hydroxytamoxifen (OHT) or to the aromatase inhibitor letrozole (15). Estrogens produced by aromatase act by binding nuclear receptor family members estrogen receptor (ER)-α and ERβ. We have demonstrated that ACCs are characterized by ERα up-regulation (12), which seems to mediate the estrogen-dependent proliferative effects (15). Classically, estrogens exert their action by binding and activating ERs that modulate directly (binding to estrogen-responsive elements) or indirectly (interacting with other transcription factors) transcription of target genes. In particular, ERα can bind activator protein-1 (AP-1) and specificity protein-1 sites present within the promoter region of the cyclin D1 gene (*CCND1*), thereby increasing its transcription (16). In addition to nuclear responses, estrogens activate rapid cellular responses known as nongenomic steroid signals (17). Through these signals estrogens are able to activate growth factor signaling. On the other hand, growth factors can stimulate ERα activity independently of estrogens. In fact IGF-I, through PI3K and ERK pathways, increases ERα phosphorylation at serine-167 and -118 (18, 19).

A number of studies have shown that estrogens and the IGF system may functionally interact and potentiate the proliferating effects of the single agents in several tumor tissues, including breast, ovary, and endometrial cancer (20–22). Estrogens increase expression of IGF-II (23) and increase IGF-I binding and IGF1R mRNA expression in MCF-7 breast cancer cells (24), whereas the antiestrogen ICI182,780 decreases IGF1R mRNA levels (25), and antiestrogens, like tamoxifen, can inhibit IGF-mediated growth (26, 27).

The aim of this study was to investigate the role of ERα in 17β-estradiol (E2)- and IGF-II-dependent H295R cell proliferation and to determine whether targeting ERα, using selective estrogen receptor modulators (SERMs), can control ACC growth *in vivo*.

Materials and Methods

Cell culture and tissues

H295R cells were obtained from Dr. W. E. Rainey (Medical College of Georgia, Augusta, GA) (28). Cells were cultured as previously described (15). Cell monolayers were subcultured onto 100-mm dishes for chromatin immunoprecipitation (ChIP) assay (8×10^6 cells/plate), 60-mm dishes for protein and RNA extraction (4×10^6 cells/plate), and 24-well culture dishes for proliferation experiments (2×10^5 cells/well) and grown for 2 d. Before experiments, cells were starved overnight in DMEM/F-12 medium containing antibiotics. Cells were treated with 17β-estradiol (100 nM) (Sigma, St. Louis, MO), 4,4′,4′-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT) (1 μM), 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) (1 μM) (Tocris Bioscience, Bristol, UK), IGF-II (100 ng/ml) (Sigma), AG1024 (AG) (10 μM) (Sigma), PD98059 (PD) (10 μM) (Calbiochem, Merck KGaA, Darmstadt, Germany), LY294002 (LY) (10 μM) (Calbiochem), GF109203X (GFX) (5 μM) (Calbiochem), OHT (10 μM) (Sigma), and IGF1R-blocking antibody αIR3 (1 mg/ml) (Abcam, Cambridge, UK). Where applicable, inhibitors were added 30 min before stimulus.

Fresh-frozen samples of adrenocortical tumors, removed at surgery, and normal adrenal cortex, macroscopically dissected from adrenal glands of kidney donors, were collected at the hospital-based Divisions of the University of Padua and Ancona (Italy). Tissue samples were obtained with the approval of local ethics committees and consent from patients, in accordance with the Declaration of Helsinki guidelines as revised in 1983. Diagnosis of malignancy was performed according to the histopathological criteria proposed by Weiss *et al.* (29) and the modification proposed by Aubert *et al.* (30). Clinical data of the six ACC patients included in this study are shown in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org. All patients were treated with mitotane. The C6 patient stopped mitotane 6 months after beginning of therapy for severe gastrointestinal side effects. The patients C1 and C2 were treated with chemotherapy EAP protocol (etoposide, doxorubicin, and cisplatin) + mitotane.

RNA extraction, reverse transcription, and real-time PCR

Cells were treated for 24 h. Total RNA was extracted with the TRizol RNA isolation system (Invitrogen, Carlsbad, CA). One microgram of RNA from each sample was used for RT-PCR with an ImProm-II reverse transcriptase system kit (Promega, Madison, WI). Quantitative PCR was performed using SYBR Green universal PCR master mix (Bio-Rad Laboratories, Hercules, CA) using *IGF1R*-specific primers: forward, 5′-AAGGCTGTGACCCTCACCAT-3′, reverse, 5′-CGATGCTGAAAGAACGTCCAA-3′, *IGF-II*-specific primers: forward, 5′-TGGCATCGTTGAGGAGTGCTGTTT, reverse, 5′-CATATTGGAAGAACTTGCCCACGG. Each sample was normalized to its 18S rRNA content as previously described (31). Human 18S rRNA primers were purchased from Applied Biosystems (Foster City, CA).

Western blot analysis

Fifty micrograms of protein were subjected to Western blot (32). Blots were incubated overnight at 4 C with the following specific antibodies: 1) anti-pIGF1R antibody (Y1135) (DA7A8) (1:500; Cell Signaling Technology, Beverly, MA); 2) anti-IGF1R antibody (C-20) (1:800; Santa Cruz Biotechnology, Santa Cruz, CA); 3) anti-pERK1/2 antibody (T202/Y204) (1:500; Cell Signaling Technology); 4) anti-ERK1/2 antibody (1:1000; Cell Signaling Technology); 5) antiphosphorylated AKT1/2/3 (Ser473)-R (1:500; Santa Cruz Biotechnology); 6) anti-AKT1/2/3 (H-136) (1:500; Santa Cruz Biotechnology); 7) antihuman P450 aromatase antibody (1:200; Serotec, Oxford, UK); 8) antisteroidogenic factor-1 (SF-1) (1:10,000; provided by Professor Ken-ichirou Morohashi, National Institute for Basic Biology, Okazaki, Japan); 9) antiphosphorylated cAMP-responsive element binding protein (pCREB) antibodies (sererine-133)] (1:1,000; Upstate Biotechnology, Temecula, CA); 10) anti-CCND1antibody (3H2043) (1:1,000; Santa Cruz Biotechnology); 11) anti-pERα (serine-118) (16J4) (1:500; Cell Signaling Technology); 12) antiphosphorylated ERα (serine-167) (D1A3) (1:500; Cell Signaling Technology); and 13) anti-ERα (F-10) antibody (1:1,000; Santa Cruz Biotechnology). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ), and immunoreactive bands were visualized with the enhanced chemiluminescence Western blotting detection system (Amersham). To assure equal loading of proteins, membranes were stripped and incubated overnight with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (FL-335) (1:2000; Santa Cruz Biotechnology).

