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Aldo-Keto Reductase 1C3 Expression in MCF-7 Cells Reveals Roles in Steroid Hormone and Prostaglandin Metabolism that may Explain its Over-Expression in Breast Cancer[1]

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Abstract

Aldo-keto reductase (AKR) 1C3 (type 5 17β-hydroxysteroid dehydrogenase and prostaglandin F synthase), may stimulate proliferation via steroid hormone and prostaglandin (PG) metabolism in the breast. Purified recombinant AKR1C3 reduces PGD_2 to 9α,11β-PGF_2, Δ^4-androstenedione to testosterone, progesterone to 20α-hydroxyprogesterone, and to a lesser extent, estrone to 17β-estradiol. We established MCF-7 cells that stably express AKR1C3 (MCF-7-AKR1C3 cells) to model its overexpression in breast cancer. AKR1C3 expression increased steroid conversion by MCF-7 cells, leading to a pro-estrogenic state. Unexpectedly, estrone was reduced fastest by MCF-7-AKR1C3 cells when compared to other substrates at 0.1 uM. MCF-7-AKR1C3 cells proliferated three times faster than parental cells in response to estrone and 17β-estradiol. AKR1C3 therefore represents a potential target for attenuating estrogen receptor α induced proliferation. MCF-7-AKR1C3 cells also reduced PGD_2, limiting its dehydration to form PGJ_2 products. The AKR1C3 product was confirmed as 9α,11β-PGF_2 and quantified with a stereospecific stable isotope dilution liquid chromatography-mass spectrometry method. This method will allow the examination of the role of AKR1C3 in endogenous prostaglandin formation in response to inflammatory stimuli. Expression of AKR1C3 reduced the anti-proliferative effects of PGD_2 on MCF-7 cells, suggesting that AKR1C3 limits peroxisome proliferator activated receptor γ (PPARγ) signaling by reducing formation of 15-deoxy-$\Delta^{12,14}$-PGJ_2 ($15dPGJ_2$).

Keywords

17β-Hydroxysteroid dehydrogenase; prostaglandin F synthase; prostaglandin D_2; estrogen receptor; peroxisome proliferator activated receptor γ

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1. Introduction

In post-menopausal women, proliferation of hormone dependent breast cancer is driven by local production of estrogens [1]. The efficacy of aromatase inhibitors against estrogen receptor α (ERα) positive breast cancer demonstrates that blockade of local estrogen production is effective for treatment and prevention of breast cancer [1,2]. Production of 17β-estradiol from Δ^4-androstenedione requires both aromatase and a reductive 17β-hydroxysteroid dehydrogenase (HSD) [2]. If aromatase acts first, the primary enzymes thought to convert estrone to 17β-estradiol are 17β-HSD types 1 and 7 [1,2]. The 17β-HSD enzyme thought to convert Δ^4-androstenedione to testosterone in breast is aldo-keto reductase (AKR) 1C3 (also known as type 5 17β-HSD or prostaglandin F synthase) [1–3]. Testosterone formed would then undergo aromatization to 17β-estradiol. It therefore appears that reductive 17β-HSDs, including AKR1C3, represent important targets for prevention or treatment of estrogen-dependent breast cancer.

Blockade of prostaglandin biosynthesis is another strategy for prevention of breast cancer. Regular use of non-steroidal anti-inflammatory drugs (NSAIDs) has been associated with a reduced risk of breast cancer [4–6]. Ibuprofen and celecoxib effectively prevent mammary tumors in rodents [7]. Celecoxib is in early clinical trials as an adjuvant for treatment of human breast cancer along with the aromatase inhibitor, exemestane [8]. NSAIDs act, in part, by inhibiting prostaglandin-dependent upregulation of aromatase, thereby limiting local production of estrogen [9]. The AKR1Cs are also inhibited by NSAIDs [10,11], and these enzymes represent alternative targets for the anti-cancer effects of these drugs.

AKR1C3 is expressed in normal breast tissue, its upregulation has been detected in 65–85% of breast cancer tissues, and its upregulation is associated with poor prognosis [12–14]. Purified recombinant AKR1C3 reduces Δ^4-androstenedione to testosterone, progesterone to 20α-hydroxyprogesterone, desoxycorticosterone to 20α-hydroxydesoxycorticosterone and, to a lesser extent, estrone to 17β-estradiol [3,15,16]. These reactions have been observed in HEK293 cells that overexpress AKR1C3 [16,17]. Because of the lower rate of estrone reduction relative to Δ^4-androstenedione and progesterone reduction, it was proposed that for breast cancer, the most important reaction catalyzed by AKR1C3 is reduction of Δ^4-androstenedione to testosterone, which would be a substrate for aromatase to form 17β-estradiol [2]. AKR1C3-mediated progesterone reduction might further contribute to a pro-estrogenic state in the breast by increasing the ratio of estrogen to progesterone.

In addition to its HSD activities, AKR1C3 is characterized as human prostaglandin (PG) F synthase. Homogenous recombinant AKR1C3 stereospecifically and efficiently converts PGH_2 to $PGF_{2\alpha}$ and PGD_2 to 9α,11β-PGF_2 [18,19]. Conversion of PGD_2 to a PGF_2 isomer by leukemia cells was reduced by the AKR1C3 inhibitor indomethacin or a shRNA targeting AKR1C3 [20]. $PGF_{2\alpha}$, acting through the F prostanoid (FP) receptor, induces cell proliferation, invasiveness, and angiogenesis in endometrial cancer [21–23]. Through depletion of PGD_2 levels, AKR1C3 prevents its spontaneous dehydration and rearrangement to form anti-proliferative and anti-inflammatory PGJ_2 isomers, including 15-deoxy-$\Delta^{12,14}$-PGJ_2 ($15dPGJ_2$). $15dPGJ_2$ covalently reacts with a key cysteine residue in PPARγ, resulting in its activation [24,25]. $15dPGJ_2$ also reacts with cysteine residues in other proteins, including DNA binding domains of nuclear factor κB (NF-κB) and ERα, resulting in the loss of transcriptional activity [26,27].

Given its over-expression in breast cancer and potential to regulate ligand access to estrogen and progesterone receptors and alter prostaglandin signaling, we hypothesized that AKR1C3 might play an important role in hormone-dependent breast cancer. To test this hypothesis, we established MCF-7 cells that stably express the AKR1C3 protein. The expression of AKR1C3

in MCF-7 cells resulted in the reduction of Δ^4-androstenedione to testosterone, progesterone to 20α-hydroxyprogesterone, estrone to 17β-estradiol and PGD_2 to 9α,11β-PGF_2. Unexpectedly, at low concentrations estrone reduction was the fastest of these reactions. Furthermore, formation of 17β-estradiol by AKR1C3 significantly increased MCF-7 cell proliferation, demonstrating that AKR1C3 likely plays an important role in the development of estrogen-dependent breast cancer. AKR1C3 expression also reduced the anti-proliferative effects of PGD_2, which suggests that it produces tumor promoting signals independent of ERα signaling.

2. Materials and Methods

2.1. Chemicals and reagents

Unlabeled estrone and 17β-estradiol were from ICN Biomedical Inc. (Aurora, OH); other unlabeled steroids were from Steraloids (Newport, RI). Unlabeled and deuterium-labeled prostaglandins were from Cayman Chemical (Ann Arbor, MI). Radiolabeled steroids and prostaglandins were from PerkinElmer Life Sciences (Waltham, MA). Media and cell culture reagents were from Invitrogen (Carlsbad, CA) except as noted. All organic solvents were from Fisher Scientific (Fair Lawn, NJ). Homogenous recombinant AKR1C3 was purified as previously described [3].

2.2. Stable transfection of MCF-7 cells

Human MCF-7 hormone dependent breast cancer cells were maintained in RPMI media supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories, Inc., Logan, UT), 2% L-glutamate, and 100 units/ml penicillin/streptomycin.

