The Stimulation of HSD17B7 Expression by Estradiol Provides a Powerful Feed-Forward Mechanism for Estradiol Biosynthesis in Breast Cancer Cells

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Our laboratory has previously cloned and purified an ovarian protein found to be a novel 17β-hydroxysteroid dehydrogenase type 7 enzyme (HSD17B7) (formerly prolactin receptor-associated protein) that converts the weak estrogen, estrone, to the highly potent estradiol. The regulation of this enzyme has not yet been explored. In this report, we show high expression of HSD17B7 in human ductal carcinoma and breast cancer cell lines and present evidence for a strong up-regulation of this enzyme by estradiol at the level of mRNA, protein expression, and promoter activity in MCF-7 cells. The effect of estradiol is mediated by estrogen receptor (ER)α, whereas ERβ prevents this stimulation. ER antagonists, ICI 182,780 and 4-hydroxytamoxifen, prevent estradiol-induced stimulation of the endogenously expressed HSD17B7, suggesting that these inhibitors not only block estradiol action but also its production. We have identified a −185-bp region of the *hsd17b7* promoter that is highly conserved among rat, mouse, and human and confers regulation by estradiol in MCF-7 cells. This region is devoid of a classical estradiol-response element but contains a nuclear factor 1 (NF1) site that is essential for estradiol action. We found that estradiol stimulates the recruitment and DNA binding of NF1 to this region of the *hsd17b7* promoter. Furthermore, knockdown of NF1 family members, NF1B, NF1A, and NF1X, completely prevents induction of this gene by estradiol. In summary, our findings demonstrate that estradiol stimulates HSD17B7 transcriptional activity in breast cancer cells through a novel mechanism requiring NF1 and strongly suggest a positive feedback mechanism to increase local estradiol synthesis causing growth of estrogen-dependent breast cancers. (*Molecular Endocrinology* 25: 754–766, 2011)

NURSA Molecule Pages: Nuclear Receptors: ER-α | ER-β; **Ligands:** 17β-estradiol | Fulvestrant | 4-Hydroxytamoxifen.

17β-Hydroxysteroid dehydrogenase type 7 (HSD17B7) is a 32-kDa microsomal protein involved in estradiol production. This enzyme was first discovered in our laboratory and named prolactin (PRL) receptor-associated protein, because it associates specifically with the cytoplasmic domain of the short form of the PRL receptor (1). Prolactin Receptor Associated Protein (known as PRAP) has been found since to be a novel isoform of 17β-hydroxysteroid dehydrogenase that is responsible for the conversion of estrone, a weak estrogen, to the more potent estradiol (2, 3). To date, 15 different isozymes of 17β-hydroxysteroid dehydrogenase have been cloned (4–8). They belong to a family of enzymes responsible for the activation/inactivation of hormones. All require nicotin-

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Abbreviations: CDT, Charcoal-dextran treated; ChIP, chromatin immunoprecipitation; Egr-1, early growth response-1; Elk-1, Ets like gene 1; ER, estrogen receptor; ERE, estradiol-response element; FBS, fetal bovine serum; HSD17B7, 17β-hydroxysteroid dehydrogenase type 7; ICI, ICI 182,780; IPTG, isopropyl-β-D-thiogalactopyranoside; NADPH, nicotinamide adenine dinucleotide phosphate; NF1, nuclear factor 1; PRL, prolactin; Q-PCR, quantitative PCR; si, small interfering; Sp1, specificity protein 1; Tam, 4-hydroxytamoxifen.

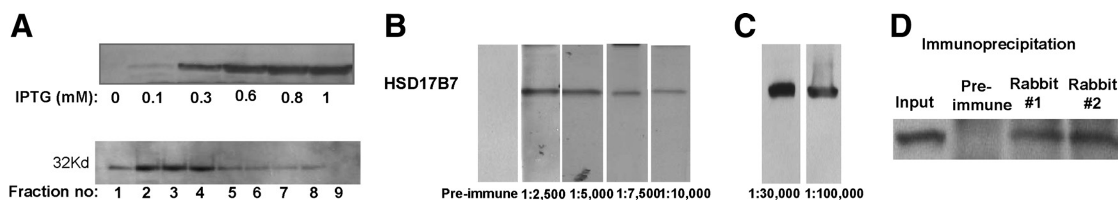

FIG. 1. Generation of HSD17B7 polyclonal antibody. A, Expression of His-tagged HSD17B7 in bacteria. Cells were grown in culture and induced to express His-HSD17B7 with increasing doses of IPTG. His-HSD17B7 proteins were purified on Talon column. The different fractions obtained were run on SDS-PAGE and stained with Coomassie blue dye. *Fraction number* denotes individual eluates obtained via sequential elution from the same set of Talon column as described in *Materials and Methods*. These eluted proteins were used to generate antibodies in two rabbits. B and C, Western blot analysis was performed with serially diluted antibodies from first (B) and fourth bleed (C) using proteins from corpora lutea known to highly express HSD17B7. D, Immunoprecipitation of luteal HSD17B7 with the two antibodies generated to native HSD17B7 protein.

amide adenine dinucleotide phosphate (NADPH) for activity and are short chain dehydrogenases/reductases, with the exception of HSD17B5. All of these enzymes, beside types 6 and 9, have been found in humans. The majority of these isoenzymes use steroids as their substrates (4, 7), and most, including HSD17B7, recognize specific substrates (2).

HSD17B7 is highly expressed in the ovarian corpus luteum of every mammalian species examined and is responsible for luteal estradiol biosynthesis in the ovary (1, 9, 10). Several HSD17B isoforms have also been found to be of importance in hormone-dependent tumors (11–13). HSD17B7 was detected by RT-PCR and immunohistochemistry in normal and pathological human breast tissue (14). The local production of estradiol in breast cancer cells is presently a subject of great interest, because it is becoming clear that locally produced estradiol can exacerbate growth of hormone-dependent breast tumors. The local mechanisms responsible for high estradiol concentrations observed in the breast are not completely understood (15) but most probably involve increased expression of enzymes involved in estradiol biosynthesis. Both P450aromatase, which converts androstenedione to estrone, and HSD17B7, which converts estrone to estradiol, are expressed in the breast (16). Although extensive efforts have been invested in defining regulatory mechanisms for P450aromatase in breast cancer (17, 18), no information is available to date as to what regulates HSD17B7 expression. Because it is estradiol, not estrone, that plays a critical role in the progression of breast cancer (15, 19–22), the control of HSD17B7 gene expression in cancer cells can be of great significance (23).

In this investigation, we show that although HSD17B7 is expressed at low levels in normal epithelial cells of breast ductal tissue, it becomes highly expressed in neighboring cancerous cells. Using breast cancer cells and a 1.16-kb HSD17B7 promoter isolated in our laboratory, we established that this enzyme is under transcriptional control by estradiol. We show that this estradiol-mediated stimulation is inhibited by 4-hydroxytamoxifen (Tam) and ICI 182,780 (ICI) and involves estrogen receptor (ER)α but not ERβ. We have also found a novel mechanism of estradiol stimulation of *hsd17b7* gene mediated by nuclear factor 1 (NF1) transcription factors.