Assessment of cell proliferation

A 3-[4,5-Dimethylthiaoly]-2,5-diphenyltetrazolium bromide (MTT) assay was conducted to detect cell proliferation. Cells were treated for 48 h in DMEM F-12 medium containing only 2% penicillin-streptomycin. When IGF1R monoclonal antibody was used, it was added 12 h before treatment. Forty-eight hours after treatment, fresh MTT (Sigma), resuspended in PBS, was added to each well (final concentration 0.33 mg/ml). After 30 min incubation, cells were lysed with 1 ml of dimethylsulfoxide (Sigma). Each experiment was performed in triplicate and the OD was measured at 570 nm in a spectrophotometer.

Chromatin immunoprecipitation assay

ChIP was performed as previously described (31). Extracted DNA was resuspended in 20 μl of Tris-EDTA buffer. A 5-μl volume of each sample and input were used for real-time PCR using primers for the *CYP19* promoter II: forward, 5′-TGATGGAAGGCTCTGAGAAG-3′, reverse, 5′-TAGCTCCTGTTGCTTCAGAGG-3′; primers for the CRE site in the *IGF1R* promoter: forward, 5′-CTCGAGAGAGGCGGGAGAGC-3′, reverse, 5′-GGAGCGGGGCCGAGGGTCTG-3′; primers for the AP-1 site in the *CCND1* promoter: forward, 5′-GAGGGGACTAATATTTCCAGCAA-3′, reverse, 5′-TAAAGGGATTTCAGCTTAGCA-3′. PCRs were performed in the iCycler iQ detection system (Bio-Rad), using 0.1 μM of each primer, in a total volume of 50 μl reaction mixture following the manufacturer's recommendations. SYBR Green universal PCR master mix (Bio-Rad Laboratories) was used for gene amplification, following the dissociation protocol. Negative controls contained water instead of DNA. Final results were calculated using the ΔΔCt method as described for the real-time experiments (30), using input cycle threshold values instead of the 18S. The calibrator used was the basal sample.

RNA interference

The ERα small interfering RNA (siRNA) and nontargeting siRNA were purchased from Ambion (Invitrogen). Cells were plated into 60-mm dishes, at 4×10^6 cells, for protein extraction and into 24-well plates, at 2×10^6 cells, for proliferation assay, and used for transfection 48 h later. siRNAs were transfected to a final concentration of 50 nM using the Lipofectamine 2000 reagent, used according to the manufacturer's recommendations (Invitrogen). ERα-specific knockdown was checked by Western analysis of proteins extracted from cells transfected for 48 h and then treated for 24 h. Proliferation was evaluated for cells transfected for 24 h and then treated for 48 h.

RIA

H295R cells were plated and maintained in complete medium for 48 h and then treated as necessary for 48 h in serum-free medium. Estradiol, aldosterone, cortisol, dehydroepiandrosterone sulfate (DHEAS), and IGF-II content of medium recovered from each well were determined using RIA kits (Diagnostic System Laboratories, Webster, TX) following the manufacturer's instructions. Results were normalized to the cellular protein content of each well.

Xenograft model

Four-week-old *nu/nu*-Forkhead box N1*nu* female mice were obtained from Charles River Laboratories Italia [Calco (LC), Italy]. All animals were maintained in groups of five or less and quarantined for 2 wk. The mice were kept on a 12-h light, 12-h dark regimen and allowed access to food and water *ad libitum*. H295R cells, 6×10^6, suspended in 100 μl PBS (Dulbecco's PBS), were injected sc in the flank region of each animal to induce tumors. Resulting tumors were measured at regular intervals using a cutimeter, and the tumor volume was calculated assuming a prolate spheroid shape, as previously described (33), using the following formula: $V = 0.52\ (L \times W^2)$, where L is the longest axis of the tumor and W is perpendicular to the long axis. The mice were treated 7 d after the cell injection, when the tumors had reached an average volume of about 60.2 mm^3. The animals were randomly assigned to be treated with vehicle or tamoxifen (TAM) (Tocris Bioscience), at a concentration of 2 mg/kg · d. For each group, the animals were killed by cervical dislocation at 31 d after cell inoculation. Drug tolerability was assessed in tumor-bearing mice in terms of the following: 1) lethal toxicity, *i.e.* any death in treated mice occurring before any death in control mice; 2) body weight loss percentage = 100 − [(body weight on day x/body weight on day 1) × 100], where x represents a day after or during the treatment period (34, 35). All animal procedures were approved by the local Ethics Committee for Animal Research.

Data analysis and statistical methods

All experiments were conducted at least three times, and the results were from representative experiments. Data were expressed as mean values ± SD, and the statistical significance between control (basal) and treated samples was analyzed with SPSS 10.0 statistical software (SPSS Inc., Chicago, IL). The unpaired Student's *t* test was used to compare two groups. $P < 0.05$ was considered statistically significant.

Results

IGF-II signaling is active in human ACC tissues and in H295R cells and is involved in cell proliferation

Because IGF1R is known to be up-regulated in human ACCs (5), we investigated the expression of genes related to IGF-II signaling in five different human ACC samples. Western analysis revealed high expression levels of IGF1R and the phosphorylated form of AKT (pAKT), compared with normal adrenal but little or no increase in the phosphorylated levels of ERK1/2 (pERK1/2) (Fig. 1A). Treatment of H295R cells, for increasing times, with IGF-II resulted in a rapid increase in IGF1R phosphorylation (Fig. 1B). Similarly, ERK1/2 and AKT were rapidly activated by IGF-II, with maximum induction observed 30 min after treatment (Fig. 1B). Specificity of these activations was confirmed by the use of kinase inhibitors, AG for IGF1R (Fig. 1C), PD for ERK1/2 (Fig. 1D), and LY for PI3K/AKT (Fig. 1E). AG blocked the activation produced by IGF-II on all kinases (Fig. 1, C–E). To confirm that the IGF-II/IGF1R pathway influences the cell cycle in ACC, we evaluated H295R cell proliferation in response to IGF-II, used alone or in combination with AG, PD, LY, and GFX, a specific PKC inhibitor (Fig. 1F). IGF-II induced cell proliferation by 1.3-fold in H295R cells, whereas the presence of AG, LY, and GFX, but not PD, blocked both basal and IGF-II-dependent cell proliferation (Fig. 1F). The most effective inhibition was observed in the presence of AG, which resulted in a 70% reduction in cell proliferation.

SF-1 and aromatase are highly expressed in human ACC tissues and regulated by IGF-II/IGF1R pathway

We have previously shown that ACCs are also characterized by aromatase up-regulation (12). We confirmed these data in our ACC samples and, additionally, showed a marked increase in the levels of pCREB and SF-1 (Fig. 2A), the two most important transcription factors regulating aromatase expression through the PII promoter (36). In H295R cells, aromatase is also highly expressed (15, 36). Here we found that IGF-II, in a dose dependent manner, up-regulated expression of aromatase and SF-1, a transcription factor involved in the regulation of aromatase expression. IGF-II was not able to modify pCREB levels (Fig. 2B). To confirm the involvement of SF-1 in the control of aromatase expression, we performed ChIP analysis that revealed increased binding of SF-1 to the human aromatase PII promoter after IGF-II treatment; this binding was decreased by AG, LY, and GFX but not PD (Fig. 2C). No changes were observed in pCREB binding levels (Fig. 2D). Consequently, IGF-II caused a significant increase in E2 production by H295R cells, which was prevented by the concomitant presence of AG, LY, and GFX but not PD (Fig. 2E). Similar effects also were produced on the other adrenal steroids aldosterone, cortisol, and DHEAS (Supplemental Fig. 1, A–C). Treatment of H295R with E2 and the ERα-specific agonist PPT caused a significant increase in cell proliferation, whereas the ERβ agonist DPN had no effect (Fig. 2F).