The cDNA encoding AKR1C3 was excised from a pcDNA-3 plasmid [28] with BamHI and ApaI. A pLNCX2 retroviral vector was linearized with StuI, and the AKR1C3 cDNA was inserted using a Rapid DNA ligation kit (Roche Diagnostics, Indianapolis, IN). *E. coli* DH5α cells were transformed with the AKR1C3-pLNCX vector, and the amplified vector was purified using a Plasmid Maxi kit (Qiagen, Valencia, CA). This vector was added to PT67 packaging cells using FuGENE 6 (Roche Diagnostics). Media from these cells was collected and added to MCF-7 cells. Stable transfectants were selected with media containing geneticin (0.5 mg/mL). Isolated clones were phenotyped by RT-PCR and immunoblot assay. Cells stably expressing AKR1C3 were maintained in RPMI media containing 0.25 mg/mL geneticin.

2.3. RT-PCR

Total RNA was isolated from cells grown to ~90% confluence using an RNeasy kit (Qiagen, Valencia, CA), according to the manufacturer's instructions, and treated with DNAse I (Qiagen) to remove genomic DNA. A GeneAmp RNA PCR Core kit (Applied Biosystems, Foster City, CA) was used to form cDNA from 1 μg RNA, according to the manufacturer's instructions. PCR amplification was performed using Taq DNA polymerase as previously described [3], except the annealing temperature was set to 68 °C for AKR1C1 and 65 °C for AKR1C2 and AKR1C3 and 25 cycles were performed. The primers used to provide isoform specific detection of the AKR1Cs were: AKR1C1 (forward) 5'GTA AAG CTT TAG AGG CCA C 3' and (reverse) 5'CAC CCA TGG TTC TTC TCG G 3'; AKR1C2 (forward) 5' GTA AAG CTC TAG AGG CCG T 3' and (reverse) 5' CAC CCA TGG TTC TTC TCG A 3'; AKR1C3 (forward) 5' GTA AAG CTT TGG AGG TCA C 3' and (reverse) 5' CAC CCA TCG TTT GTC TCG T 3'.

2.4. Immunoblot analysis

Cells were lysed in protease inhibitor-containing RIPA buffer by sonication. Samples were centrifuged at 9,000 *g* and the protein concentration in the supernatant was determined using the Bradford reagent (Bio-Rad Hercules, CA) according to the manufacturer's instructions. Each sample, containing 30 μg total protein, was separated by 10% SDS/PAGE and transferred onto a nitrocellulose membrane. AKR1C3 was detected with a mouse monoclonal antibody against AKR1C3 [13] at 1:1000 dilution, visualized with an horse radish peroxidase-conjugated sheep anti-mouse antibody (1:2000) using the ECL reagent (GE Healthcare, Piscataway, NJ).

2.5. Radiometric determination of steroid metabolism

For determination of estrone reduction by purified recombinant AKR1C3, reaction mixtures containing 0.2 μCi [^{3}H]-estrone, varied unlabeled estrone to obtain a final concentration of 3.75 to 30 μM, 1 mM NADPH, and 4% ethanol in 100 mM potassium phosphate buffer (pH 7.0, 200 μL total volume) were incubated at 37 °C for 1 hour. Reactions were initiated with the addition of NADPH and were terminated with the addition of 1 mL of cold water-saturated ethyl acetate. The organic fraction was evaporated to dryness under reduced pressure, and redissolved in 50 μL ethyl acetate. The details of substrate and product separation are given below.

For determination of radiolabeled steroid metabolism by MCF-7 and MCF-7-AKR1C3 cells, 1×10^{6} cells were plated in 6 well dishes and incubated overnight. In order to limit background estrogen signaling, media was replaced with 2 mL phenol-free RPMI with 1% charcoal stripped FBS (HyClone Laboratories, Inc.). After a 30 min equilibration, [4-^{14}C]-estrone (0.013 μCi, 0.1 μM final concentration), [4-^{14}C]-progesterone (0.010 μCi, 0.1 μM), or [4-^{14}C]-Δ^{4}-androstenedione (0.096 μCi, 0.1 μM) and unlabeled steroid (if necessary) in DMSO were added to attain a final concentration of 0.1 or 5 μM steroid and 0.25% DMSO. After incubation for 0, 4, 6, 24, or 48 h, 1 mL media was removed from cells and extracted twice with 2 mL cold ethyl acetate. Organic fractions were pooled, dried under reduced pressure, redissolved in 100 μl ethyl acetate. The details of substrate and product separation are given below.

For both recombinant enzyme and cell experiments, samples were applied to LK6D Silica thin layer chromatography (TLC) plates (Whatman Inc., Clifton, NJ). TLC plates were developed using a methylene chloride/ethyl acetate (80:20 v/v) solution for estrone or progesterone metabolites or a methylene chloride/ethyl ether (110:10 v/v) solution for Δ^{4}-androstenedione metabolites and counted with a Bioscan System 200 plate reader (Washington, DC). Identification of products was determined by co-migration on TLC with nonradioactive reference steroids (25 μg each) visualized with an acetic acid/sulfuric acid/anisaldehyde (100:2:1, v/v/v) solution and heating. Peaks were integrated as a percent of total radioactivity, assuming that each steroid was recovered with equal efficiency. The specific radioactivity of the substrate was used to calculate nmoles of steroid product formed. Independent measurements showed that the recovery of the steroids from media following extraction was >95%.

2.6. Radiometric determination of PGD_2 metabolism

For determination of PGD_2 metabolism by MCF-7 and MCF-7-AKR1C3 cells, 1×10^{6} cells were plated in 6 well dishes and incubated overnight. Because initial prostaglandin metabolism experiments were not successful in the presence of serum, and MCF-7 cells required either serum or phenol red (an estrogen) for survival, media was replaced with 2 mL serum-free RPMI (with phenol red) containing [5,6,8,9,^{12},^{14},^{15}-^{3}H]-PGD_2 (0.17 μCi, 0.5 nM) and unlabeled PGD_2 to attain a final concentration of 0.1 or 5 μM along with 0.1% ethanol. After incubation for 0, 3, 6, or 24 h, 1 mL media was removed from cells and acidified with 5 μL of 20% formic

acid. Twenty microliters of acidified media was added to 5 mL of UltimaGold scintillation fluid (PerkinElmer Life Sciences). The remaining media was extracted twice with 2 mL cold ethyl acetate and organic fractions were pooled and dried under reduced pressure. Twenty microliters of the aqueous fraction was added to 5 mL scintillation fluid and the initial media and aqueous fractions were analyzed with a TriCarb 2100 (Packard Instrument, PerkinElmer Life Sciences) scintillation counter. Dried extracts were redissolved in 200 μl of ethyl acetate and applied to LK6D Silica TLC plates. TLC plates were developed using the organic layer from an ethyl acetate/2,2,4-trimethyl pentane/water/acetic acid (110:50:100:20 v/v/v/v) solution and were counted with the Bioscan System 200 plate reader. Identification of products was determined through inclusion of nonradioactive reference prostaglandins visualized as above. This method did not separate 9α,11β-PGF_2 from $PGF_{2\alpha}$. Peaks on the radiochromatogram were integrated and quantitated as described above, except that the results were adjusted to account for the percentage of radioactivity that remained in the aqueous fraction following extraction.

2.7. LC-MS determination of PGD_2 metabolism

MCF-7 and MCF-7-AKR1C3 cells were treated with 0.1 μM PGD_2 as described above, except that only unlabeled PGD_2 was used. After 6 h, 1 mL media was removed, deuterated internal standards (1 ng each of PGD_2-d_4, PGE_2-d_4, 15dPGJ$_2$-d_4, $PGF_{2\alpha}$-d_4, and 9α,11β-PGF_2-d_4) were added, media was acidified with 5 μL formic acid (20%), and extracted twice with 1 mL ethyl ether. Organic fractions were combined and dried under nitrogen. Eicosanoids were derivatized with pentafluorobenzyl bromide and analyzed using liquid chromatography-electron capture atmospheric pressure chemical ionization/mass spectrometry (LC-ECAPCI/MS) as previously described [29]. Multiple reaction monitoring (MRM) was performed on the following transitions: *m/z* 351 → *m/z* 271 (PGD_2 and PGE_2), *m/z* 355 → *m/z* 275 (PGD_2-d_4 and PGE_2-d_4), *m/z* 315 → *m/z* 271 (15dPGJ$_2$), *m/z* 319 → *m/z* 275 (15dPGJ$_2$-d_4), *m/z* 353 → *m/z* 193 ($PGF_{2\alpha}$ and 9α,11β-PGF_2), and *m/z* 357 → *m/z* 197 ($PGF_{2\alpha}$-d_4 and 9α,11β-PGF_2-d_4).