Results

Purification of His-tagged HSD17B7 in its native form

When HSD17B7 was first discovered, our laboratory cloned its cDNA and generated a polyclonal antibody to the denatured form of the HSD17B7 protein, which has limited use (9). To generate a polyclonal antibody to the functional HSD17B7 that has a folded structure, we subcloned its cDNA into a prokaryotic N-terminal His-tag expression vector (pPro-Ex-HT). As shown in Fig. 1A, *upper panel*, we were able to induce the expression of HSD17B7 in response to isopropyl-β-D-thiogalactopyranoside (IPTG) in BL21 bacterial cells. The native HSD17B7 was eluted in several fractions and was subjected to coomassie blue staining (Fig. 1A, *lower panel*). The three fractions that contained the highest amount of protein were used for immunizations. We established by Western blot analysis the antibody specificity and titer (Fig. 1B). After the fourth immunization, sera from both rabbits showed very high reactivity with HSD17B7, even when the dilution factor was as high as 1:100,000 (Fig. 1C). Results shown in Fig. 1D indicate that this antibody, generated against the native protein, can immunoprecipitate HSD17B7.

HSD17B7 is expressed in rodent mammary gland and human breast cancer tissue

In the present investigation, we detected HSD17B7 not only in ovarian corpus luteum but also in HC-11 cells (Fig. 2A), a mouse mammary gland-derived epithelial cell line, and in a human cell line derived from the pleural effusion of a breast cancer patient, MCF-7. Cell extracts from nonsteroidogenic tissue (liver, brain, skeletal muscle, and heart) served as negative controls (Fig. 2A). HSD17B7 was also found in mouse mammary tissue (Fig. 2B). Similar to previous reports, HSD17B7 was detected in various human breast cancer cell lines (5, 8, 24) (data

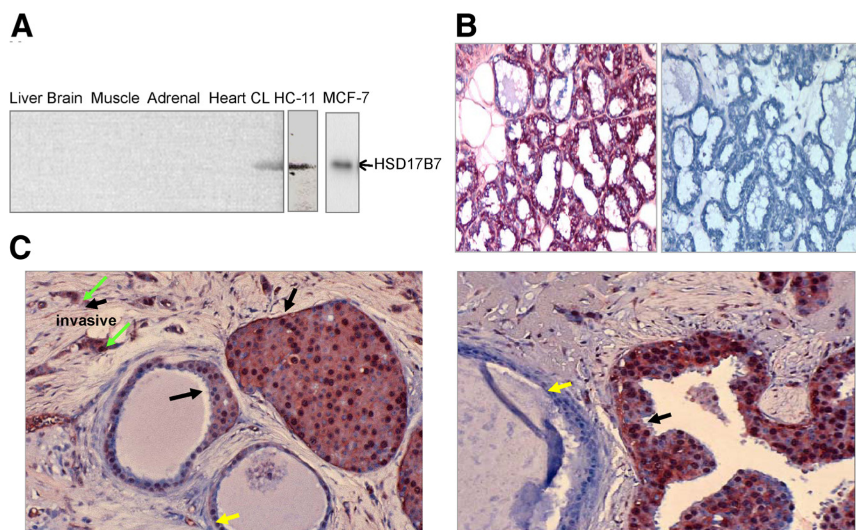

FIG. 2. Expression of HSD17B7 protein in mouse mammary gland and human breast carcinoma tissue. A, Western blot analysis was performed with whole-cell extracts isolated from corpora lutea (CL) (positive control) and nonsteroidogenic tissues (negative control) as well as mouse mammary gland-derived epithelial cells (HC-11) and human breast cancer cell line (MCF-7). B, Mouse mammary gland tissue was prepared for immunohistochemistry as described in *Materials and Methods*, and HSD17B7 expression (*left panel*) was detected using the specific antibody to HSD17B7 as indicated by the *red color*. Negative control using antirabbit IgG and hemotoxylin staining was performed in adjacent cut (*right panel*). C, Immunohistochemical detection of HSD17B7 protein in human ductal carcinoma showing extensive expression in pathological ducts (*black arrows*) and low expression in adjacent normal ducts (*yellow arrows*). Immunohistochemical detection of HSD17B7 protein in ductal carcinoma in human tissues demonstrates extensive expression in both invasive (*green arrow*) and *in situ* (*black arrow*) carcinoma. HSD17B7 is not expressed in adjacent normal ducts (*yellow arrow*).

not shown). HSD17B7 protein expression was examined in different human breast cancer tissues (provided courtesy of Northwestern University and the Specialized Program of Research Excellence Center at the University of Chicago). Immunohistological studies (Fig. 2C) show HSD17B7 to be highly expressed in ductal carcinoma. The detection of HSD17B7 protein in ductal carcinoma in human tissues demonstrated extensive expression in both invasive (Fig. 2C, *green arrow*) and *in situ* (Fig. 2C, *black arrow*) carcinoma. HSD17B7 enzyme was either not detectable or barely detectable in neighboring adjacent ducts undergoing hyperplasia (Fig. 2C, *yellow arrows*).

Estradiol stimulates the expression of HSD17B7 in MCF-7 cells

Because previous work has indicated a role for estradiol in the stimulation of HSD17B7 in the corpus luteum (1) and to better understand the regulation of HSD17B7 in human breast cancer, we examined whether estradiol influences *hsd17b7* gene expression in the ER-positive breast cancer cell line, MCF-7. Immunocytochemical (Fig. 3A) and Western blot analysis (Fig. 3B) revealed low expression of HSD17B7 in untreated MCF-7 cells. Estradiol treatment induced a remarkable increase in the level of HSD17B7 expression in these cells (Fig. 3B). We also found using both RT-PCR (Fig. 3C, *left panel*) and quantitative PCR (Q-PCR) (Fig. 3C, *right panel*) that estradiol markedly stimulates the endogenous expression of HSD17B7 mRNA. This stimulation involves ER, because ER antagonists, ICI and Tam, blocked the effect of estradiol on HSD17B7 protein (Fig. 3B) and mRNA (Fig. 3C) levels. Results generated with 1.16-kb *hsd17b7* promoter construct (3) suggest that estradiol-mediated stimulation of *hsd17b* gene expression in MCF-7 cells may be at the level of transcription (Fig. 3D). Although stabilization of the mRNA could be another possibility. An artificial estradiol-responsive promoter (2xERE pS2) was used as a positive control (Fig. 3D, *right panel*). The specific role of ER in estradiol-induced transcriptional activity was further assessed using either ICI or Tam, both of which acted as antagonists and reversed the stimulatory effect of estradiol (Fig. 3D, *left panel*), further substantiating the finding that estradiol up-regulation of *hsd17b7* is an ER-mediated event.

Characterization of estradiol up-regulation of *hsd17b* promoter activity and the role of ERα and ERβ

Time-course (Fig. 4A) and dose-response (Fig. 4B) analysis of estradiol stimulation of the *hsd17b7* promoter indicated that this stimulation is highly sensitive to low doses of estradiol and becomes apparent within 8 h of treatment. MCF-7 cells endogenously express ERα and respond to estradioltreatment with an increase in transcriptional activity of the *hsd17b7* promoter. To examine the effect of ERβ expression on the regulation of *hsd17b7* by estradiol, MCF-7 cells were transfected with two different amounts of ERβ expression vector (Fig. 4C), both of which completely blocked regulation of *hsd17b7* by estradiol, suggesting that ERβ represses the activity of endogenous ERα on this gene. Interestingly, overexpression of ERα, which was carried out as a control, showed that 50 ng/well of ERα could increase the regulation of *hsd17b7* by estradiol, indicating that the level of ERα in these cells may be a limiting factor. This was confirmed (Fig. 4D) by transfection of increasing amounts of ERα, where estradioltreatment causes increase in transcriptional activity of the *hsd17b* promoter.