ERα silencing blocks E2- and IGF-II-dependent H295R cell proliferation

Results indicated in Fig. 2F demonstrate that ERα was involved in E2-dependent H295R cell proliferation, confirming our previous data (15). We wanted to verify whether this receptor was also involved in IGF-II-dependent cell proliferation. The use of a specific ERα siRNA resulted in silencing of gene expression in H295R cells (Fig. 3A); under these conditions a reduction in basal (70%), E2- (77%), and IGF-II-induced (72%) cell proliferation was observed (Fig. 3B). We then examined the

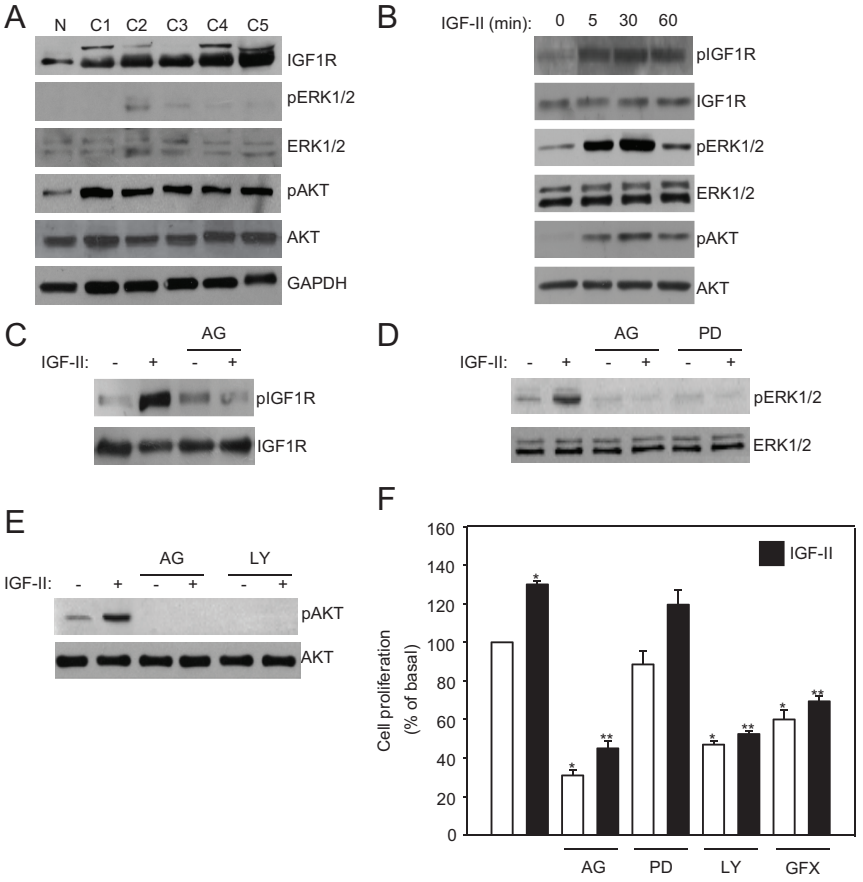

FIG. 1. IGF-II signaling is active in human ACC tissue and in H295R cells and is involved in cell proliferation. A, Immunoblot analyses for IGF1R, pAKT, and pERK1/2 were performed on 50 μg of total proteins extracted from human normal adrenal tissues (N) and ACCs (C1–C5). B, Western blot analyses of phosphorylated IGF1R, pERK1/2, and pAKT were performed on 50 μg of total protein extracted from H295R cells, untreated or treated, for the indicated times, with IGF-II (100 ng/ml). GAPDH was used as a loading control. Results are representative of three different experiments. C, Immunoblot analysis for phosphorylated IGF1R was performed on 50 μg of total protein extracted from H295R cells, untreated or treated for 10 min, with IGF-II (100 ng/ml) and AG (10 μM) alone or in combination. IGF1R was used as a loading control. Results are representative of three different experiments. D, Immunoblot analysis for pERK1/2 was performed on 50 μg of total protein extracted from H295R cells, untreated or treated for 10 min, with IGF-II (100 ng/ml) and AG (10 μM) and PD (10 μM), alone or in combination. Total ERK1/2 was used as a loading control. Results are representative of three different experiments. E, Immunoblot analysis for pAKT was performed on 50 μg of total protein extracted from H295R cells, untreated or treated for 10 min, with IGF-II (100 ng/ml), AG (10 μM) and LY (10 μM), alone or in combination. Total AKT was used as a loading control. Results are representative of three different experiments. F, H295R cells were left untreated or treated for 48 h with AG (10 μM), PD (10 μM), LY (10 μM), GFX (5 μM), alone or in combination with IGF-II (100 ng/ml). H295R proliferation was evaluated by an MTT assay. Results are representative of three independent experiments. Statistically significant differences are indicated. *, $P < 0.05$ compared with basal; **, $P < 0.01$, compared with IGF-II.

ability of a specific IGF1R monoclonal antibody (αIR3) (37) to control E2- and IGF-II-dependent H295R cell proliferation. The presence of IGF1R blocking antibody decreased IGF1R protein levels (Fig. 3C). Additionally, basal and IGF-II-dependent cell proliferation decreased by 50% in the presence of αIR3, whereas the same antibody was able to inhibit E2-induced cell growth by only 25% (Fig. 3D). The data shown in Fig. 3 suggest that the inhibition of IGF1R activation can only partially reverse H295R cell growth because of the involvement of E2 dependent and IGF1R-independent mechanisms.

E2 and IGF-II increase cyclinD1 expression via ERα

To clarify how ERα is capable of regulating IGF-II-dependent cell growth, we evaluated whether, in our cell model, IGF-II via IGF1R was able to activate ERα in a ligand-independent manner. One hour of treatment with IGF-II caused a dose-dependent increase in ERα phosphorylation on both serine-118 and serine-167 (Fig. 4A). Furthermore, the concomitant presence of AG, LY, and GFX, but not PD, was able to reverse this activation (Fig. 4B). High phosphorylation levels were also found in ACC tissues in which we confirmed higher ERα expression (Fig. 4C). To correlate ERα activation with cell cycle progression, we evaluated the effects of E2 and IGF-II on *CCND1*, a gene regulated by ERα, after activation with E2 or through IGF1R transductional pathways (38), which we found overexpressed in human ACC tissues (Fig. 4D). Treatment with IGF-II, E2, and PPT caused an increase in CCND1 protein levels (Fig. 4E). Furthermore, both IGF-II and E2 increased ERα binding to the AP-1 site of the *CCND1* promoter (Fig. 4F).