Individual eicosanoids were quantitated by comparing the areas of the peaks corresponding to the unlabelled compounds to the areas of the peaks for the deuterated internal standards. Calibration curves were established by adding known concentrations of unlabelled prostanoids and a set amount (1 ng) of deuterated standards to the media minus cells.

2.8. Cell proliferation

Proliferation in response to estrogens was determined with the MTT assay. MCF-7 and MCF-7-AKR1C3 cells were maintained in phenol-free RPMI containing 5% charcoal-stripped FBS for 72 h. Cells (1×10^4) were then plated into 96 well plates containing 200 μL phenol-free RPMI with 5% FBS and grown overnight. Estrone and 17β-estradiol were added in ethanol to obtain the indicated concentration of estrogen and 0.25% ethanol. Cells were incubated for 6 days then stopped with the addition of 0.1 mg MTT reagent (Molecular Probes, Eugene, OR) in 20 μL phosphate buffered saline. Cells were incubated 4 h, prior to removal of supernatant. Crystals were dissolved with 150 μL DMSO and read at 570 nm, with 630 nm as a reference using a Synergy 2 plate reader (Biotek, Winooski, VT). Cell numbers were normalized to ethanol-treated control cells and statistics were performed using a two-sided Student's t-test.

Proliferation in response to prostaglandins was measured by bromo-deoxyuridine (BrDU) incorporation with a bioluminescent detection kit (Roche Diagnostics). The BrDU assay was used in place of the MTT assay because in the absence of serum the latter assay lacks sufficient sensitivity to detect proliferation of MCF-7 cells. It was necessary to perform these assays in the absence of serum since serum limits the uptake of PGD_2 into these cells. Cells (1×10^4) were maintained in normal RPMI media with 10% FBS and passaged into 96 well plates containing 200 μL of the same media. Twenty-four hours after passaging, cells were washed

twice with phosphate buffered saline and media was replaced with serum-free normal RPMI media. After an additional 24 h, cells were treated with PGD_2, 9α,11β-PGF_2, or 15dPGJ_2 in 0.1% DMSO. Cells were incubated for 3 days, BrDU labeling reagent was added and cells were incubated an additional 24 h. Cell numbers were determined using luminescence on the Synergy 2 plate reader according to the manufacturer's instructions. Cell numbers were normalized to ethanol-treated control cells and statistics were performed using a two-sided Student's t-test.

3. Results

3. 1. Steady state kinetic parameters for the reduction of estrone catalyzed by recombinant AKR1C3

We have previously reported kinetic constants for the reduction of Δ^4-androstenedione to testosterone (K_m 6.6 μM; k_{cat} 0.16 min^{-1} and k_{cat}/K_m = 24 min^{-1} mM^{-1}) [10] and the reduction of progesterone to 20α-hydroxyprogesterone (K_m = 2.8 μM; k_{cat} = 1.04 and k_{cat}/K_m = 370 min^{-1} mM^{-1}) [16]. However, kinetic constants for the reduction of estrone to 17β-estradiol have not been previously reported due to the low turnover number and the difficulty in obtaining reliable steady state kinetic constants. In the present study we were able to circumvent these problems and measure a K_m =9.0 μM and a k_{cat} = 0.068 min^{-1} yielding a k_{cat}/K_m =×min^{-1} mM^{-1}. These measurements predict that *in vitro* the conversion of estrone to 17β-estradiol is the least favorable of the reactions monitored. By contrast we an others have shown that 11-keto reduction of prostaglandin D_2 is a highly favored reaction yielding a K_m = 1.1 μM and a k_{cat} of 1.4 min^{-1}, yielding a k_{cat}/K_m = 1270 min^{-1} mM^{-1}. [18] To determine whether these reactions could occur in a breast cancer cell environment and whether there would be consequences on cell phenotype a stably transfected cell line was sought.

3.1. Development of an MCF-7 cell line that stably expresses AKR1C3

A pLNCX retroviral vector was used to stably express AKR1C3 in MCF-7 cells. RT-PCR using isoform-specific primers indicated that these new MCF-7 cell lines expressed AKR1C3, but not AKR1C1 or AKR1C2 (Figure 1A). Expression of AKR1C3 protein was observed in each of eight transfected clonal lines by Western blot analysis using an isoform-specific monoclonal antibody (Figure 1B). Based on a standard curve for recombinant AKR1C3, AKR1C3 accounted for approximately 0.01% to 0.1% of total protein in the lysate. Clone #5, which exhibited high AKR1C3 expression, was selected for in depth study (henceforth referred to as MCF-7-AKR1C3 cells).

3.2. AKR1C3 reduces ketosteroids in MCF-7 cells

In parental MCF-7 cells, 0.1 and 5 μM [^{14}C]-Δ^4-androstenedione was primarily metabolized by 5α-reductase to 5α-androstanedione, with testosterone and androsterone formed as minor metabolites (Figure 2). The MCF-7-AKR1C3 transfectants formed much higher levels of testosterone from both concentrations of Δ^4-androstenedione. The testosterone peak accounted for 23% and 11% of total radioactivity at 24 h with 0.1 and 5 μM Δ^4-androstenedione, respectively. 5α-DHT was also observed as a product in MCF-7-AKR1C3 cells, accounting for 5% of the total radioactivity by 24 h when 0.1 μM Δ^4-androstenedione was used as precursor. 5α-DHT would be formed through the sequential action of 5α-reductase and AKR1C3.

When MCF-7 cells were incubated with 0.1 μM [^{14}C]-progesterone, there was substantial endogenous metabolism, with 54% of the parent compound metabolized by 24 h (Figure 3). The primary products formed were polar compounds, possibly glucuronides, that did not migrate on the TLC plate. 5α-Dihydroprogesterone and 5α-pregnane-20α-ol-3-one were also detected and reflect significant 5α-reductase activity in these cells. At 5 μM [^{14}C]-

progesterone, these products were formed by parental cells at much lower levels, and 20α-hydroxyprogesterone was detected as a very minor metabolite. The MCF-7-AKR1C3 cells metabolized both concentrations of progesterone at a much higher rate. With 0.1 μM progesterone, 49% was reduced by 6 h and 64% of 5 μM progesterone was reduced by 24 h. The initial primary product formed by MCF-7-AKR1C3 transfectants was 20α-hydroxyprogesterone, which underwent further metabolism to 3α- and 5α-reduced products.

When [^{14}C]-estrone was incubated with parental MCF-7 cells, substantial endogenous 17-ketosteroid reductase activity was observed. This activity resulted in 53% conversion of 0.1 μM estrone to 17β-estradiol by 24 h (Figure 4). No other metabolic pathways were observed. MCF-7-AKR1C3 cells converted 0.1 μM estrone to 17β-estradiol but at a much faster rate. By 6 h, 82% of the estrone had been converted into 17β-estradiol by MCF-7-AKR1C3 cells. However, when reactions were performed with 5 μM estrone, expression of AKR1C3 had a more modest impact on 17β-estradiol formation, increasing the amount of estradiol at 24 h from 10% of the total to 19%.

3.3. AKR1C3 reduces PGD_2 to 11β-PGF_2 in MCF-7 cells

When incubated in cell free media, [^{3}H]-PGD_2 spontaneously dehydrated to form PGJ_2 and 15dPGJ_2, which accounted for 60% and 8% of the total radioactivity by 24 h, respectively (data not shown). In parental MCF-7 cells, [^{3}H]-PGD_2 disappeared at a similar rate, but only very low levels of PGJ_2 products were detected, with most of the radioactivity staying in the aqueous phase after extraction (Figure 5). These results were consistent with conjugation of PGJ_2 and 15dPGJ_2 to glutathione or other cellular nucleophiles. No 9α,11β-PGF_2 formation was observed in parental cells or cell-free media. In incubations with MCF-7-AKR1C3 cells, [^{3}H]-PGD_2 disappeared at a faster rate, which corresponded to formation of 9α,11β-PGF_2 as a major metabolite, accounting for 35% and 18% of total radioactivity at 24 h with 0.1 and 5 μM PGD_2 as substrate, respectively. Lower levels of PGJ_2 and aqueous metabolites were formed by MCF-7-AKR1C3 cells relative to parental cells at both concentrations of substrate.

