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The Farnesoid X Receptor Regulates Transcription of 3β-Hydroxysteroid Dehydrogenase Type 2 in Human Adrenal Cells

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Abstract

Recent studies have shown that the adrenal cortex expresses high levels of farnesoid X receptor (FXR), but its function remains not known. Herein, using microarray technology, we tried to identify candidate FXR targeting genes in the adrenal glands, and showed that FXR regulates 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2) expression in human adrenocortical cells. We further demonstrated that FXR stimulated HSD3B2 promoter activity and have defined the cis-element responsible for FXR regulation of HSD3B2 transcription. Transfection of H295R adrenocortical cells with FXR expression vector effectively increased FXR expression levels and additional treatment with chenodeoxycholic acid (CDCA) caused a 25-fold increase in the mRNA for organic solute transporter alpha (OSTα), a known FXR target gene. HSD3B2 mRNA levels also increased following CDCA treatment in a concentration-dependent manner. Cells transfected with a HSD3B2 promoter construct and FXR expression vector responded to CDCA with a 20-fold increase in reporter activity compared to control. Analysis of constructs containing sequential deletions of the HSD3B2 promoter suggested a putative regulatory element between -166 and -101. Mutation of an inverted repeat between -137 and -124 completely blocked CDCA/FXR induced reporter activity. Chromatin immunoprecipitation assays further confirmed the presence of a FXR response element in the HSD3B2 promoter. In view of the emerging role of FXR agonists as therapeutic treatment of diabetes and certain liver diseases, the effects of such agonists on other FXR expressing tissues should be considered. Our findings suggest that in human adrenal cells, FXR increases transcription and expression of HSD3B2. Alterations in this enzyme would influence the capacity of the adrenal gland to produce corticosteroids.

Keywords

FXR; HSD3B2; Adrenal gland; Bile acids

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1. Introduction

Cholesterol is an essential component of both steroid hormone and bile acid biosynthesis. Many parallels can be drawn between the mechanisms regulating cholesterol conversion to steroid hormones in the adrenal and those involved in bile acid biosynthesis in the liver. These similarities include the presence of several nuclear transcription factor homologues that play critical regulatory roles in these two tissues. The orphan nuclear receptors steroidogenic factor 1 (NR5A1, SF1) in the adrenal and liver receptor homologue-1 (NR5A2, LRH-1) in the liver act by binding to a DNA response element (AAGGTCA) to increase transcription of genes encoding enzymes involved in steroid hormone biosynthesis (Parker and Schimmer,1995) and bile acid production (Lu et al.,2000), respectively. In addition, the orphan nuclear hormone repressor homologues DAX-1 (NR0B1, dosage-sensitive sex reversal – adrenal hypoplasia congenital critical region on the X chromosome, gene 1) and SHP (NR0B2, short heterodimer partner) are also expressed in the adrenal (Zanaria et al.,1994) and liver (Lee et al.,1998). These factors act, respectively, to repress the ability of SF1 and LRH-1 to increase gene transcription (Makishima et al.,1999; Goodwin et al.,2000), thus decreasing steroidogenesis in the adrenal and bile acid biosynthesis in the liver.

In the liver a bile acid receptor, FXR (NR1H4, farnesoid X receptor), has been identified as a key regulator of bile acid biosynthesis, by controlling levels of cholesterol metabolism (Makishima et al.,1999). FXR acts as a heterodimer with the ubiquitous retinoid X receptor (NR2B1, RXR) (Forman et al.,1995b; Seol et al.,1995; Makishima et al.,1999), binding with highest affinity to IR-1 sites (inverted repeat spaced by 1 nucleotide) on target genes in response to ligand activation (Laffitte et al.,2000). FXR response elements (FXRE) are found in the promoters of genes regulating bile acid biosynthesis such as ileal bile acid binding protein (IBABP) (Grober et al.,1999) and SHP (Goodwin et al.,2000; Lu et al.,2000) but not on the rate-limiting enzyme in the conversion of cholesterol to bile acids, namely CYP7A1 (Chiang et al.,2000). FXR is involved in a negative feedback loop in which elevations in bile acids (natural FXR ligands) activate FXR and activate SHP gene transcription. The increase in SHP expression decreases bile acid biosynthesis by inhibiting LRH-1 induction of bile acid metabolizing enzymes including CYP7A1. This signaling pathway has been substantiated *in vivo* with a murine model that lacks expression of FXR (Sinal et al.,2000). This mouse model exhibits increased levels of hepatic and serum cholesterol and triglycerides, as well as serum bile acids, indicating that the FXR is an integral intermediate in bile acid biosynthesis and homeostasis in the liver.