E2 bound to ERα increases IGF1R activation and expression

We also evaluated whether E2 binding ERα, through a rapid, nongenomic signaling, was able to activate IGF1R signaling in a ligand-independent manner as seen in normal and malignant cells of various origins (39). We treated H295R cells for increasing times with E2 (Fig. 5A) and found that IGF1R, ERK1/2, and AKT were rapidly phosphorylated. To demonstrate that the effect was directly dependent on ERα, we also used PPT, which was able to reproduce the effects seen with E2 (Fig. 5B). Both ERK1/2 and Akt phosphorylation are mediated by IGF1R because the presence of AG decreased both E2 and PPT effects (Fig. 5C). We further investigated the ability of estrogen to interact with IGF-II signaling by evaluating E2 effects on IGF1R expression. H295R cells were

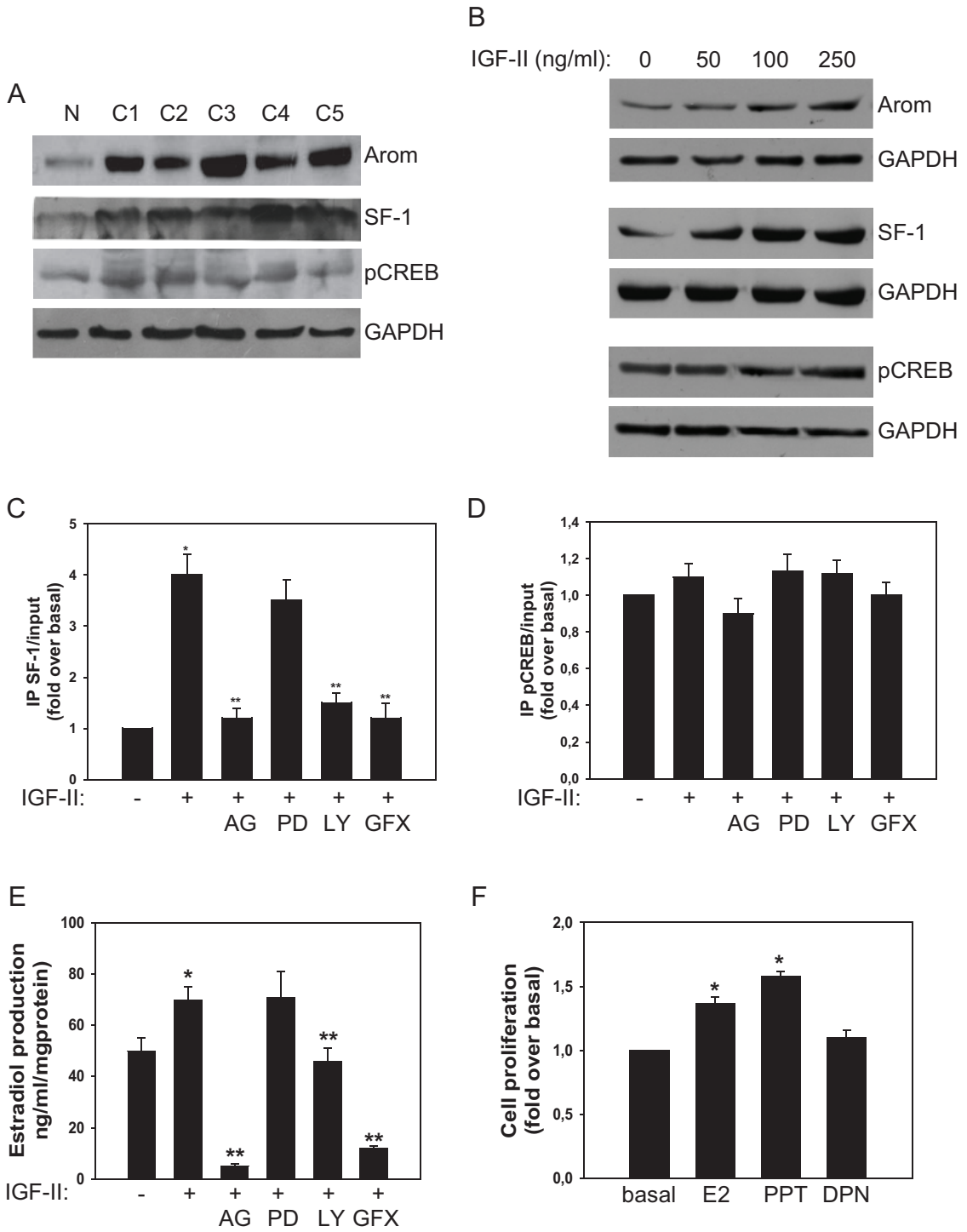

FIG. 2. SF-1 and aromatase are highly expressed in human ACC tissues and regulated by the IGF-II/IGF1R pathway. A, Immunoblot analyses of aromatase (Arom), SF-1, and pCREB in human normal adrenal tissues (N) and ACCs (C1–C5). B, Immunoblot analyses of Arom, SF-1, and pCREB in H295R cells treated with IGF-II at the indicated doses. C and D, H295R cells were treated with AG (10 μM), PD (10 μM), LY (10 μM), and GFX (5 μM), in combination with IGF-II (100 ng/ml). *In vivo* binding of SF-1 (C) or pCREB (D) to the aromatase PII promoter was examined using a ChIP assay after 24 h treatment. E, E2 content in H295R culture medium was determined by RIA and normalized to the cell culture well protein content. F, H295R cells were left untreated (basal) or treated for 48 h with E2 (100 nM), PPT (1 μM), and DPN (1 μM). H295R cell proliferation was evaluated by an MTT assay. Results are representative of three independent experiments. *, $P < 0.001$ compared with basal; **, $P < 0.001$ compared with IGF-II.

treated with E2 and PPT for 24 h, and IGF1R expression was evaluated at both mRNA and protein levels. Results obtained demonstrated a significant increase in IGF1R levels in response to E2 and PPT (Fig. 5, D and E). In an attempt to define how estrogens could induce IGF1R expression, we evaluated whether E2 and PPT could increase levels of pCREB, a transcription factor controlling IGF1R gene transcription (40). We found that in our cell line,