Because our TLC conditions did not separate 9α,11β-PGF_2 from $PGF_{2\alpha}$, a method utilizing pentafluorobenzyl derivatization of prostaglandins followed by chiral normal phase HPLC and analysis with ECAPCI-MRM/MS was used to identify and quantify the products formed from 0.1 μM PGD_2 by MCF-7 and MCF-7-AKR1C3 cells (Figure 6). As expected, MCF-7-AKR1C3 cells stereospecifically reduced PGD_2 to 9α,11β-PGF_2 with no detectable $PGF_{2\alpha}$. Quantification of PGD_2, 15dPGJ_2, and the PGF_2 isomers was obtained through inclusion of deuterium labeled internal standards. Standard curves were established using cell-free media that demonstrated no interference from media to the assay and a linear relationship ($R^2 > .995$) between the observed and predicted ratios of labeled to unlabeled prostaglandins across the concentrations tested. The concentrations of products detected by LC-MS were consistent with those observed with radiolabeled substrate.

3.4. Expression of AKR1C3 increases estrogen-dependent cell proliferation in MCF-7 cells

We next explored whether the conversion of estrone to 17β-estradiol catalyzed by AKR1C3 would alter proliferation of MCF-7 cells. Treatment with picomolar concentrations of estrone and 17β-estradiol increased the number of parental MCF-7 cells by up to 2.2 fold over ethanol-treated controls at 6 days. Expression of AKR1C3 significantly increased the responsiveness of the cells to both estrogens, with up to a 7 fold increase in the number of the MCF-7-AKR1C3 cells following estrogen treatment as compared to ethanol-treated controls (Figure 7A). Estrone was almost as effective as 17β-estradiol at stimulating proliferation in both parental and AKR1C3 expressing MCF-7 cells. However, the effect of both estrogens was consistently much greater in the AKR1C3 transfectants. These results suggest that a rapid equilibrium between estrone and 17β-estradiol was reached in these cells. In parental cells, this equilibrium

is established by reductive (whose expression is type 7 > type 1 > type 12) and oxidative (primarily type 4) 17β-HSD isoforms present in MCF-7 cells [30, 31]. Selective inhibitors of 17β-HSDs 1, 7, and 12 all inhibit the reduction of estrone by parental MCF-7 cells by about 30%, suggesting that all of these enzymes play a role [30]. In transfected cells, AKR1C3 (type 5) provides an additional reductive 17β-HSD that could alter the equilibrium in favor of 17β-estradiol, thus stimulating proliferation.

3.5. Expression of AKR1C3 reduces the anti-proliferative effects of prostaglandins

We next explored how AKR1C3 expression would affect cell proliferation in the presence of PGD_2, 9α,11β-PGF_2, and $15dPGJ_2$ (Figure 7B–D). Each prostaglandin had little effect on proliferation of parental and AKR1C3-expressing MCF-7 cells at concentrations below 1 μM, suggesting that they lack cell surface receptors for these prostaglandins. Consistent with this hypothesis, we were unable to detect the FP receptor by Western blot (data not shown) or RT-PCR analyses (Supplemental Figure). $15dPGJ_2$ inhibited proliferation of parental MCF-7 cells at concentrations of 1 μM and higher. PGD_2 inhibited proliferation starting at a concentration of 2.5 μM. Expression of AKR1C3 eliminated the anti-proliferative effect of 2.5 μM PGD_2 and had a small, but significant effect at 10 μM PGD_2. Surprisingly, AKR1C3 also significantly limited the inhibition of proliferation by 2.5 μM $15dPGJ_2$. Micromolar concentrations of 9α, 11β-PGF_2 stimulated mild MCF-7 cell proliferation, which was not affected by AKR1C3 expression.

4. Discussion

We demonstrate that AKR1C3 reduces steroid hormones and PGD_2 when expressed in MCF-7 breast cancer cells. This study allows a comparison of the roles of AKR1C3 in these metabolic pathways in a relevant breast cancer cell model and allows examination of its effects on cell proliferation. We made the unexpected finding that AKR1C3 expression has a dramatic effect on the rate of estrone conversion to 17β-estradiol, with consequences for cell proliferation. As predicted, we also observed that AKR1C3 expression led to metabolism of PGD_2 to 9α,11β-PGF_2, which prevented its rearrangement to reactive and anti-proliferative products of the PGJ_2 series. These data provide a functional correlate to overexpression of AKR1C3 in breast cancer patients and its association with poor prognosis [12–14,32].

Unexpectedly, in cultured MCF-7-AKR1C3 cells, expression of AKR1C3 has the most significant effect on reduction of estrone to 17β-estradiol. In AKR1C3 expressing cells, estrone was reduced the fastest at the more physiologically relevant substrate concentration (0.1 μM). Based on this observation and the lack of an effect at 5 μM estrone, we predict that AKR1C3 is a high affinity, low capacity enzyme towards estrone *in vivo*. Reduction of estrone by MCF-7 cells expressing AKR1C3 was much higher than what was expected based on the activity of homogenous recombinant AKR1C3. Our observation of rapid 0.1 μM estrone reduction by AKR1C3 expressing MCF-7 cells is in contrast to what was observed in HEK-293 cells by Dufort et al [17]. These investigators found that estrone reduction occurred at a much slower rate than reduction of Δ^4-androstenedione and progesterone. This discrepancy could be explained by the presence of an oxidative 17β-HSD in their HEK-293 cells that would catalyze the conversion of 17β-estradiol back to estrone and limit the effect of AKR1C3. Consistent with this hypothesis, we have observed higher rates of oxidation of 17β-estradiol to estrone in HEK-293 cells relative to MCF-7 cells (Duan and Penning, unpublished data). The importance of estrone to 17β-estradiol conversion due to AKR1C3 expression is further supported by the ability of this reaction to confer a proliferative phenotype to estrogen-dependent breast cancer cells. Stimulation of estrogen-dependent cell proliferation provides an explanation for AKR1C3 over-expression in hormone-dependent breast cancer.

Progesterone is also an important substrate for AKR1C3 in breast. At 5 μM, progesterone was the best substrate assayed, and there was also high activity at the lower concentration. Wiebe et al have reported that progesterone and 20α-hydroxyprogesterone similarly inhibit proliferation of MCF-7 cells [33], which we have also observed (data not shown). Although 20α-hydroxyprogesterone is anti-proliferative, it does not stimulate progesterone receptor dependent gene transcription. By reducing nuclear progesterone receptor *trans*-activation, AKR1C3 activity is expected to increase expression of proteins such as aromatase, Her-2/neu, COX-2 and matrix metalloproteinases that could promote development of breast tumors *in vivo* [34,35].

While AKR1C3 mediated Δ^4-androstenedione reduction rates were low relative to other substrates, this is still likely an important AKR1C3-mediated reaction, as it is the primary 17β-HSD expressed in breast that catalyzes this reaction [36]. Due to the lack of aromatase activity [37], reduction of Δ^4-androstenedione to testosterone would not have a significant effect on MCF-7 cell proliferation *in vitro*, but will likely be important to proliferation of breast cancer *in vivo.* One study recently detected higher levels of expression of AKR1C3 in stromal cells, particularly undifferentiated fibroblasts, surrounding breast tumors, rather than in tumor cells themselves [38]. This study also found that levels of AKR1C3 in isolated primary fibroblasts were upregulated by MCF-7 cell conditioned media. These undifferentiated fibroblasts exhibit the highest levels of aromatase expression in breast. Therefore, in mammary fibroblasts conversion of Δ^4-androstenedione to testosterone by AKR1C3 will allow subsequent conversion by aromatase to 17β-estradiol, which could stimulate proliferation of adjacent tumor cells.

We have demonstrated that AKR1C3 catalyzes 11-ketoprostaglandin reduction in MCF-7 cells. This is the first time that keto-steroid and keto-prostaglandin reduction by AKR1C3 has been directly compared within a cell using quantitative mass-balance measurements. Given the high rate of PGD_2 reduction by purified recombinant AKR1C3 relative to steroid hormone reduction [18,19], conversion of PGD_2 to 9α,11β-PGF_2 was slower than expected. At 0.1 μM, PGD_2 reduction by MCF-7-AKR1C3 cells was faster than Δ^4-androstenedione reduction, but slower than reduction of either estrone or progesterone. The lower than predicted rate of PGD_2 metabolism may be a consequence of its negative charge, which would limit diffusion into cells. A two step model for metabolism of prostaglandins by 15-hydroxyprostaglandin dehydrogenase has been proposed, in which transport into cells via the prostaglandin transporter can be rate limiting [39]. A similar situation likely occurs with PGD_2 metabolism by AKR1C3, and expression of the prostaglandin transporter could be a determining factor in the kinetics of this reaction in MCF-7-AKR1C3 cells.