While the importance of FXR in bile acid biosynthesis in the liver is well elucidated, parallel studies to define the role of FXR in steroid hormone biosynthesis in the adrenal have not been undertaken. The organic solute transporter genes alpha and beta (OSTα and OSTβ) recently have been identified as FXR targets in the human adrenal carcinoma cell model-H295R and mouse adrenal glands (Lee et al.,2006b). Potential FXR response elements are also found in human OSTα and OSTβ promoter regions. It has been suggested that the function of OSTα / OSTβ heterodimer is to facilitate the uptake of conjugated bile acids into adrenals and export conjugated steroid intermediates from the adrenal into circulation (Lee et al.,2006b).

The enzyme 3β-hydroxysteroid dehydrogenase/$\Delta^{5 \to 4}$-isomerase type II (HSD3B2) is a critical enzyme in the synthesis of aldosterone and cortisol. It is found at high levels in the adrenal cortex and gonads (Rheaume et al.,1991), converting Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids. This enzyme exhibits a zonal pattern of expression in the human adrenal cortex, with high levels in both zona glomerulosa and zona fasciculata but low levels in zona reticularis (Suzuki et al.,2000). The zonal expression pattern of HSD3B2 coincides with its role in steroid synthesis pathway, which leads to biosynthesis of cortisol and aldosterone but not dehydroepiandrosterone (DHEA) / dehydroepiandrosterone sulfate (DHEA-S). Furthermore,

regulation of this enzyme in the adrenal is known to vary throughout the human lifespan; it is suppressed in the fetal adrenal, resulting in large quantities of DHEA-S and very little cortisol being produced (Mason et al.,1993). Although several key transcriptional regulators including NGFI-B (NR4A1), GATA family members and SF1 have been identified (Bassett et al., 2004; Martin et al.,2005), the exact mechanism for the time- and space-regulated expression pattern of HSD3B2 remains unclear.

In the current study, we demonstrated that activation of FXR in human adrenal cells resulted in the induction of HSD3B2 gene. In addition, we used mutation and deletion promoter constructs in transient transfection and chromatin immunoprecipitation assays to confirm the functionality of the FXRE in the HSD3B2 gene. Taken together, we identify HSD3B2--one of the key genes involved in the steroid synthesis in human adrenal, as a novel FXR target gene.

2. Materials and Methods

2.1 RNA extraction and reverse transcription

Human adrenal gland, testis, and liver were obtained through the Cooperative Human Tissue Network (Philadelphia, PA). Human ovary and placenta were obtained from Parkland Memorial Hospital (Dallas, TX). The use of these tissues was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center at Dallas, Texas. Total RNA was extracted from tissues (Chirgwin et al.,1979) and purity and integrity of the RNA was checked spectroscopically using Nano-drop (NanoDrop Technologies, Wilmington, DE). Deoxyribonuclease I (Ambion Inc, Austin, TX)-treated total RNA (2 μg) was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following the manufacturer recommendations and stored at -80°C.

2.2 Real-time RTPCR (qPCR)

Primers for the amplification of the target sequence were based on published sequences for the human FXR (GenBank accession number: <u>NM_005123</u>), human OSTα: (Genbank accession number: <u>NM_152672</u>) and human HSD3B2 (GenBank accession number: <u>NM_000198</u>). The primer sequences used were:

FXR:	Forward: 5'-CTGTGTGTTGTTTGTGGAGACAGA-3'
	Reverse: 5'-ACAGCGTTTTTGGTAATGCTTCT-3'.
HSD3B2:	Forward: 5'-GCGGCTAATGGGTGGAATCTA-3'
	Reverse: 5'-CATTGTTGTTCAGGGCCTCAT-3'
	Probe: TGATACCTTGTACACTTGTGCGTTAAGACCCA.