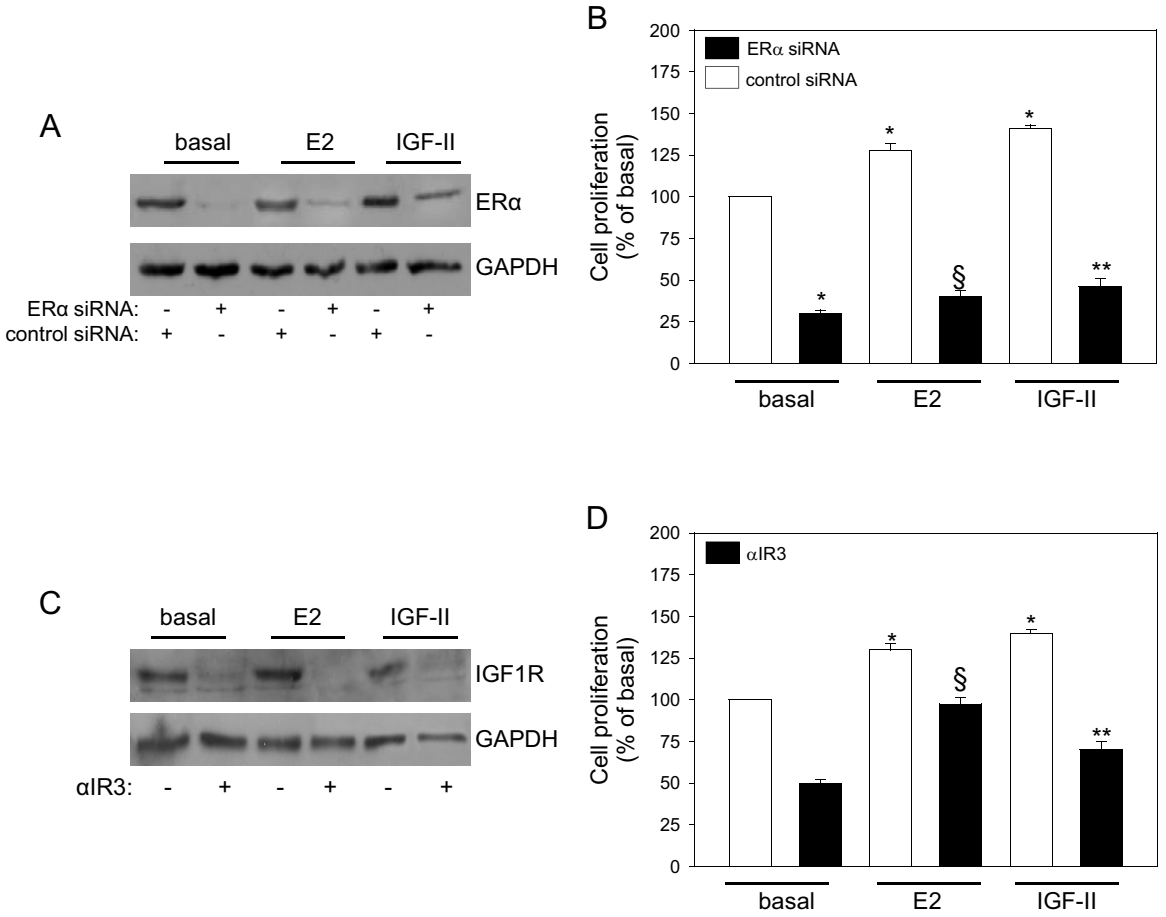

FIG. 3. ERα silencing blocks E2- and IGF-II induced H295R cell proliferation. A and B, H295R cells were transfected with ERα siRNA or a nontargeting siRNA (control siRNA). Twenty-four (A) or 48 h (B) after transfection, cells were treated for an additional 48 h (A) or 24 h (B) with IGF-II, E2 or vehicle (basal). C and D, Where indicated, H295R cells were incubated overnight with αIR3 before being treated for an additional 48 (C) or 24 h (D) with IGF-II, E2, or vehicle (basal). Immunoblot analyses of ERα (A) and IGF1R (C) were performed on 50 μg of total protein. GAPDH was used as a loading control. Results are representative of three independent experiments. B and D, Reduction of IGF-II- and E2-induced H295R cell proliferation by ERα (B) and αIR3 (D) siRNA and evaluation by an MTT assay. *, $P < 0.0001$ compared with basal; **, $P < 0.001$ compared with IGF-II; §, $P < 0.01$ compared with E2.

pCREB was strongly activated by E2 and PPT treatment, an effect abrogated by the presence of AG (Fig. 5C). These data suggested that ERα, via IGF1R activation, leads to phosphorylation of downstream targets involved in IGF1R regulation. Moreover, ChIP experiments clarified that E2 enhanced, by 2-fold, pCREB binding to the *IGF1R* promoter (Fig. 5F). Collectively these data suggest that E2, through ERα, increases IGF1R activation and expression.

OHT-inhibiting ERα reduces IGF1R expression, H295R cell proliferation, and ACC growth *in vivo*

We used OHT, which has been shown to inhibit ERα-dependent cell proliferation (15) to evaluate whether it was able to control IGF1R expression. Western blot analyses indicated that OHT reduces both E2- and PPT-induced IGF1R levels (Fig. 6A). OHT was also able to inhibit both E2- and IGF-II-dependent cell proliferation (Fig. 6B), and this was associated with a decreased CCND1 expression (Fig. 6C). We then examined whether targeting ERα could control ACC growth *in vivo*. For this purpose, H295R cells were used to generate xenograft tumors in athymic nude mice, which were subsequently treated with TAM. All H295R-injected mice (n = 40 from two independent experiments) developed a detectable tumor. Tumors from each group, control and TAM treated, were followed up and measured during the 31-d experiment. Tumor volume was calculated for each mouse at each time point. Figure 6D shows the tumor growth rate in the two treatment groups. Data were plotted as mean tumor volume in each group over time. TAM administration resulted in a statistically significant decrease in tumor volume, starting after 12 d of treatment. Specifically, tumor mass size decreased by 46.7% compared with the beginning of treatment and by 56.7% compared with control mice (*, $P < 0.01$). A trend of growth inhibition was observed thereafter. At d 16 a reduction by 65% was observed compared with the beginning of treatment and by