The stereospecific LC-MS method for detecting PGF_2 isomer formation by AKR1C3 provides an important tool for studying the contribution of AKR1C3 to endogenous eicosanoid signaling. Because this method allows the separate detection of $PGF_{2\alpha}$ and 9α,11β-PGF_2, it definitively shows the stereospecificity of PGD_2 reduction in these cells. Because $PGF_{2\alpha}$ is the stereospecific product of PGH_2 reduction by AKR1C3, its absence in MCF-7-AKR1C3 cells suggests that there was not enough endogenous PGH_2 in these cells for that reaction to occur. Detection of PGF_2 metabolites by LC-MS has several advantages over other methodologies. The addition of radiotracers does not permit detection of eicosanoids formed endogenously under physiological conditions. Antibody-based approaches to prostaglandin detection are limited by difficulties in obtaining specificity to a single prostanoid in a biological matrix and generally overestimate prostaglandin levels. Utilizing this LC-MS method, we can examine how expression of AKR1C3 modifies endogenous metabolism of prostaglandins formed in response to inflammatory stimuli.

AKR1C3 expression limited the anti-proliferative effects of PGD_2 on MCF-7 cells. This observation is consistent with our hypothesis that AKR1C3 reduces PGD_2 concentrations and prevents its spontaneous dehydration to form PGJ_2 products. Surprisingly, AKR1C3 expression also reduced the anti-proliferative effects of 2.5 μM $15dPGJ_2$. This could suggest that AKR1C3 limits the anti-proliferative effects of PGD_2 through an additional mechanism. Given the similar structures of PGD_2 and $15dPGJ_2$, AKR1C3 will likely bind $15dPGJ_2$, which could reduce its available concentration and keep it from reaching its molecular targets. AKR1C3 also has several cysteine residues located on its surface, which could react with $15dPGJ_2$. However, there are no cysteines located near the prostaglandin binding pocket in the AKR1C3·PGD_2·$NADP^+$ structure (PDB 1RY0). Although the mechanism is not entirely clear, the prevention of the anti-proliferative effects of PGD_2 and $15dPGJ_2$ suggests that AKR1C3 acts as a regulator of PPARγ in cells.

AKR1C3-mediated formation of PGF_2 isomers from PGH_2 and PGD_2 could play an important role in cancer progression. $PGF_{2\alpha}$ isomers stimulate proliferation, angiogenesis, and tumor invasiveness [21–23]. We did not observe FP receptor expression in MCF-7 cells, which likely explains their limited proliferative response to 9α,11β-PGF_2. While physiological concentrations of 9α,11β-PGF_2 did not increase the proliferation of MCF-7 cells, PGF_2 isomer formation by AKR1C3 would be proliferative in FP receptor expressing tumors. In addition, the formation of PGF_2 isomers could contribute to development of breast tumors *in vivo* by stimulating production of aromatase, vascular endothelial growth factor, matrix metalloproteinases, and other factors by stromal cells that express the FP receptor, including preadipocytes and vascular smooth muscle cells [40,41].

Given the effects of AKR1C3 expression on steroid hormone metabolism, its inhibition might compliment aromatase inhibitors by further shutting down estrogen signaling in breast. Interestingly, celecoxib, which has shown promise as an adjuvant to exemestane treatment, inhibits AKR1C3 at the plasma concentrations attained *in vivo* [11,42]. The anti-cancer effects of celecoxib are in part due to COX-2 inhibition, but there is evidence for COX-independent mechanisms [43], which could include inhibition of AKR1C3 mediated steroid hormone and prostaglandin reduction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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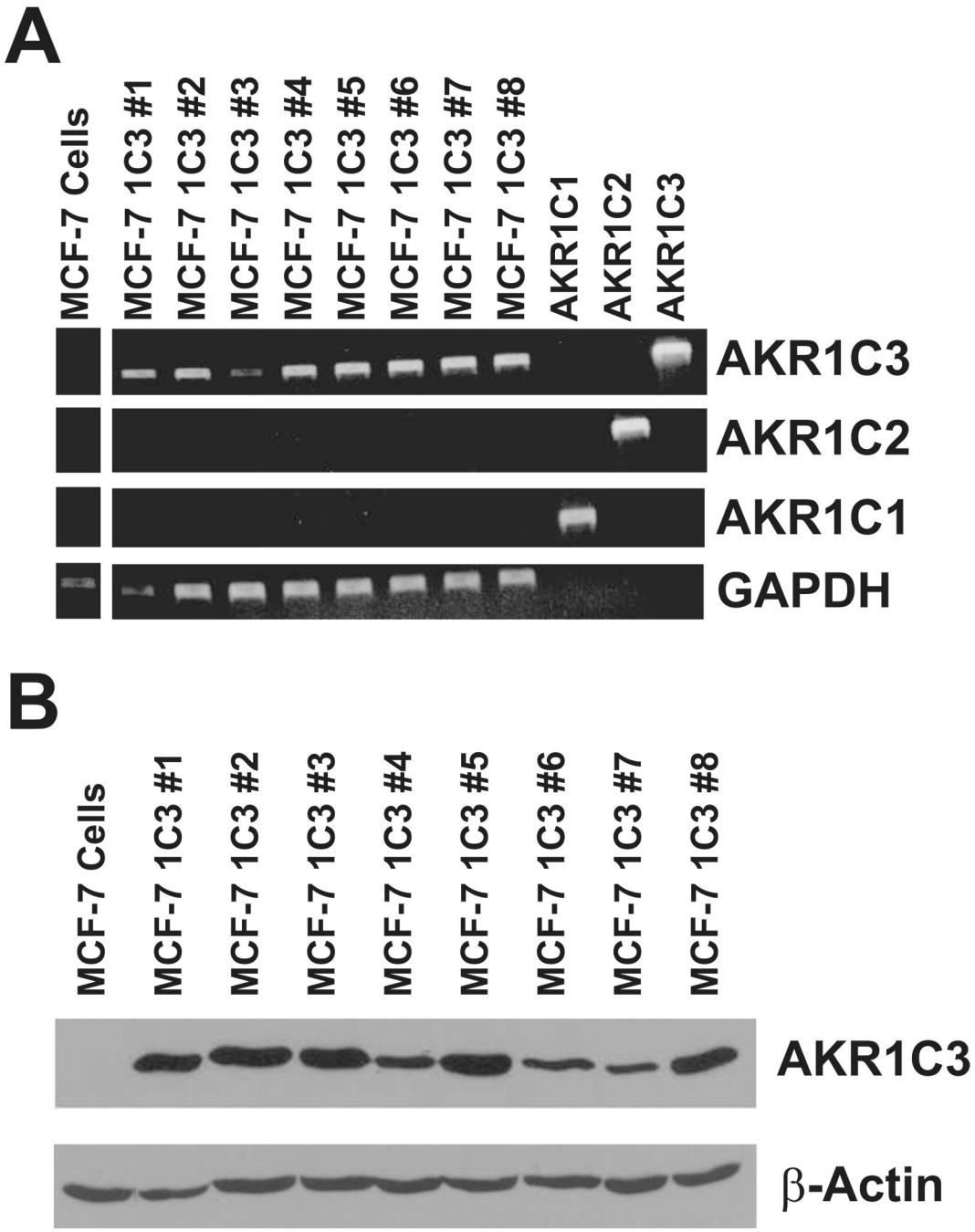

Fig. 1.

Expression of AKR1C isoforms in parental and AKR1C3 transfected MCF-7 cells. (A) Measurement of AKR1C isoform expression by RT-PCR using primers specific for AKR1C1, AKR1C2, AKR1C3 in MCF-7 parental cells and eight cell lines stably expressing AKR1C3. Left panel shows lack of AKR1C expression in the parental cells. The right panel shows expression of AKR1C3, but not AKR1C1 or AKR1C2, in the stably transfected cell lines. The last three lanes show the detection of cDNA from plasmids containing AKR1C1, AKR1C2, and AKR1C3, respectively, as positive controls. GAPDH was also amplified in each of the cell lines for normalization. (B) Detection of AKR1C3 protein by immunoblot with an isoform

specific monoclonal antibody in parental MCF-7 cells and AKR1C3 expressing cells. An antibody against β-actin was used as a loading control.