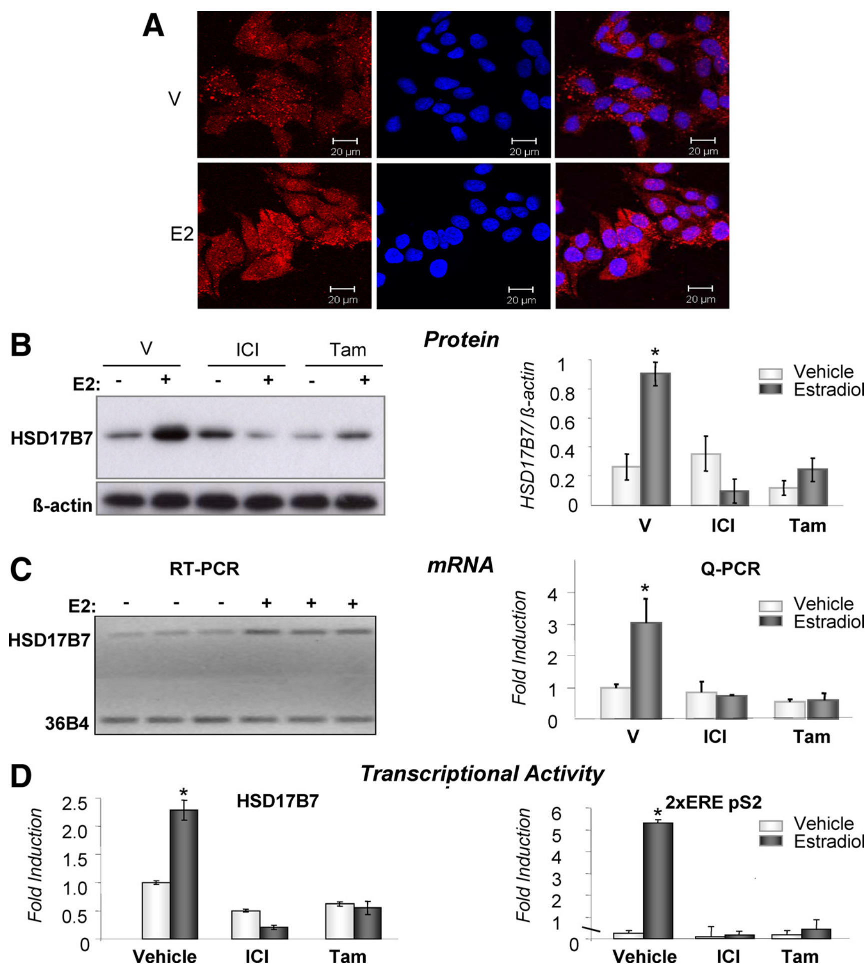

FIG. 3. Estradiol (E2) up-regulates HSD17B7 expression and transcriptional activity in MCF-7 cells. A, MCF-7 cells were cultured on chamber slides, treated with either estradiol (10^{-9} M) or vehicle (V) (0.1% ethanol) and prepared for immunocytochemistry as described in *Materials and Methods*. Cells treated with vehicle (*upper panel*) or estradiol (*lower panel*) were stained with polyclonal antibody to HSD17B7 (1:200, final dilution). Nuclei were stained with 4′,6-diamino-2-phenylindole. *Red*, HSD17B7; *blue*, nucleus. *Scale bars*, 20 μm. B, MCF-7 cells were cultured with either estradiol or vehicle, in the presence or absence of ICI or Tam for 24 h. HSD17B7 protein levels were examined by Western blotting, and densitometer data are shown in *right panel*. C, mRNA expression was determined by either RT-PCR (*left panel*) or Q-PCR (*right panel*). D, *left panel*, 1.16-kb *hsd17b7* (0.5 μg/well) promoter was transfected into MCF-7 cells using Lipofectamine 2000. 2xERE pS2 promoter (0.5 μg/well) was used as control (*right panel*). Cells were treated with or without inhibitors, and luciferase activity was determined. Results in each panel are the mean ± SEM from three independent experiments performed in triplicate (*, $P < 0.05$).

Localization of the *hsd17b7* promoter region that is responsive to estradiol

To determine the response elements necessary for estradiol stimulation of the *hsd17b7* promoter, we have generated truncated constructs by 5′ deletion. After transfection with 1.16-kb and 5′ truncated *hsd17b7* promoters, MCF-7 cells were treated for 24 h with 10^{-9} M estradiol. Estradiol stimulated the 1.16-kb as well as two truncated *hsd17b7* promoters (Fig. 5A). However, deletion of the region between −185 and −115 bp abolished the effect of estradiol, suggesting that this 70-bp region is necessary for estradiol-mediated stimulation. ICI and Tam antagonized estradiol stimulation of the −185-bp fragment of the *hsd17b7* promoter (Fig. 5B). Analysis of this region of the promoter with the Transcription Element Search Software revealed that this region is highly conserved among human, rat, and mouse. To narrow down the site important for estradiol action, we performed further truncations and internal deletions of the −185-bp promoter (Fig. 5C). Internal deletion of the piece of DNA between −140 and −115 bp (Fig. 5C) significantly reduced, but did not completely prevent, stimulation of the promoter by estradiol, suggesting that multiple estradiol-responsive regulatory regions may exist in this promoter.

Recent studies have shown that the mechanism of estradiol activation of some target genes can involve interaction of ERα with other DNA-bound transcriptional factors, such as specificity protein 1 (Sp1) proteins (25–28). A number of estrogen-responsive genes contain Sp1 sites adjacent to an imperfect estradiol-response element (ERE) or to an ERE half-site (29). At these sites, ERα is able to bind directly to DNA and/or enhance Sp1 binding to its recognition site (reviewed in Refs. 25 and 30). Within the −185- to −115-bp region of the *hsd17b7* promoter, we found one half-ERE as well as two Sp1 sites. Interestingly, all three sites were found in the −185-bp fragment. However, mutation of the ERE half-site and Sp1-binding sites either alone or in combination did not have any effect on estradiol stimulation of this promoter (Fig. 5D). We tested several different mutations to each Sp1 and half-ERE sites, and each one had the same results. These results indicating that although these sites in other genes can mediate estradiol action do not confer responsiveness of *hsd17b7* to estradiol.