Validated OSTα primer-probe mix was obtained from Applied Biosystems. PCR reactions were performed in the ABI Prism 7000 Sequence Detection System (Applied Biosystems) in a total volume of 20 μl reaction mixture following the reaction parameters recommended by the manufacturer. The SYBR Green Universal PCR Master Mix 2X (Applied Biosystems), 50 nM of each primer, and 5 μl of each first-strand cDNA sample were combined in each reaction vessel for FXR detection. Standard curves were prepared using a human FXR construct. Negative controls contained water instead of first-strand cDNA. The Taqman 2X Master Mix (Applied Biosystems), 900 nM of each primer, 250 nM of probe and 5 μl of each first-strand cDNA sample were combined in each reaction vessel for HSD3B2 detection. Samples were normalized on the basis of the 18S ribosomal RNA content (μg). The quantification of the 18S RNA in each sample was performed using a TaqMan Ribosomal RNA Reagent Kit (Applied Biosystems) following the manufacturer recommendations.

2.3 Preparation of reporter constructs and expression vectors

The 5' flanking DNA from the human HSD3B2 gene (-963), CYP11A1 (-1680), CYP17 (-1124) and StAR (-1300) was inserted upstream of the firefly luciferase gene in the reporter vector pGL3 Basic (Promega, Madison, WI). Deletion constructs were prepared using available restriction sites (*Nhe I* for -585; *Bgl II* for -345; *Hind III* for -52) or by introduction of a *Kpn I* site at the appropriate location by PCR for all other deletion constructs (Bassett et al.,2004). Mutations to putative FXR and NGFI-B binding sites in the HSD3B2 promoter were created using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's recommendations. In the NGFI-B response element mutant construct, the sequence 5'-AAAGGTCA-3' (-131/-124) was changed to 5'-AgAatTCA-3', and in the FXR response element mutant construct, the sequence 5'-TAACCTAA-3' (-137/-130) was changed to 5'-TAgaCTAA-3'. For all transfections, the pGL3 basic promoter-less vector was used as a control to measure basal activity. Human FXR expression vector was constructed by inserting the 1551bp human FXR gene (accession number GI4826979, 309bp-1860bp) into the multiple cloning site of pcDNA3.1 zeo vector (Invitrogen, Carlsbad, CA).

2.4 Cell culture and transfection

The human adrenocortical cell line H295R was used for all transfection experiments and was routinely cultured in Dulbecco's modified Eagles/Ham F12 (DME/F12) medium (Gibco, Carlsbad, CA) supplemented with 10 % cosmic calf serum (Hyclone, Logan, UT) and antibiotics including 1% Penicillin/ Streptomycin (Gibco), 0.1% Gentamicin (Sigma-Aldrich, St. Louis, MO). Transient transfection assays were performed using Transfast (Promega) in a ratio of 4 μl Transfast per microgram of DNA and the indicated amounts of expression vectors. To ensure constant amounts of DNA for each transfection, pcDNA 3.1 zeo empty vector was added to each well as needed. Luciferase activity was tested using the Luciferase assay system (Promega) and values were expressed as arbitrary light units normalized to the β–galactosidase activity of each sample, and compared to basal condition for the fold change. Electrical transfection assays were performed using the Nucleofector system (AMAXA, Gaithersburg, MD). Cells were cultured 80-90 % confluence in growth medium before being trypsinized and resuspended in Nucleofector Solution R at a ratio of 5 million cells per 100 μl solution. Indicated amount of FXR vectors were added to the solution and mixture was run under program T20 in the Nucleofection System. Cells were allowed to recover for 48 h prior to treatment and RNA was isolated for further analysis.

2.5 Microarray analysis

Total RNA isolated from AMAXA transfected H295R cells (basal and GW4064 treated) were used on 2 genomic expression arrays. In summary, RNA was hybridized to an Affymetrix human HG-U133+2 oligonucleotide microarray set containing 54,675 probe sets presenting around 40,500 independent human genes. The arrays were scanned at high resolution using an Affymetrix GeneChip Scanner 300 from Microarray Core Facility, Medical College of Georgia (Augusta, GA). Results were analyzed using GeneSpring GX 7.3.1 software (Silicon Genetics, Redwood City, CA) for different expression of steroidogenesis genes between basal and FXR agonist treatment. From this gene list, 42 genes involved in steroid biosynthesis and metabolism was prepared as previously described (Bassett et al.,2005).