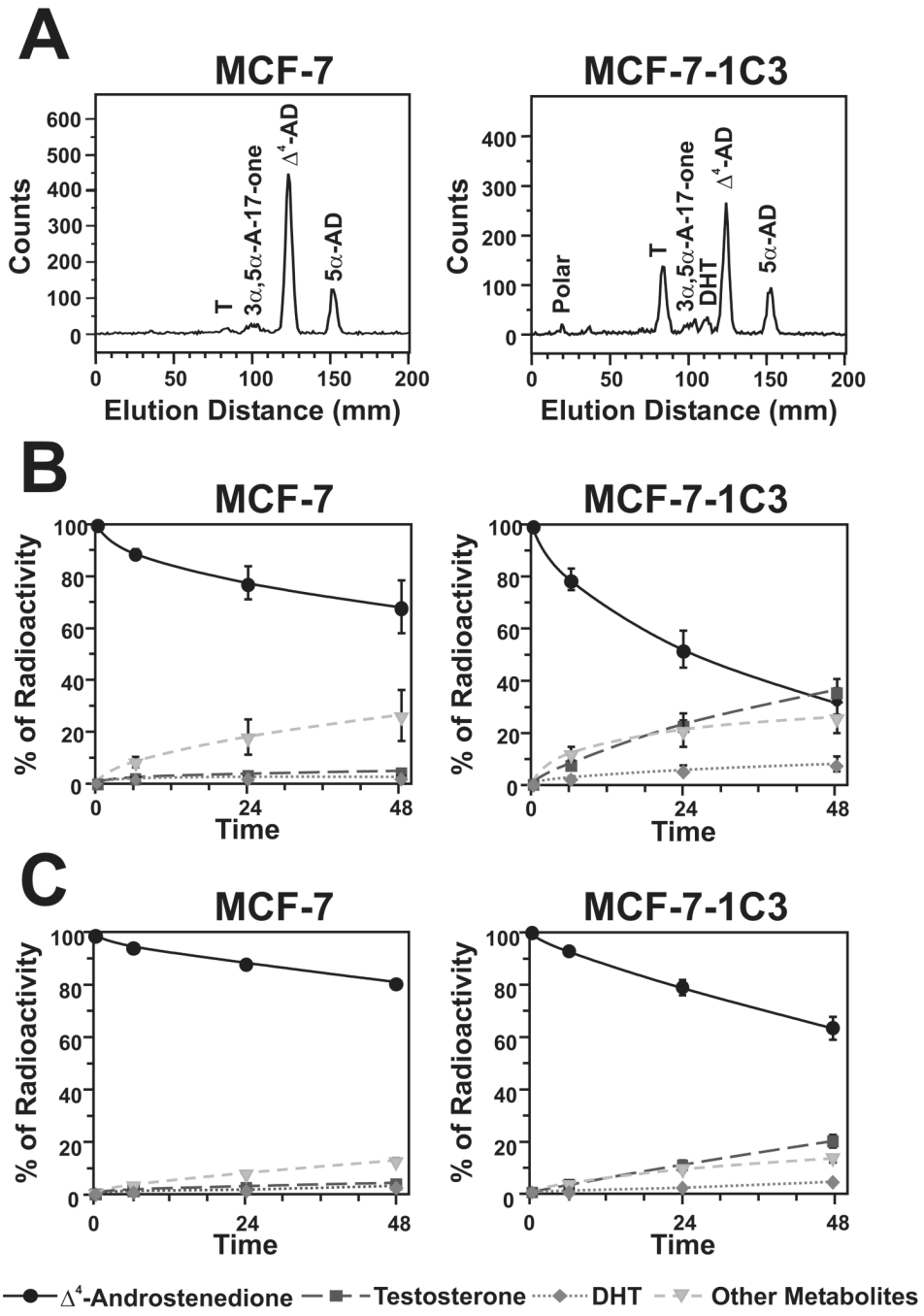

Fig. 2.

Expression of AKR1C3 increases conversion of Δ^4-androstenedione to testosterone by MCF-7 cells. (A) Representative metabolic profiles at 24 h demonstrating metabolism of 0.1 μM [^{14}C]-Δ^4-androstenedione by MCF-7 and MCF-7-AKR1C3 cells. Time courses of metabolism of (B) 0.1 μM and (C) 5 μM [^{14}C]-Δ^4-androstenedione by MCF-7 and MCF-7-AKR1C3 cells. Results are from three independent experiments performed in triplicate. Abbreviations used: T, testosterone; 3α,5α-A-17-one, androsterone; DHT, 5α-dihydrotestosterone; Δ^4-AD, Δ^4-androstenedione; 5α-AD, 5α-androstanedione.

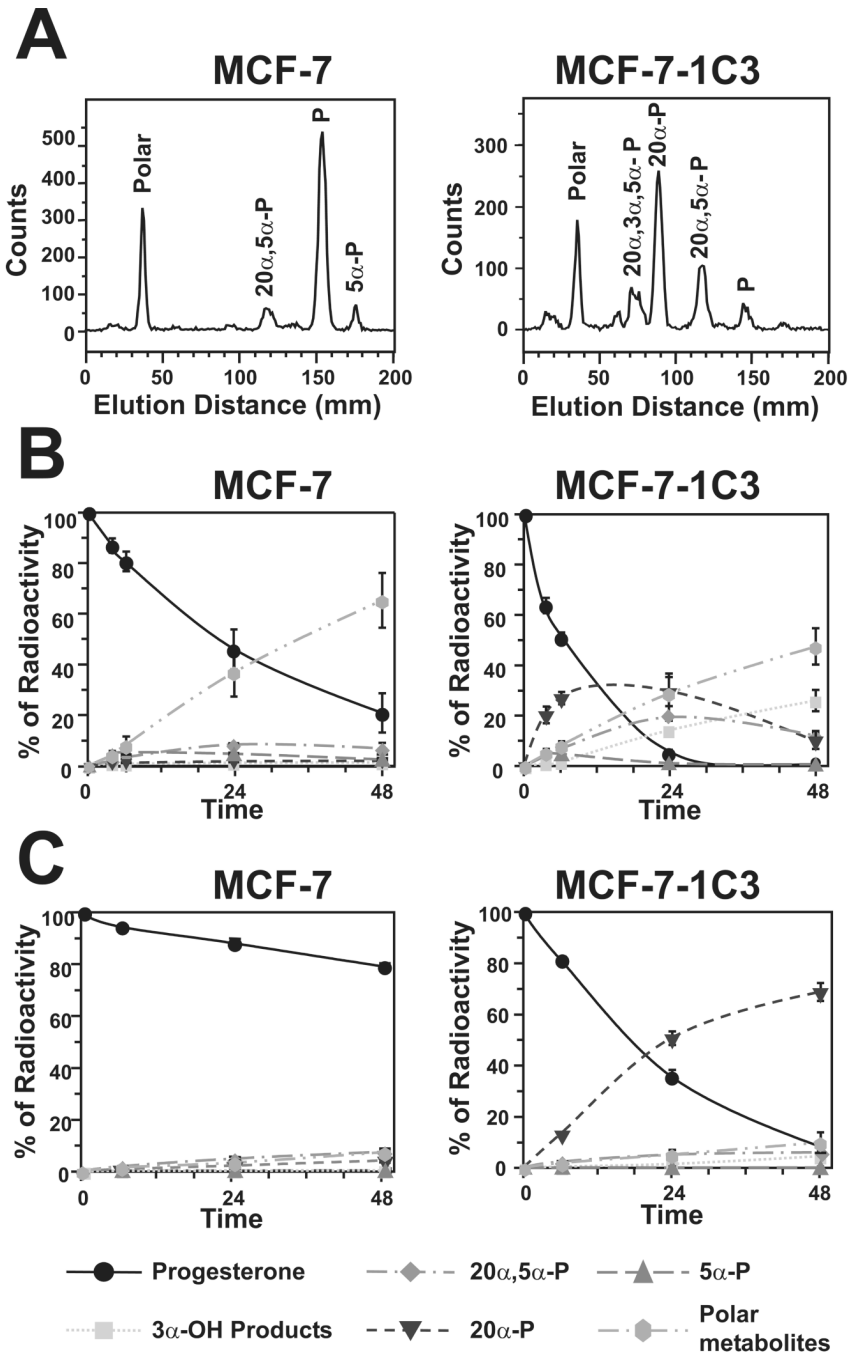

Fig. 3.