Transcription factors necessary for estradiol stimulation of hsd17b7

To examine whether the −185- to −140-bp region does in fact bind protein in an estradiol-stimulated manner, a gel shift mobility assay was performed with an oligo spanning −160 to −140 bp (Fig. 6). Similar data using an oligo spanning −185 to −140 was obtained (data not shown). We found that estradiol stimulated the formation of a protein/

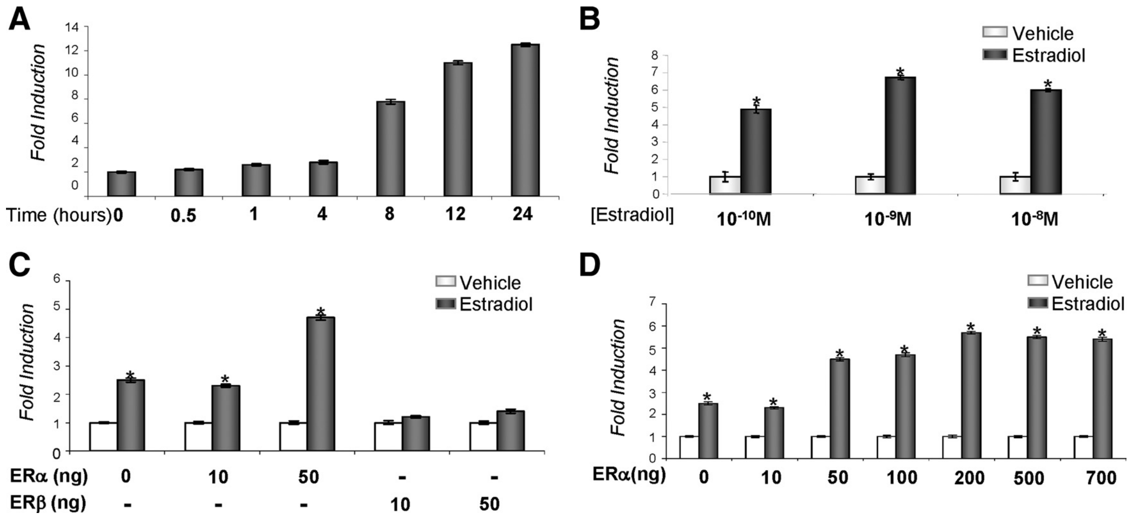

FIG. 4. Characterization of estradiol up-regulation of *hsd17b7* promoter activity and the role of ERα and ERβ. MCF-7 cells were transfected with *hsd17b7* promoter (0.5 μg/well). Five hours after transfection, time course (A) and dose response of estradiol action (B) were examined. C, *hsd17b7* promoter (0.5 μg/well) was transfected with different doses of either ERα or ERβ expression vectors, and reporter activity was measured. D, *hsd17b7* promoter (0.5 μg/well) was cotransfected with increasing doses of *ERα* expression vector. In all transfections, the total amount of DNA was balanced with the appropriate empty vector. After transfection, cells were treated for 24 h with estradiol, and reporter activity was measured. Results are the mean ± SEM from three independent experiments performed in triplicate (*, $P < 0.05$).

DNA complex in a time-dependent manner and that this complex was blocked by excess unlabeled oligo, indicating that transcription factor binding in response to estradiol is specific for this nucleotide sequence. No supershift was seen with Sp1 or ERα antibodies, confirming the importance of this region but the lack of Sp1/ERE involvement. Although we had expected that estradiol mediates its action through Sp1/ERE sites, these data suggest that the regulation of *hsd17b7* by estradiol must involve another, potentially novel regulatory mechanism.

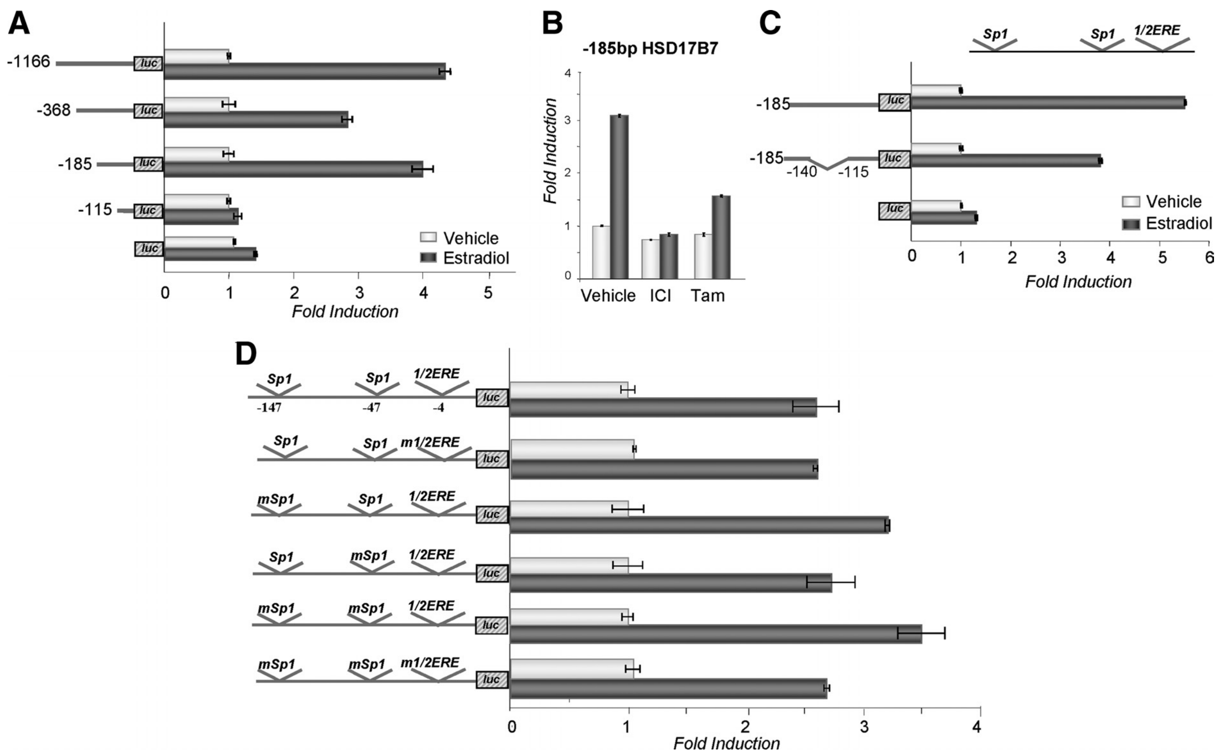

FIG. 5. Localization of estrogen response element in *hsd17b7* promoter. A, Truncated constructs were generated by 5′ deletion and transfected (0.5 μg/well) into MCF-7 cells. B, Cells transfected with −185-bp promoter were cultured in the presence or absence of ICI or Tam for 24 h. C, Internal deletion in the context of −185-bp promoter was examined in the presence or absence of estradiol. D, Mutation analysis of Sp1 and half-ERE putative binding sites in the −185-bp *hsd17b7* promoter. In each case, cells were harvested after a 24-h treatment with estradiol, and reporter activity was measured. Results are the mean ± SEM for three independent experiments performed in triplicate (*, $P < 0.05$).

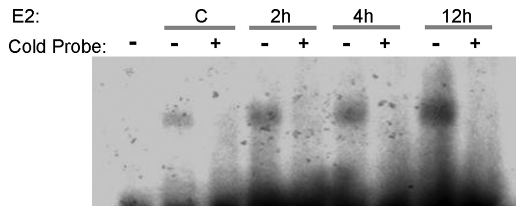

FIG. 6. Protein/DNA complex stimulated by estradiol (E2). Gel shift mobility assay was performed with either cold or ^{32}P-labeled oligo spanning −160 to −140 bp with nuclear extracts from MCF-7 cells treated with estradiol at different time points. DNA/protein complexes were run on acrylamide gel as described in *Materials and Methods*. The findings in this gel shift are representative of three independent experiments. C, Control.