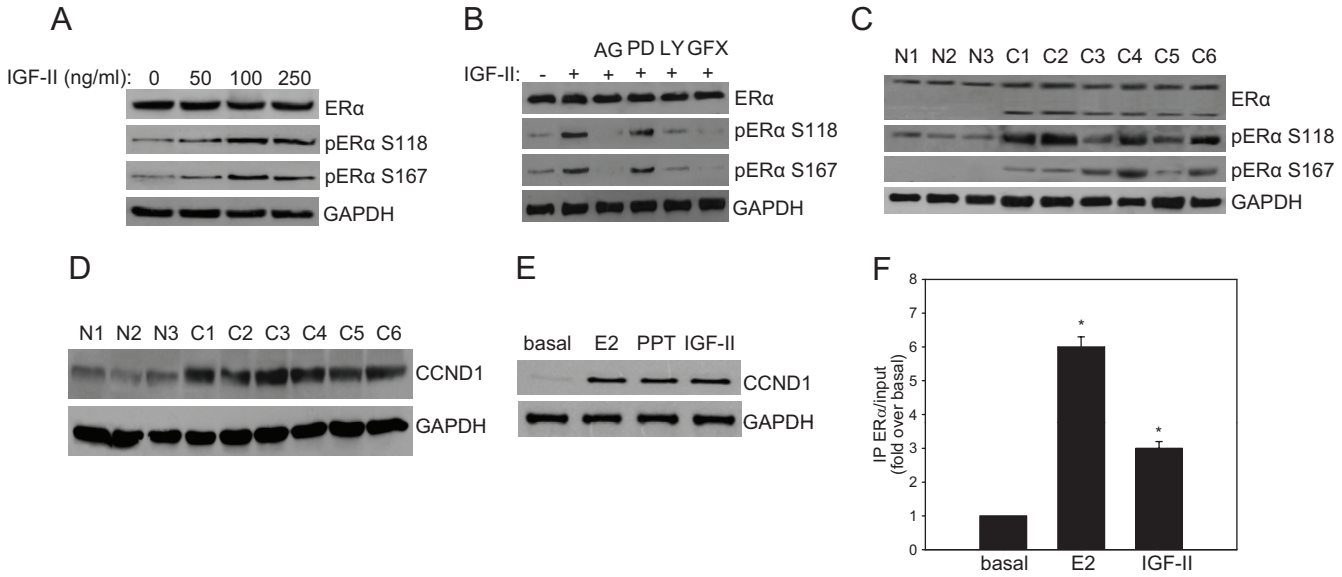

FIG. 4. E2 and IGF-II increase ERα recruitment to cyclin D1 promoter. A, Immunoblot analyses of phosphorylated ERα serine-118 (S118) and phosphorylated ERα serine-167 (S167) were performed on 50 μg of total protein extracted from H295R cells untreated (basal) or treated for 1 h with increasing doses (nanograms per milliliter) of IGF-II. B, Immunoblot analyses of phosphorylated ERα S118 and phosphorylated ERα S167 were performed on 50 μg of total protein extracted from H295R cells untreated (basal) or treated for 1 h with of IGF-II (100 ng/ml) alone or combined with AG (10 μM), PD (10 μM), LY (10 μM) and GFX (5 μM). C and D, Immunoblot analyses for phosphorylated ERα serine 118 (phosphorylated ERα S118), phosphorylated ERα serine 167 (phosphorylated ERα S167) (C) and CCND1 (D) in human normal adrenal tissues (N1–N3) and ACCs (C1–C6). E, Immunoblot analyses of CCND1 were performed on 50 μg of total protein extracted from H295R cells untreated (basal) or treated for 24 h with E2 (100 nM), PPT (1 μM), or IGF-II (100 ng/ml). GAPDH was used as a loading control. F, *In vivo* binding of ERα to the CCND1 promoter, after 1 h treatment, was examined using a ChIP assay. *, $P < 0.001$ compared with basal.

73.4% compared with untreated control mice ($P < 0.001$). At d 20 comparable percentages of inhibition were observed. The reduction of mass size was persistent up to d 24 with an inhibition of 70% compared with the beginning of treatment in TAM groups and of 80.6% compared with untreated control mice (**, $P < 0.001$) (Fig. 6D).

The drug was well tolerated without lethal toxicity or body weight loss during treatment (data not shown).

Discussion

The current therapy for adrenocortical carcinoma includes the use of mitotane, a drug with cytotoxic effects controlling steroid secretion, which, however, shows modest efficacy on metastatic disease (41). In addition, an anti-IGF1R monoclonal antibody has been used in phase I clinical trials for the treatment of refractory ACC. However, no objective responses were seen in the refractory ACC patients (10). The main purpose of this study was to demonstrate that ERα represents a good target to control ACC growth and that SERMs blocking ERα activity are effective in controlling ACC growth *in vivo*. We hypothesized that in ACC, ERα can be activated in a ligand-dependent manner by E2 and in a ligand-independent manner by IGF-II/IGF1R. In addition, both E2 and IGF-II can activate pathways that influence the proliferative effects of the other hormone: IGFII increases local estrogen production, whereas E2 increases IGF1R expression and its ligand independent phosphorylation.

In the first part of our study, we investigated whether IGF-II signaling regulates estrogen production in ACC. Our data on human ACC samples demonstrated an increase in the expression of proteins associated with the IGF pathways, particularly IGF1R and the phosphorylated form of AKT, but not ERK1/2. A similar expression pattern was found in H295R cells in response to IGF-II treatment. Evaluation of proliferative behavior demonstrated that IGF-II increased cell proliferation, which was blocked by inhibitors for IGF1R, PKC and AKT but not for ERK1/2. These data suggest that ERK1/2 do not seem to be involved in IGF-II/IGF1R-dependent adrenal tumor cell proliferation.

We also evaluated the effect of IGF-II/IGF1R activation on the major steroids produced by H295R cells (aldosterone, cortisol, and DHEAS). Our results showed a moderate increase in the production of these hormones after IGF-II treatment that was completely blocked by the use of IGF1R inhibitor AG and partially inhibited by LY and GFX. The presence of PD seemed to increase steroid hormones production. These data indicate that activation of IGF-II/IGF1R-dependent signaling pathways can contribute to modulate steroid production observed in most ACCs. The presence of a different steroid secretion pattern in different subtypes of ACC could depend on the differ-