Expression of AKR1C3 increases conversion of progesterone to 20α-hydroxyprogesterone by MCF-7 cells. (A) Metabolic profiles at 24 h demonstrating metabolism of 0.1 μM [^{14}C]-progesterone by MCF-7 and MCF-7-AKR1C3 cells. Time courses of metabolism of (B) 0.1 μM and (C) 5 μM [^{14}C]-progesterone by MCF-7 and MCF-7-AKR1C3 cells. Results are from three independent experiments performed in triplicate. Abbreviations used: P, progesterone; 20α,5α-P, 5α-pregnan-20α-ol-3-one; 20α-P, 20α-hydroxyprogesterone; 5α-P, 5α-dihydroprogesterone.

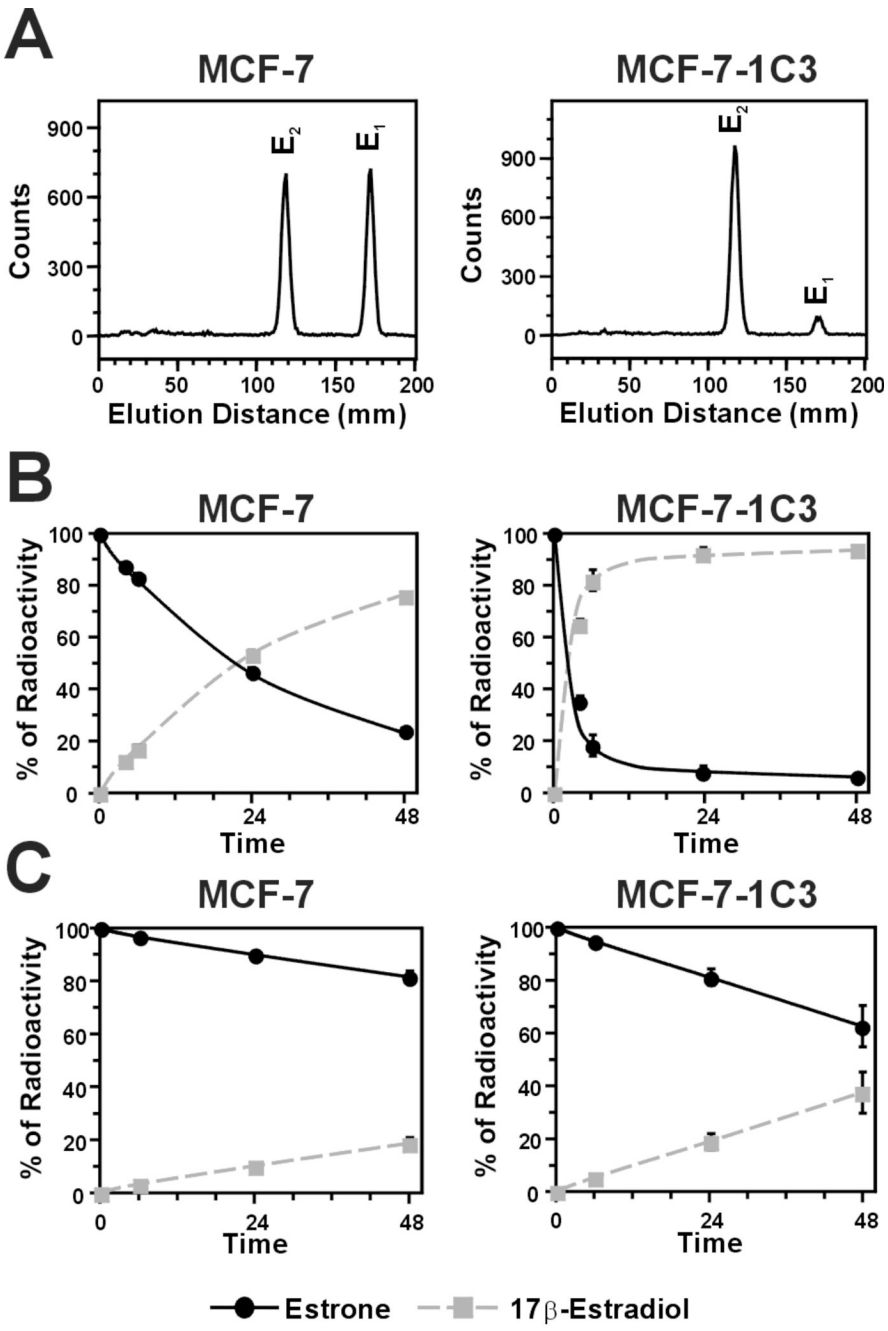

Fig. 4.

Expression of AKR1C3 increases conversion of estrone to 17β-estradiol by MCF-7 cells. (A) Metabolic profiles at 24 h demonstrating metabolism of 0.1 μM [^{14}C]-estrone by MCF-7 and MCF-7-AKR1C3 cells. Time courses of metabolism of (B) 0.1 μM and (C) 5 μM [^{14}C]-estrone by MCF-7 and MCF-7-AKR1C3 cells. Results are from three independent experiments performed in triplicate. Abbreviations used: E_1, estrone; E_2, 17β-estradiol.

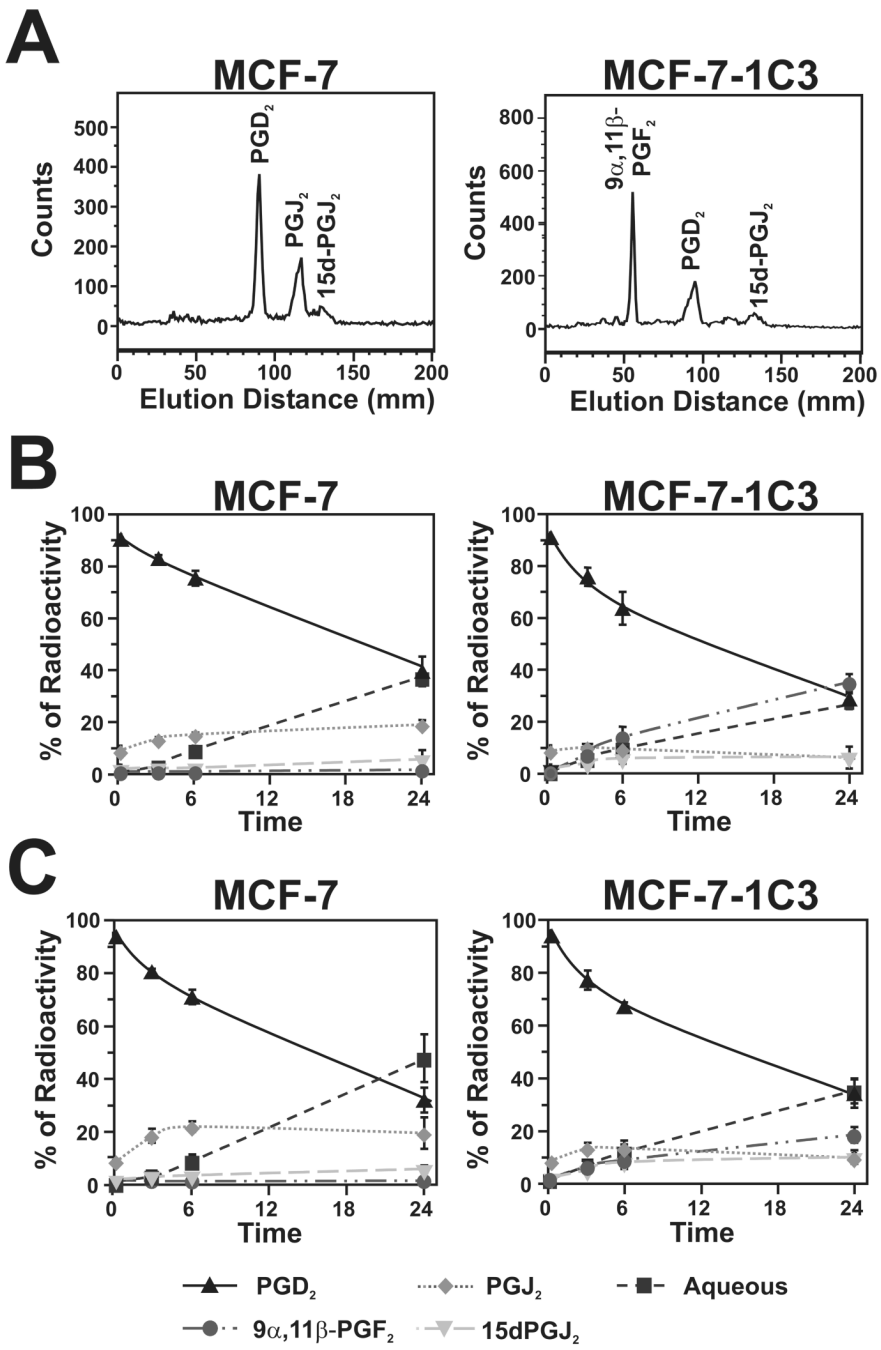

Fig. 5.