Further analysis of the −185- to −140-bp proximal region of *hsd17b7* revealed putative binding sites for Ets like gene 1 (Elk-1), early growth response-1 (Egr-1), and NF1. As shown in Fig. 7A, Elk-1 protein levels were up-regulated by estradiol. ICI and Tam reversed this response. Egr-1, a known target of estradiol in MCF-7 cells (reviewed in Ref. 25), was also stimulated by estradiol in our hands. Mutation of the response elements to both Elk-1 and Egr-1 in the −185-bp promoter did not prevent this stimulation (Fig. 7B). Supershift assays using antibodies to Sp1, Elk-1, and Egr-1 established that these transcription factors are not part of the protein-DNA complex stimulated by estradiol (data not shown). These results suggest that although Elk-1 and Egr-1 are up-regulated by estradiol, their binding to their respective DNA sites does not appear to play a role in estradiol-induced stimulation of the *hsd17b7* promoter.

The NF1 family is composed of four members encoded by distinct genes, NF1A, NF1B, NF1C, and NF1X (31), producing distinct protein products, which can form homo- or heterodimers (32). In addition, all four NF1 mRNAs can be differentially spliced to yield a multitude of proteins with subtle differences in their transactivation capacities (33–35). NF1 transcription factors are ubiquitously expressed in most tissues and possess a constitutive DNA-binding capa-

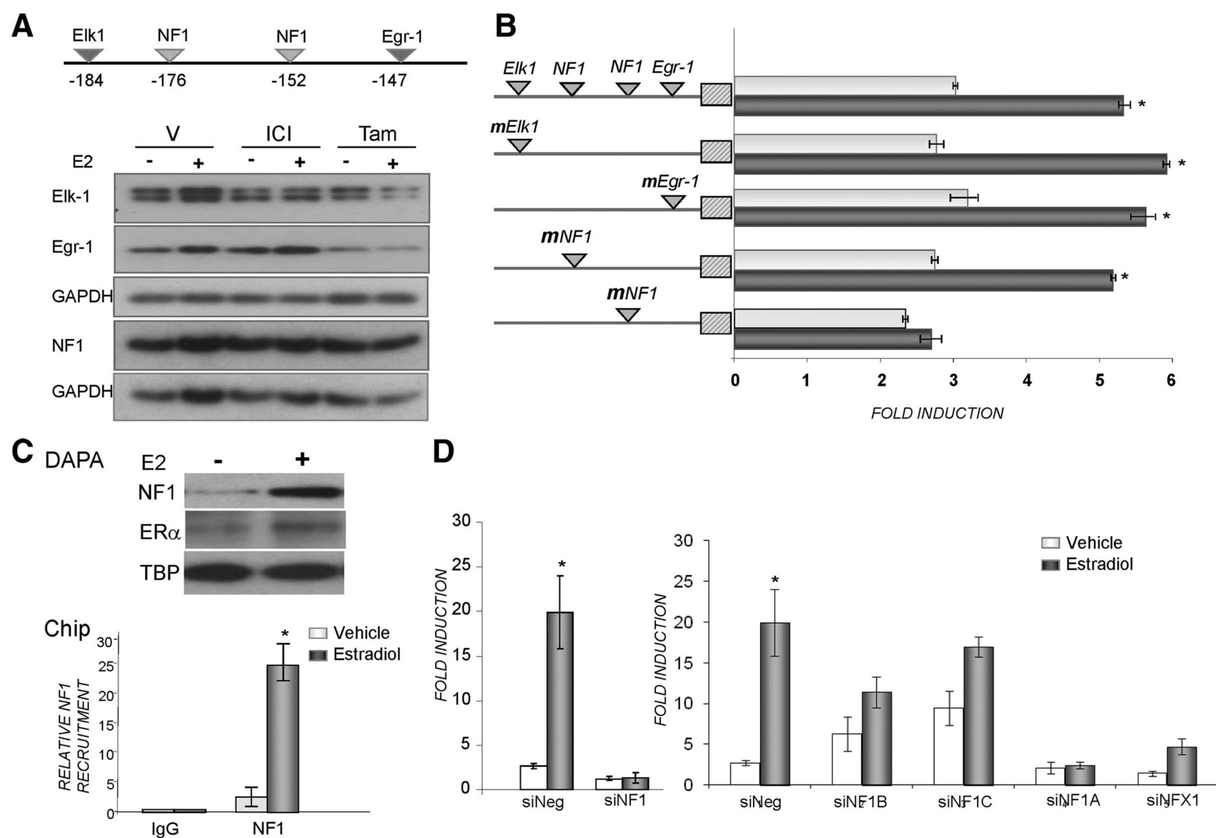

FIG. 7. Transcription factors necessary for estradiol (E2) stimulation of *hsd17b7*. A, MCF-7 cells were treated with either estradiol or vehicle (V) in the presence or absence of ICI or Tam for 24 h. Cell extracts were subjected to Western blot analysis with Elk-1, Egr-1, and NFI antibodies. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for loading control. B, −185-bp *hsd17b7* promoter constructs containing either intact or mutated Egr-1, Elk-1, and NF1 sites were transfected into MCF-7 cells. Cells were treated with estradiol and harvested after 24 h. Reporter activity was measured. C, *upper panel*, Protein-protein interactions were determined using DNA affinity precipitation assay analysis. Nuclear extract from MCF-7 cells treated with either estradiol or vehicle were incubated with biotinylated-labeled oligos containing NF1-binding sites. The precipitated DNA/protein complexes were subjected to immunoblotting with specific antibodies to pan NF1 proteins. The blot was reprobed for ERα and Tata-binding protein. C, *lower panel*, ChIP assay was performed in MCF-7 cells treated with either estradiol or vehicle using pan NF1 antibody. A nonspecific IgG antibody served as negative control. Q-PCR was then carried out on immunoprecipitated chromatin for 70-bp region containing the NF1 in the *hsd17b7* gene (*, $P < 0.05$). D, MCF-7 cells were cotransfected with −185-bp promoter and with NF1(s) siRNA. A negative siRNA was used as control. Results are the mean ± SEM from three independent experiments performed in triplicate (*, $P < 0.05$).

bility. These proteins act as regulators in viral DNA replication, gene transcription, cell proliferation, and development. It is difficult to pinpoint which NF1 proteins may be involved in the regulation of *hsd17b7* gene due to their high degree of sequence homology, functional similarity, and complex interactions as well as the large number of NF1 family proteins. Therefore, in this study two different pan antibodies to NF1 were used, and similar results were obtained with both. Western blot analysis using whole-cell extracts from MCF-7 cells treated with or without estradiol indicated that estradiol does not affect total NF1 expression (Fig. 7A). Densitometer analysis is included in Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org. Mutation of the distal NF1 site (Fig. 7B) also did not prevent estradiol stimulation of the *hsd17b7* promoter. However, mutation of the proximal NF1 site (Fig. 7B) led to total inhibition of estradiol stimulation.