2.6 Immunohistochemistry Assay

Immunohistochemical analysis for FXR was performed employing the streptavidin-biotin amplification method using a U.T.R. Vectastain Kit (Vector Laboratories, Burlingame, CA). Polyclonal rabbit anti-human FXR antibody (NB 400-153; 1:200 dilution) was purchased from Novus Biologicals (Littleton, CO). Antigen retrieval for immunostaining was performed using a previously described method (Nakamura et al.,2007). The antigen-antibody complex was

visualized with 3.3'-diaminobenzidine (DAB) solution [1 mM DAB, 50 mM Tris-HCl buffer (pH 7.6), and 0.006 % H_2O_2]. Immunostaining for HSD3B2 was performed with the IDetect super stain system (Alk Phos)-Fast Red (Labs biotechnology, London, Canada) using a polyclonal antibody (Sasano et al.,1990) in adjacent tissue sections to further characterize these positive cells in human adrenal. Negative controls, in which primary antibody was replaced with PBS were also performed.

2.6 Chromatin immunoprecipitation

H295R cells were transfected by Nucleofection kit as indicated above, and allowed to recover for 48 h. Cells were incubated in DME/F12 medium supplemented with 0.1 % calf serum medium with or without CDCA (10 μM) or GW4064 (1 μM) for an additional 24 h. Chromatin immunoprecipitation was performed using EZ-ChIP (Upstate, Charlottesville, VA) following the manufacturer recommendations. DNA was diluted into 20 μl of nuclease-free water and 5 μl was used for each PCR reaction of 32 cycles. The PCR primer sequences utilized were as follows:

HSD3B2 promoter:

Forward 5'-TCACAGATTGCAGATCCCAGACAG-3',

Reverse 5'-ACTCCTGCCACATACTCATTGCTC-3';

OSTα promoter:

Forward 5'-TGCCCTTTCACCTTTCCTGCCATT-3',

Reverse 5'-TCTCTCCCAAGAGTCAAGCTGCA-3'.

Products were then run on 4 % agarose gel for detection of promoter region amplification.

2.7 Electrophoretic mobility shift assay

H295R cells were transfected by Nucleofection kit as indicated above, and allowed to recover for 48 h. H295R nuclear extracts were prepared by previous method (Schreiber et al.,1989). IRDye 700 labeled oligonucleotides flanking the -146/-114 region of HSD3B2 promoter were ordered from LI-COR Inc (Lincoln, Nebraska) and annealed at 94°C for 3 min following the manufacturer instructions (extra GC were added to both ends to help annealing and prevent space obstruction). *In vitro* synthesized FXR and RXR were produced using PROTEINscrip II kit (Ambion, Austin, TX). To prevent RXR homodimer formation, FXR and RXR were mixed in 2:1 ratio and 3 μl of final mix were applied in each reaction. Nuclear extract (1 μg) and annealed oligo (50 nM) were incubated at room temperature for 30 min in 20 μl reaction mixture [20 mM HEPES (pH 8.0), 80 mM KCL, 1mM EDTA, 10% glycerol, 1mM dithiothreitol, 0.5 mg/ml bovine serum albumin and 10 ng/μl poly dI-dC as non-specific competitor]. For competition analysis, indicated amounts of non-labeled oligonucleotides were added to the reaction simultaneously. Electrophoresis was performed on 4% native polyacrylamide gel with 1X tris-glycine running buffer at 4°C for 1.5 h. Shifted oligonucleotides were detected using Odyssey Infrared Imaging System (LI-COR Biosciences). Sequences used in EMSA assay:

HSD3B2 FXRE:

5' IRDye 700- GCTTAGAGATATAACCTAAAGGTCACTATTATTCTG-3'

Wild type competitive oligo:

5'- GCTTAGAGATATAACCTAAAGGTCACTATTATTCTG-3'

CYP11B2 Ad1 oligo (used as a nonspecific DNA):

5'-CCGGTTCTCCCATGACGTGATATGTTTCGTAC- 3'

2.8 Statistical analysis

Results are given as mean ± SD or mean ± SEM as are indicated. Individual experiments were repeated at least three times. Paired t-tests with a confidence interval of 95% were performed for group comparisons using Graphpad Prism 3.0 software (Graphpad Software Inc. San Diego, CA). 1-way ANOVA followed by Dunnett's method was applied for analysis of three or more groups of samples.