Expression of AKR1C3 increases conversion of PGD_2 to 9α,11β-PGF_2 by MCF-7 cells. (A) Metabolic profiles at 24 h demonstrating metabolism of 0.1 μM [^{3}H]-PGD_2 by MCF-7 and MCF-7-AKR1C3 cells. Time courses of metabolism of (B) 0.1 μM and (C) 5 μM [^{3}H]-PGD_2 by MCF-7 and MCF-7-AKR1C3 cells. Results are from three independent experiments performed in at least duplicate.

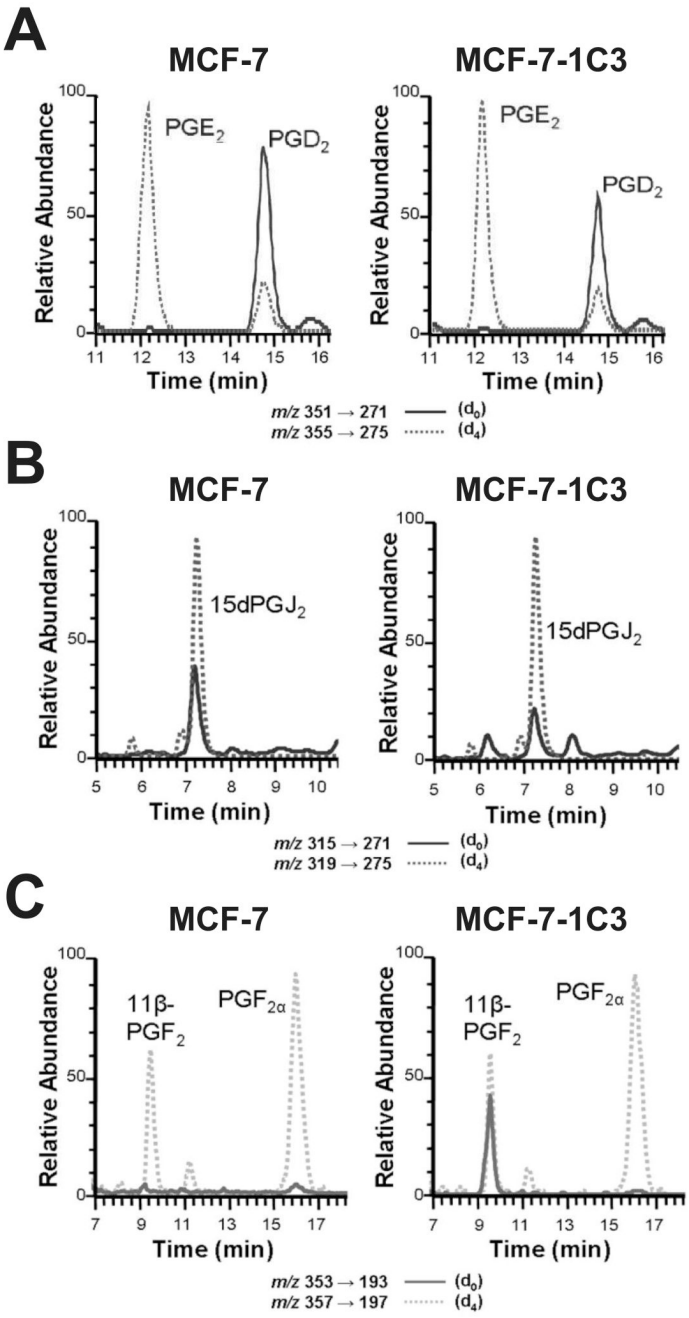

Fig. 6.

LC/MS analysis confirmed that AKR1C3 stereospecifically generates 11β-PGF_2 from PGD_2 in MCF-7 cells. Extracts of media from cells incubated with PGD_2 for 6h were monitored for the detection of pentafluorobenzyl derivatives of (A) PGE_2 and PGD_2, (B) $15dPGJ_2$, and (C) 9α,11β-PGF_2 and $PGF_{2\alpha}$. Deuterated standards (dashed lines) for each prostaglandin were added prior to extraction.

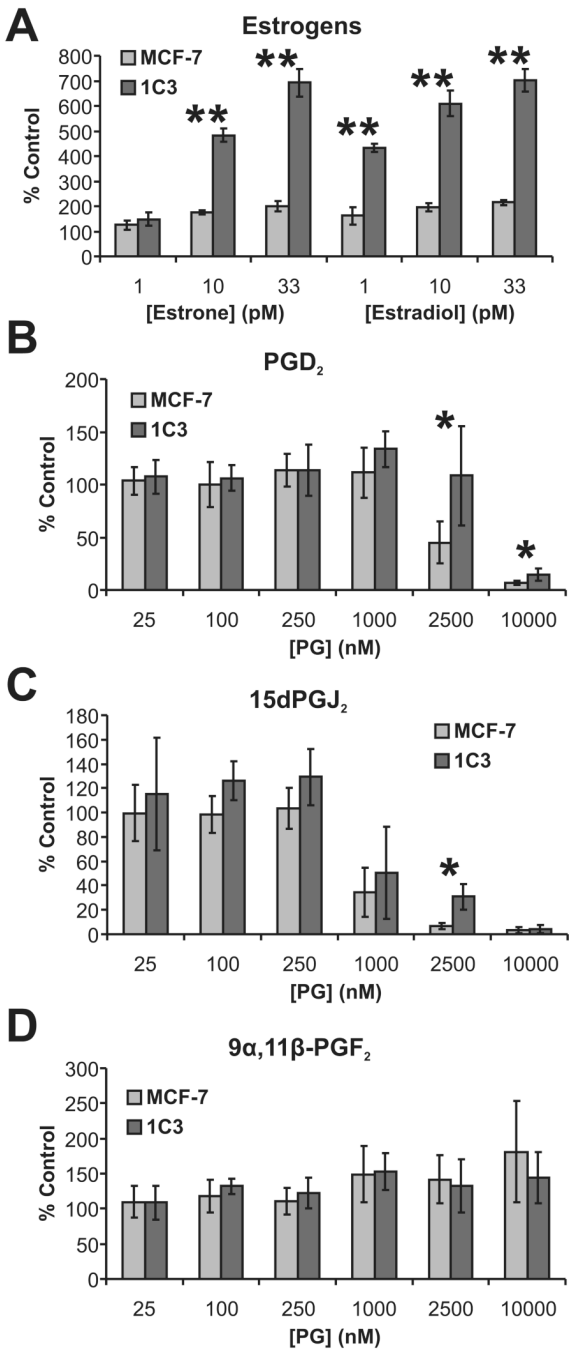

Fig. 7.

Expression of AKR1C3 modifies proliferation of MCF-7 cells in response to estrogens and prostaglandins. (A) AKR1C3 expression increased proliferation of MCF-7 cells in response to estrone and 17β-estradiol as measured with the MTT assay. (B-D) Proliferation of MCF-7 and MCF-7-1C3 cells in response to (B) PGD_2, (C) $15dPGJ_2$, and (D) $9\alpha,11\beta\text{-}PGF_2$ as measured by BrDU incorporation. Results are from at least three independent experiments performed in triplicate. * $p < 0.05$; ** $p < 0.01$.