To examine whether NF1 can bind to the proximal NF1 response element, short biotinylated DNA oligos containing the proximal NF1 sequence were used for DNA affinity precipitation assay. Mutated NF1 oligos were used as control as shown in Supplemental Fig. 2. We found that estradiol induced NF1 and ERα binding to the DNA region (Fig. 7C). Studies using chromatin immunoprecipitation (ChIP) assay and primers that amplify a region of 70 bp, from -185 to -115 bp, suggest that estradiol stimulated the recruitment of NF1 to the DNA motif, further suggesting that NF1 is a major transcription factor responsible for estradiol stimulation of *hsd17b7* promoter. The importance of NF1-mediated estradiol induction of *hsd17b7* was further confirmed using RNA interference method to knockdown NF1 or individual NF1 family members (Fig. 7D). MCF-7 cells were cotransfected with -185-bp promoter and small interfereing (si)NF1 or siControl (siNegative) oligos. siRNA to total NF1 inhibited NF1 expression (Supplemental Fig. 2) and abolished the estradiol up-regulation of *hsd17b7* (Fig. 7D). Further studies using siRNA to each member of NF1 complex (NF1B, NF1C, NF1A, and NFX1) showed that knockdown of NF1B, NF1A, and NFX1 abolish the stimulation by estradiol. Taken together, these results demonstrate that the ability of estradiol to stimulate expression of *hsd17b7* requires a novel mechanism involving NF1 recruitment to an essential NF1 response element in the gene promoter.

Discussion

Local estradiol production plays a pivotal role in the development and progression of hormone-dependent breast cancers (15, 22, 36). Human breast cancer tissues contain all the enzymes involved in the last steps of estradiol biosynthesis, including the rate-limiting step aromatase, estrone sulfatase, and 17β-hydroxysteroid dehydrogenase (37, 38). Sulfotransferases, which convert estradiol into the biologically inactive estrogen sulfates, are also present in this tissue (39). There is a great deal of interest in determining the role these enzymes play in producing estradiol in cancer cells, because this hormone plays a significant role in etiology of hormone-dependent breast cancer. HSD17B family of enzymes control the last step in the formation of highly potent estradiol, are highly expressed in many breast cancer cases, and are of great significance in the pathology of the breast (15, 22, 36). To date, three HSD17B enzymes have been identified to play a significant role in the production of the highly active estradiol, and these are HSD17B1 (17, 40–45), HSD17B7 (8, 11), and HSD17B12 (14). Before the discovery of HSD17B7 and HSD17B12, HSD17B1 was the only enzyme known to convert estrone to estradiol. It was originally cloned from human placenta (46) and found to be expressed in many breast cancer cases (17, 40–45, 47). This enzyme was thought to be the main player in the local production of estradiol in breast cancer (42, 44). However, Labrie and co-workers (14) reported that HSD17B7 and HSD17B12, and not HSD17B1, are commonly expressed in human breast cancer. HSD17B12 is the last discovered enzyme shown to catalyze the conversion of estrone to estradiol (48, 49). It is ubiquitously expressed with the highest levels in liver, muscle, and kidney cells. It is also expressed in ovarian, mammary gland, and breast cancer cells (48, 49). There is a great deal of interest in determining the role that these three enzymes play in producing estradiol in cancer cells and in generating antagonists to these enzymes (12, 13, 24, 49, 50). However, there is currently no information about what stimulates their expression in cancer cells. In this investigation, we found that the expression of *hsd17b7* gene is up-regulated by estradiol, suggesting that estradiol can sustain its own production in breast cancer cells. Recent studies by Kinoshita and Chen (51) and Andò and co-workers (52) show that estradiol amplifies its own signal by up-regulating aromatase activity in MCF-7 cells, and this estradiol/ER-mediated effect is suppressed by antiestrogens (51, 52). Therefore, by regulating expression of enzymes responsible for its own metabolism (biosynthesis and degradation), estradiol can create a potent feed-forward loop to further increase local estrogen levels and drive growth of hormone-dependent breast tumors.

Estradiol induces different mechanisms of action in breast cancer, and central to these mechanisms are two types of ERs, ERα and ERβ. Both receptors are coexpressed in the majority of human breast cancers. It is now becoming apparent that progression of disease is dependent not only on individual expression and action of ERα

and ERβ (53) but also on the interaction and ratio of the two receptors (54–56). It is well established that ERα has mitogenic activity in human breast cancer. In our studies, endogenous expression of ERα leads to up-regulation of *hsd17b7* gene, and overexpression of this receptor further up-regulated estradiol action. Similar results to ours have previously been reported for other estradiol-responsive genes activated through nuclear or nongenomic pathways of estrogen action (31, 57), and this is apparently related to limiting levels of ERα in the cells. The repression of *hsd17b7* promoter's activity by ERβ is also in agreement with its repressive action on other genes (16, 36, 54–56, 58, 59). Interestingly, ERβ expression in breast cancer is correlated with positive prognosis (35), and the mechanism of ERβ-mediated *hsd17b7* repression deserves further investigation, because this receptor may prevent positive estradiol feed forward in breast cancer.

Given the fact that estradiol stimulates the growth of ER-positive breast cancer, antiestrogens have been established as an important endocrine therapy for breast cancer treatment (60). Tamoxifen, one of the antiestrogens, is broadly used to prevent estradiol/ER–mediated stimulation of cell growth in breast cancer, and many genomic studies have been done to show its effect on different genes (34, 36). From the results of this investigation, tamoxifen appears to antagonize not only estradiol action but also the local production of estradiol in ERα-expressing breast cancer cells.

It is clear from this investigation that estradiol induction of *hsd17b7* transcriptional activity involves ER and resides within a small −185-bp promoter that is devoid of classical palindromic sequences of ERE sites. We identified a variety of putative binding sites in the *hsd17b7* promoter region. However, only three, Sp1, Elk-1, and Egr-1, are known to regulate estradiol-responsive genes (25, 30, 61). To our dismay, mutation of these sites in the −185-bp promoter region did not prevent estradiol stimulation of the *hsd17b7* promoter. This, however, does not eliminate them as factors involved in regulation of *hsd17b7*, because they might act indirectly through protein-protein interactions.

Interestingly, despite the lack of estradiol stimulation of NF1 expression, we found the proximal NF1 site in *hsd17b7* promoter to be essential for estradiol-mediated activation. Estradiol markedly enhanced the occupancy of NF1 at the proximal site of the promoter. Studies with other steroid receptors have demonstrated that NF1 is recruited after displacement of the transcriptional repressive complex (62). In our studies, in the uninduced state, NF1 sites might not be accessible for estradiol/ER complex to bind to *hsd17b7* gene. Estradiol binding to ER and other coactivators might stabilize and facilitate NF1 binding to the basal transcriptional machinery resulting in *hsd17b7* promoter activation. Because each member of NF1 protein family has similar palindromic DNA-binding specificities, it makes it difficult to attribute specific molecular functions to individual NF1 proteins. Nevertheless, our results suggest a key role for NF1-B, NF1A, and NFX1. NF1 transcription factors may also have a broader role in the regulation of *hsd17b7* in tissues other than breast cancer. Indeed, deletion of *hsd17b7* by our laboratory (63), and disruption of three out of four members of the NF1 family (64), caused severe neurological defects and fetal death (65, 32, 35).

In addition to estradiol, PRL is thought to induce breast cancer (66, 67). Interestingly, both PRL and estradiol are involved in either stabilizing or stimulating HSD17B7. The association of the short form of the PRL receptor with HSD17B7 leads to the tyrosine phosphorylation of the enzyme by Janus kinase 2 and its stabilization, enhancing the cells' capacity to convert estrone into estradiol (1, 68). Furthermore, PRL stimulation of ER expression (69–72) might render the cell more sensitive to estradiol.