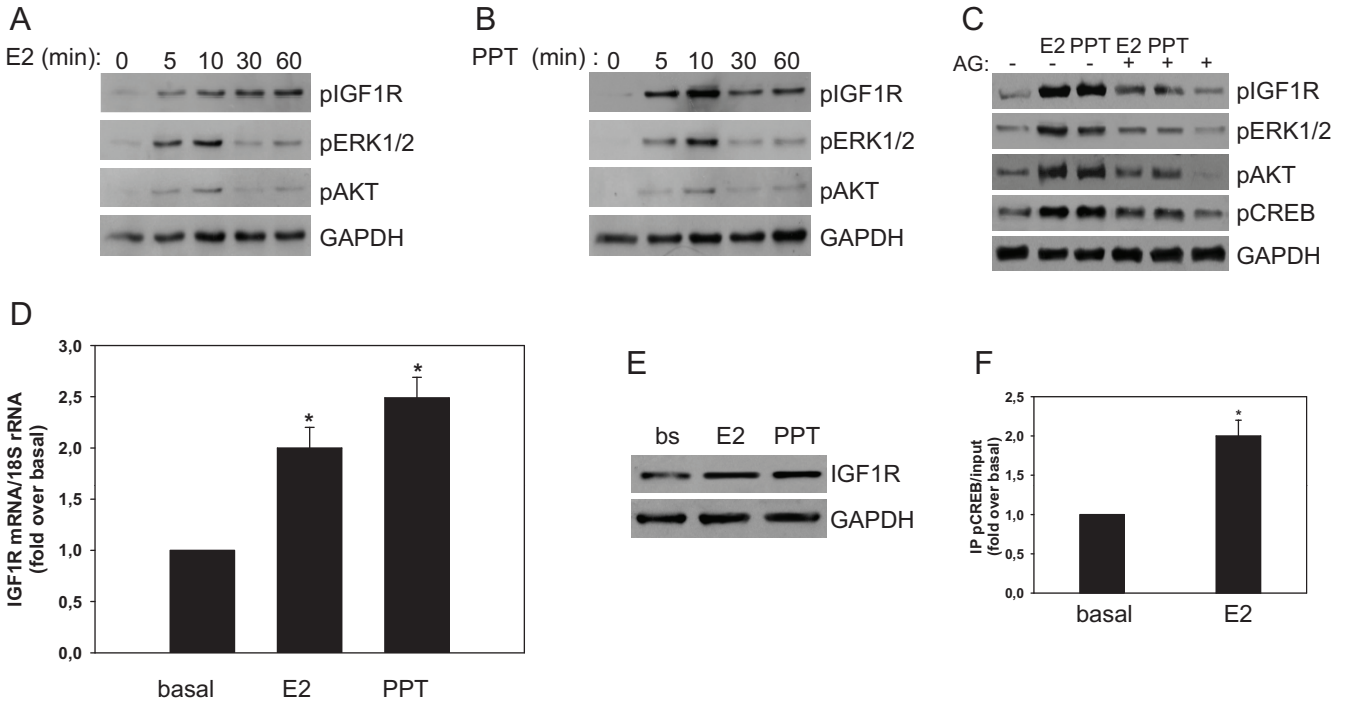

FIG. 5. E2, binding ERα, increases IGF1R activation and expression. A and B, Immunoblot analysis for phosphorylated IGF1R, pERK1/2, and pAKT in H295R cells treated for the indicated times with E2 (100 nM) (A) and PPT (1 μM) (B). C, Immunoblot analysis for phosphorylated IGF1R, pERK1/2, pAKT, and pCREB in H295R cells were left untreated [basal (bs)] or treated with E2 (100 nM) and PPT (1 μM), alone or combined with AG (10 μM) for 1 h. D, Total RNA was extracted, and real-time RT-PCR was used to analyze IGF1R mRNA levels. E, Western blot analysis of IGF1R was performed on 50 μg of H295R total protein. F, After chromatin immunoprecipitation, using an anti-pCREB antibody, total DNA was extracted and real-time RT-PCR was used to analyze pCREB binding to an IGF1R promoter after 24 h treatment with E2 (100 nM). *, $P < 0.01$ compared with basal.

ential expression and activity of transcription factors and cofactors (*i.e.* SF-1, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1, fos, jun, GATA, nerve growth factor IB) contributing to adrenal steroidogenic enzyme expression (42–45).

In H295R cells IGF-II increased aromatase expression controlling SF-1 levels and its binding to the PII promoter, the promoter used in H295R cells (36), with a mechanism similar to what we have previously demonstrated in tumor Leydig cells (32). MAPKs do not seem to play a role in aromatase expression (Fig. 2C); in fact, they do not influence SF-1 binding to the aromatase promoter and do not influence E2 production. In contrast, the inhibitors LY and GFX decrease SF-1 binding to the PII promoter and consequently E2 production (Fig. 2E). A very recent publication reported SF-1 staining in 158 of 161 analyzed ACCs (98%), indicating that SF-1 is a valuable immunohistochemical marker for this type of tumor (46). Our results showing the up-regulation of SF-1 in ACCs are in agreement with this study. IGF-II, then, by regulating aromatase expression, increases E2 production. Binding of locally produced estrogen to ERα could activate an autocrine/intracrine mechanism involved in H295R cell proliferation. A role for estrogen could be hypothesized by the observation that many studies describe a female predominance among adrenocortical cancer patients (ratio about 1:1.5) (47, 48). However, several studies have indicated that in patients with estrogen-dependent breast, endometrial, and ovarian cancers, especially in postmenopausal women, intratumoral estrogens derived from *in situ* aromatization could function as an autocrine growth and a mitogenic factor and could impart a growth advantage to these cancer cells, regardless of serum concentration of estrogens (49). Furthermore, in some tissues or cells in adenocarcinoma of the prostate, it has also been observed that biosynthesis takes place without release into the extracellular space as intracrine activity (50). In an intracrine system, serum concentrations of hormones do not necessarily reflect the local hormonal activities in the target tissues. We believe that ACCs could have the characteristic of an intracrine/autocrine regulated tumor similarly to tumors of the breast, ovary, and endometrium. The involvement of the ERα subtype in this mechanism was suggested by the ability of the selective ERα agonist PPT, but not the ERβ agonist DPN, to induce cell proliferation. DPN was also unable to induce IGF1R phosphorylation and mRNA levels (data not shown), suggesting that ERβ activation, by itself, is not sufficient to mediate E2 effects on H295R cell growth.