In summary, all the results taken together suggest the working hypothesis (Fig. 8) that there is a powerful feedback mechanism between estradiol and HSD17B7, the enzyme that converts estrone to estradiol. Estradiol stimulates the transcriptional activity of *hsd17b7* in cancer cells by activating ERα and recruiting at least one transcription factor, NFI, to a specific NF-1-binding site within the short −185-bp promoter region. Because HSD17B7 is stabilized by its association with the PRL receptor and because it is stimulated by estradiol, HSD17B7 might well be one common molecule used by PRL and estradiol to exacerbate breast tumorigenesis through a common signaling cascade.

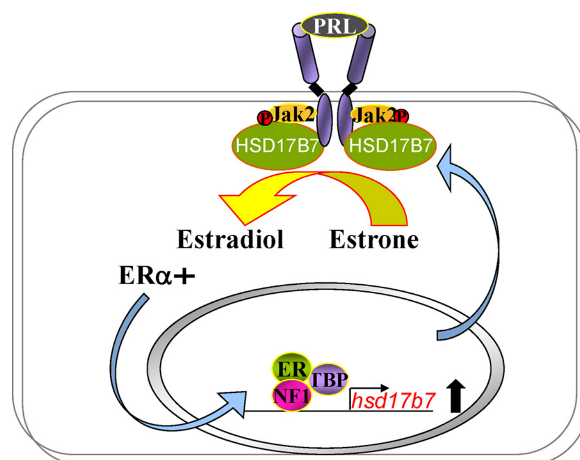

FIG. 8. Proposed model for HSD17B7 enzyme in cancer cells. HSD17B7 is expressed in breast cancer cells, where it can cause the conversion of estrone produced by breast fat cells to the potent estradiol. In turn, estradiol stimulates HSD17B7 gene expression through acting through ERα and a defined region of the promoter containing NF1-binding site, stimulates HSD17B7 gene expression, leading to a positive feed-forward loop. Because HSD17B7 binds to and is stabilized by the PRL receptor, and is stimulated by estradiol, this enzyme may well be one common molecule used by PRL and estradiol to induce breast cancer. Jak2, Janus kinase 2.

Materials and Methods

Materials

Benita Katzellenbogen generously supplied the human pCMV-ERα and pCMV-ERβ expression vectors and 2xERE pS2 reporter vector. MCF-7 cells were provided by Randall Jaffe.

Purification of His-tagged HSD17B7

DH5α bacterial cells transformed with HSD17B7 in the pProEx-HT plasmid were grown in a 500 ml culture of lysogeny broth medium at 37 C overnight; 10 ml of this overnight culture were placed into 1 liter of fresh, prewarmed lysogeny broth and grown at 30 C for 4 h. IPTG was then added to a final concentration of 0.6 mM to induce expression of His-tagged HSD17B7. The time of incubation with IPTG to maximal expression of HSD17B7 was shown to be 2–3 h, so incubation was stopped 3 h after the addition of IPTG. The culture was centrifuged to obtain the bacterial pellet, which was then frozen at −80 C overnight. Talon resin (CLONTECH, Mountain View, CA) was used for purification under native conditions according to the manufacturer's protocol. Because HSD17B7 is a microsomal protein, we used the nondenaturing detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (10 mM), cofactor NADPH (40 μM), and very stringent salt washes to purify this protein. The pellet was resuspended in lysis buffer [50 mM sodium phosphate (pH 8.0), 300 mM NaCl, and 10 mM imidazole] with lysozyme (1 mg/ml) and with 1 μM NAD+ (NADPH) as cofactor. This was incubated for 30 min on ice and then subjected to sonication to lyse the cells. It was then centrifuged (10,000 × *g* for 30 min) to sediment the cellular debris. The supernatant was then incubated overnight at 4 C with slurry containing the Talon resin to bind the His-tagged HSD17B7. The next morning, the mixture was poured into a column and the unbound fraction collected. Wash buffer [50 mM sodium phosphate (pH 8.0), 300 mM NaCl, and 20 mM imidazole] was used to elute weakly bound proteins, and finally elution buffer [50 mM sodium phosphate (pH 8.0), 300 mM NaCl, and 250 mM imidazole] was used to elute the fusion protein.

Generation of polyclonal antibody to native HSD17B7 enzyme

Needle injections and serum collection were performed at University of Illinois at Chicago Biological Research Laboratory according to institutional guidelines for animal care and handling. Female New Zealand white rabbits (The Jackson Laboratory, Bar Harbor, ME) were used for immunization as described previously (9). The specificity of the antibody was tested by probing a Western blot containing HSD17B7 protein purified as described above and whole-cell extract from d 15 of pregnancy. The antibody recognized a single band of the correct size for HSD17B7 (Fig. 1).

Tissue preparation and histology

Mammary gland and breast cancer tissue were sectioned as previously described (63) and were incubated overnight at 4 C with a primary polyclonal antibody to HSD17B7 (1:150 dilution) and then incubated with a secondary biotinylated goat antirabbit IgG according to manufacturer's instructions (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Peroxidase activity was developed with Nova Red solution (Vector Laboratories), and sections were counterstained with hematoxylin (Vector Laboratories). Appropriate controls were performed by preabsorbing HSD17B7 antibody in 0.1 M sodium phosphate buffer (1:150 dilution) with a corpora lutea-enriched microsomal fraction overnight at 4 C.

Cell culture and treatments

MCF-7 cells, which are estrogen-sensitive cells derived from the pleural effusion of a breast cancer patient, were cultured in DMEM (Invitrogen, Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) and 1× pen/strep solution (CellGro, Herndon, VA) at 37 C in a 5% CO_2, humidified atmosphere. At least 3 d before the experiments, maintenance media were replaced by treatment media, consisting of phenol red-free DMEM supplemented with 5% charcoal-dextran-treated (CDT) serum (CDT-FBS; HyClone) and antibiotics. To examine the effects of estradiol on HSD17B7 mRNA expression, estradiol dose-response experiments were performed. Unless listed otherwise, estradiol treatments were 10^{-9} M for 24 h, whereas the concentration of estradiol inhibitors, Tam and ICI, was 100-fold higher than that of estradiol. The control group always received ethanol (0.1%) as vehicle. After treatment, cells were washed twice with cold PBS and stored at −80 C before RNA and protein were extracted or luciferase assay was performed.

Transfection

For transient transfections, 10^5 cells were seeded in 24-well plates and cultured in phenol red-free media containing 5% CDT-FBS. In general, cells were 80–90% confluent at the start of transfection. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Unless otherwise mentioned, 0.5 μg of the promoter construct were added for optimum transfection efficiency. Expression vectors were transfected at different doses as indicated in each experiment. In all transfections, the total amount of DNA was balanced with the appropriate empty vector. After treatment, cells were washed twice with cold PBS and stored at −80 C for reporter assays.

Reporter assays

Luciferase activity was measured by first preparing cell lysates in 1× reporter lysis buffer (Promega, Madison, WI). Luciferase activity driven by the *hsd17b7* promoter constructs was measured by combining the lysate with firefly luciferase assay substrate (Promega) according to manufacturer's protocol. Luciferase activity was normalized to total protein levels in each well, because β-galatosidase and Renilla activities are regulated by estradiol.

Immunocytochemistry

MCF-7 cells were grown on Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY) with either estradiol or vehicle for 24 h and processed for immunocytochemistry as described previously (32). A polyclonal antibody to HSD17B7 (1:200) and Cy3-conjugated donkey antirabbit IgG (1:800; Jackson ImmunoResearch, West Grove, PA) were used as primary and secondary antibodies, respectively.

RNA isolation and reverse transcription

RNA was isolated from cells using TRIzol reagent (Invitrogen) as described previously (3). One microgram total RNA was reverse transcribed in a total volume of 20 μl using 200 U reverse transcriptase, 0.3 μg oligo-$(dT)_{18}$, and 1 mM deoxynucleotide triphosphate (Invitrogen). The resulting cDNA was then diluted to a total volume of 100 μl by adding diethylpyrocarbonate-treated H_2O.

Real-time PCR

To generate standard curves for real-time PCR, a series of dilutions from 1:5 to 1:500 were prepared from RT products; 5 μl aliquots of standards or diluted RT products were combined with 1× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 50 nM forward and reverse primers. HSD17B7 and ribosomal phosphoprotein expression was detected using 5′-CTG GAA TGG CTC CGG GCT TTG C-3′ (forward) and 5′-CCT GCC CTC GGA GAC GGC GTCG-3′ (reverse), and 5′-GTG TTC GAC AAT GGC AGC AT-3′ (forward) and 5′-GAC ACC CTC CAG GAA GCG A-3′ (reverse) primers, respectively. Reactions were carried out on an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA) for 40 cycles (95 C for 15 sec, 60 C for 1 min) after initial 10-min incubation at 95 C. Relative expression levels were calculated using the ΔΔCT (cycle threshold) method with the ribosomal phosphoprotein mRNA used as an internal control.

Western blottings

Whole-cell extracts from MCF-7 cells were prepared by lysing cells in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1% sodium dodecyl sulfate) containing 1× protease inhibitor (Sigma, St. Louis, MO) and 100 mM sodium orthovanadate as described previously (73). Antiserum to the native HSD17B7 (developed as described above and named AS-07 or AS-08) was used to examine HSD17B7 expression at 1:10,000 dilution, anti-Elk-1 (sc-355; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at 1:1,000, anti-Egr-1 (sc-189; Santa Cruz Biotechnology, Inc.) was used as 1:1000, and anti-NF-1 was used at 1:1000 (sc74445, sc-5567, and sc-870). β-Actin (ab8227) or glyceraldehyde-3-phosphate dehydrogenase (ab9485), purchased from Abcam (Cambridge, MA), was used as an internal control. After a series of washes, blots were incubated with a donkey antirabbit secondary antibody linked to horseradish peroxidase for 1 h. Densitometry was performed with ImageQuant version 3 software (Molecular Dynamics, Sunnyvale, CA). The intensity of the HSD17B7 signal was normalized to that of respective internal control.

Mutation of *hsd17b7* promoter

The first set of mutations made to the *hsd17b7* promoter consisted of four nucleotides, respectively, being changed to abolish ERE, Sp1, NF1 sites, or their combinations. Using −185-bp *hsd17b7* promoter as a template, promoter mutations were made via PCR using the Quick-Change II site-directed mutagenesis kits (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The presence of correct mutations was confirmed by DNA sequencing. Primers for the mutations are included in Supplemental Table 1.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from cultured cells as described (2, 3). Protein concentration in nuclear extracts was determined using the bicinchoninic acid protein assay (Pierce, Madison, WI). Cold competitor probes were added to a final concentration of 2.5 pmol. Samples were run on a 4% nondenaturing polyacrylamide gel in 0.5× Tris borate EDTA buffer at 200 V for 2–3 h. The gels were then dried and analyzed by autoradiography.

DNA affinity precipitation assay

5′-Biotin end-labeled sense and antisense oligonucleotides corresponding to the wild-type NF-1-binding sites of the *hsd17b7* promoter were custom made by Integrated DNA Technologies, Inc. (Carolville, IA). MCF-7 cell nuclear extracts (50 μg) were incubated with 0.2 μg of biotinylated probe in binding buffer [60 mM KCl, 12 mM HEPES (pH 7.9), 4 mM Tris-HCl, 5% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol, and 1× protease inhibitor cocktail] on ice for 45 min. The DNA-protein complexes were then incubated with 40 μl of Strepavidin M270 Dynabeads (Invitrogen), which was preequilibrated in the binding buffer for 1 h. The incubation was continued for 1 h at 4 C with gentle rotation. DNA-protein complexes were then washed six times with the binding buffer. Next, 36 μl of 2× protein sample buffer were added to the avidin-precipitated DNA-protein complex, which was then boiled for 5 min to dissociate the complexes. Western blot analysis was performed with the specific antibodies to NF1 (sc-5567; Santa Cruz Biotechnology, Inc.), ERα (RT-1641-P0; Thermo Scientific, Rockford, IL) and Tata-binding protein (ab818; Abcam).

ChIP assays

MCF-7 cells were seeded in 10-cm plates and grown in phenol red-free media containing 5% CD-stripped calf serum. When 90% confluent, cells were treated either vehicle or estradiol and cross-linking was carried out with 1.5% formaldehyde for 15 min at room temperature. Cells were lysed and chromatin sonicated as previously described (74). NF1 antibodies (sc-5567x and sc-870x) from Santa Cruz Biotechnology, Inc. or rabbit polyclonal IgG antibody were preincubated with protein A-coated magnetic beads (Dynabeads; Invitrogen) overnight. Decross-linking was carried out overnight at 65 C. DNA was purified using Qiaquick (QIAGEN, Valencia, CA) columns and eluted in prewarmed water. Using Q-PCR, input was diluted (1:20, 1:100, 1:250, and 1:2000) to generate a standard curve. Primers for ChIP Q-PCR are 5′CTG GAA TGG CTC CGG GCT TTG C-3′ (forward) and 5′CCT GCC CTC GGA GAC GGC GTC G-3′ (reverse). These primers amplify 70-bp region, from −185 to −115 bp, a region deleted in our promoter studies. Data shown represent the mean ± SEM from at least three independent determinations.

Small interfering RNA transfection

RNAi experiments were performed with Silencer Select Predesigned oligos purchased from Ambion, targeting total NF1 (s9478 or s9481), NFIB (s9496 or s9495; Ambion), NFIC (s9497 or s194650; Ambion), NF1A (s9503 or s9501; Ambion), or NFX1 (s9525 or s9526; Ambion) mRNAs and siNegative nontargeting siRNA no. 1 (Santa Cruz Biotechnology, Inc. and Ambion). MCF-7 cells (100×10^4 per well of 24-well plate) were transfected in Opti-MemI (Invitrogen) with 20 pmol of each siRNA oligo using Attractene reagent (QIAGEN) and 0.15

μg of *hsd17b7* reporter plasmid. After a 24-h transfection, media were changed to treatment media. Luciferase reporter activity was determined 48 h after transfection.

Statistical analysis

Normalized data from real-time-PCR and promoter studies were analyzed using one-way ANOVA followed by Tukey test. Student's *t* test was also used when appropriate. A level of $P < 0.05$ was considered statistically significant.

Acknowledgments

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