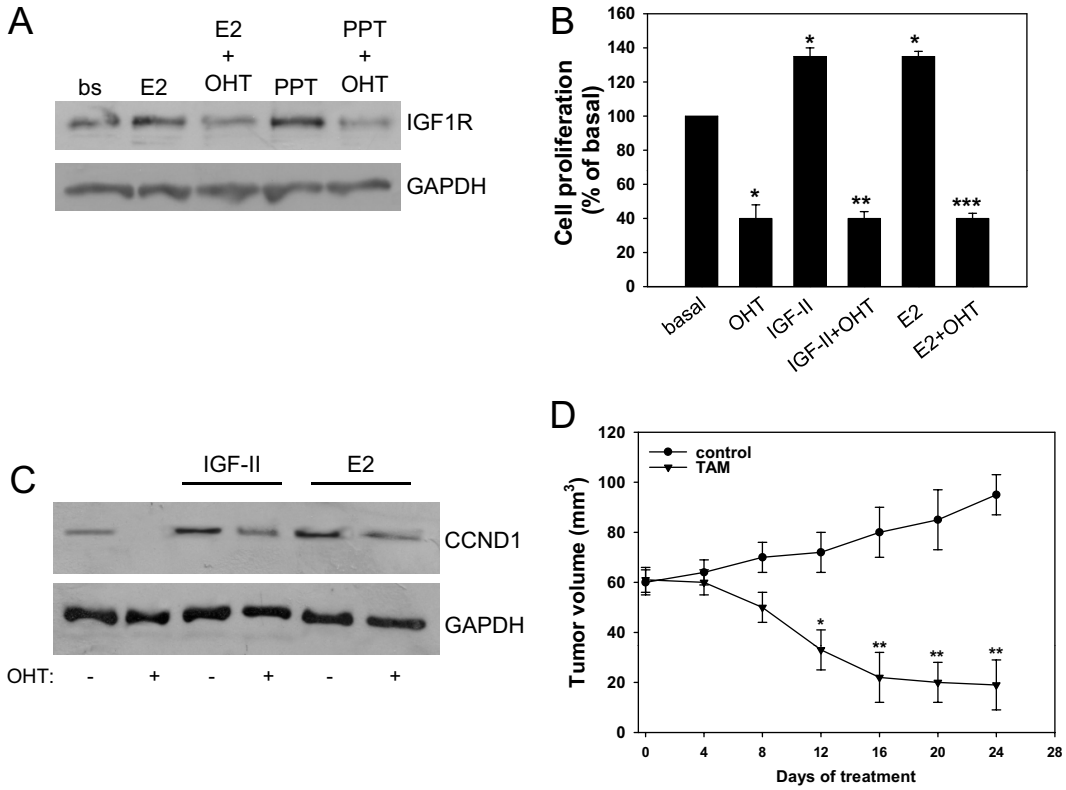

FIG. 6. OHT-inhibiting ERα reduces IGF1R expression, H295R cell proliferation, and ACC growth *in vivo*. A, Western blot analyses of IGF1R were performed on 50 μg of total protein extracted from H295R cells left untreated [basal (bs)] or treated for 24 h with E2 (100 nM) and PPT (1 μM) alone or combined with OHT (10 μM). B, H295R cells were left untreated (basal) or treated for 48 h with E2 (100 nM) and IGF-II (100 ng/ml), alone or combined with OHT (10 μM). H295R cell proliferation was evaluated by an MTT assay. Results are representative of three independent experiments. C, Immunoblot analysis of cyclin D1 was performed on 50 μg of total protein extracted from H295R cells untreated (basal) or treated for 24 h with E2 (100 nM) and IGF-II (100 ng/ml), alone or combined with OHT (10 μM). GAPDH was used as a loading control. *, $P < 0.001$ compared with basal; **, $P < 0.001$ compared with IGF-II; ***, $P < 0.001$ compared with E2. D, 6×10^6 H295R cells were injected sc in the flank region of mice, and the resulting tumors were grown to an average of 60.2 mm^3 at 7 d after inoculation. Tumor volumes were calculated, as indicated in *Materials and Methods*. Values represent the mean ± SD of measured tumor volume over time in the control group (*filled circles*, n = 20) and in the TAM-treated group (*filled triangles*, n = 20). Data were pooled from mice treated in two independent experiments. *, $P < 0.01$ compared with control at the same day of treatment; **, $P < 0.001$ compared with control at the same day of treatment.

We obtained further demonstration of ERα involvement in H295R cell proliferation by using a specific ERα siRNA that allowed for a reduction in basal and E2-induced cell proliferation. Furthermore, silencing ERα decreased IGF-II-dependent cell proliferation, indicating the requirement for this receptor in IGF-II induced H295R cell growth. This outcome was explained by the observation that IGFII caused ERα phosphorylation of serine-118 and serine-167, as previously demonstrated for other cell types (18, 51, 52). Phosphorylated ERα are active and regulate CCND1 expression, increasing cell proliferation. Importantly, analysis of ACC tissues revealed the presence of both phosphorylated forms of ERα and elevated CCND1 expression. Importantly, in ACC samples we detected the presence of the shorter ERα isoform of 46 kDa that mediates membrane initiated rapid estrogen signaling (53). The expression of this isoform in tumor samples further supports the involvement of rapid estrogen signaling in ACC growth.

It was shown for different tissues that IGF-I also required ERα to induce cell proliferation, similar to what we observed using IGF-II. For example, it was shown, *in vivo*, in the uterus of ERα knockout mice that the loss of ERα resulted in the inability of IGF-I to induce uterine nuclear proliferative responses (54). The existence of a cross talk between IGF1R and ER signaling pathways was also shown *in vitro* in MCF-7 breast cancer cells in which ERα silencing resulted in decreased IGF-I-induced G_1-S phase progression and decreased expression of CCND1 and cyclin E (22). IGF-I and IGF-II produce similar effects in different cell types, probably because they bind to the same receptor, IGF1R.

ERα is also involved in an E2-dependent increase in cyclin D1 expression. In fact, E2 as well as PPT increases ERα recruitment to the AP-1 site of the *CCND1* promoter. These data confirmed our hypothesis of a direct involvement of ERα in IGF-II- and E2-dependent H295R cell proliferation.

On the other hand, we observed that acute treatment with E2 or PPT caused phosphorylation of IGF1R, ERK1/2, and AKT that was inhibited by AG, demonstrat-

ing that estrogens can induce ligand-independent IGF1R activation. This result was probably due to the ability of estrogens to activate rapid nongenomic actions of activated ERα (39) that directly interacts with the Src homology 2 region of Src, the p85α regulatory subunit of PI3K, Shc, and IGF1R (51, 55, 56), confirming the ability of E2 to exhibit IGF-II-like activity in ACC. We also observed that E2- and PPT-dependent cAMP-responsive element binding protein (CREB) phosphorylation was inhibited by AG. This result was probably due to the ability of estrogens to activate an additional mechanism downstream of IGF1R that cannot be directly induced by IGF-II because IGF-II alone did not influence CREB phosphorylation (Fig. 2B). This mechanism could involve protein kinase A activation because we detected an increase in protein kinase A activity after 1 h stimulation with estradiol (data not shown). We are currently investigating the mechanism of ERα and IGF1R interaction in H295R cells, which could be one of the possible mechanisms explaining why IGF1R monoclonal antibodies recently entered phase I clinical trials for the therapy of ACC but failed to give objective responses in refractory ACC patients (10). We have also evaluated the possibility for E2 to exert indirect effects, depending on increased IGF-II expression, which then activated IGF1R. However, the evaluation of IGF-II mRNA levels by real-time RT-PCR and protein levels by RIA, excluded this hypothesis. In fact, treatment with E2 and PPT did not modify *IGF-II* mRNA expression and production (data not shown).

We also showed that treatment of H295R cells with E2 caused an increase in *IGF1R* mRNA and protein expression. This mechanism was mediated by an increase in CREB phosphorylation and in its binding to the *IGF1R* promoter. These results correlate well with data on ACC, demonstrating increased CREB phosphorylation and IGF1R overexpression, and explain the role played by the increased pCREB levels in ACC. Our data are in agreement with reports indicating the ability of E2 to regulate *IGF1R* expression in prostate cancer (57). The use of PPT, a specific ERα agonist, confirms the role for this estrogen receptor isoform in mediating the estrogen effects on IGF1R. The involvement of ERα in this process was further demonstrated by the use of a SERM, OHT. This SERM, by blocking ERα, reduces the IGF1R expression. OHT reduced IGF-II- and E2-dependent CCND1 expression and cell proliferation, confirming our previous data (15). Based on these *in vitro* data, we decided to treat H295R xenograft tumors in nude mice with TAM, a precursor of OHT used for breast cancer therapy. TAM reduced H295R xenograft tumor, indicating its effectiveness *in vivo*.

In conclusion, results from this study demonstrate the important role played by ERα in mediating the mitogenic activity of E2 and IGF-II in ACC. Both E2 and IGF-II induce many comparable responses in H295R cells including activation of IGF1R/AKT signaling and CCND1 expression. Our experiments clearly demonstrate that an anti-IGF1R antibody can inhibit basal and IGF-II-induced cell proliferation but not E2-dependent cell growth. Targeting ERα is, instead, effective in controlling E2- and IGF-II-dependent cell proliferation, indicating a central role for ERα in the mechanisms controlling ACC cell proliferation. These data support the possibility of using antiestrogens for the purpose of controlling adrenocortical carcinoma cell proliferation.

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