3. Results

3.1 The human adrenal expresses high levels of FXR

To determine the levels of FXR expression in human steroidogenic tissues, qPCR analysis was performed. FXR expression was relatively high in both the adult (0.12 attomoles/µg 18S RNA) and fetal (0.097 attomoles/µg 18S RNA) adrenal, although it was still less than half the value seen in liver (0.29 attomoles/µg 18S RNA). FXR was also present in the ovary (0.043 attomoles/µg 18S RNA) but not detectable in the testes or placenta (Fig. 1). FXR protein expression was also examined in the human adult adrenal gland using immunohistochemical analysis. As indicated from the results, FXR protein was expressed in the nuclei of zona fasciculata (ZF) cells, while in zone reticularis (ZR) cells the expression of FXR was primarily in the cytoplasm (Fig.2A). In accordance, HSD3B2 protein was present in ZF but not ZR (Fig.2B). No immunoreactivity was detected in the negative controls prepared in the absence of FXR antibody (Fig.2C & 2D).

3.2 Effects of FXR on gene regulation in adrenocortical cells

Although much has been elucidated about the FXR-mediated effects of bile acid biosynthesis in the liver (Makishima et al.,1999; Sirvent et al.,2004; Claudel et al.,2005; Westin et al., 2005; Cai and Boyer,2006; Lee et al.,2006a; Ma et al.,2006; Modica and Moschetta,2006), little is known about the role of FXR in the adrenal gland and specifically in steroidogenesis regulation. To determine the candidate FXR target genes, H295R cells were transfected with FXR expression vectors using the AMAXA electroporation system, which provides a much higher transfection efficiency than traditional methods(Hamm et al.,2002). RNA was then isolated and used for Affymetrix microarray analysis. As shown in Fig. 3A, OSTα was the top gene stimulated by treatment of FXR ligand GW4064. In panel B, when the gene analysis was narrowed to a gene list of 42 steroidogenic enzymes, HSD3B2 was the most responsive FXR target (3.8-fold increase). The ability of FXR ligand to increase HSD3B2 mRNA was confirmed using qPCR (Fig. 3C).

3.3 Effects of FXR on endogenous HSD3B2 expression

The effects of FXR on HSD3B2 were further tested using qPCR and compared to the previously reported FXR target gene OSTα (Fig.4). Data from three experiments demonstrated that GW4064 caused a 12-fold increase in HSD3B2 mRNA levels. The effects were also observed if the natural ligand CDCA was used to activate FXR. Both ligands had effects that were concentration-dependent. GW4064 had a greater stimulatory effect for both FXR-mediated HSD3B2 and OSTα mRNA expression (Fig. 4A). FXR-mediated effects on HSD3B2 gene expression were also time-dependent with the 24 h CDCA treatment causing maximal stimulation (Fig. 4B).

3.4 Effect of FXR on steroidogenesis gene promoter constructs

Transient transfection analyses were performed in H295R adrenocortical cells. As shown in Fig.5, there was a strong FXR-mediated induction of the HSD3B2 reporter construct in H295R

cells, and this effect was further increased upon treatment with ligand. However, there appeared to be no significant effect on the StAR, CYP11A or CYP17 promoter constructs.

Fig. 6 further illustrated the positive concentration-dependent increase of both FXR expression vectors (0.03 μg- 0.3 μg) and the FXR ligand, CDCA (0.1 μM - 20 μM) on HSD3B2 reporter activity. The maximal induction of reporter activity was approximately 20-fold over basal (untreated cells with empty vector) using 0.3 μg FXR vectors and 10 μM CDCA.

3.5 Definition of a FXR response element in HSD3B2 promoter

FXR response elements (FXRE) have been characterized as inverted repeats (IR) of a nuclear receptor half site (Forman et al.,1995a). To date at least 38 FXRE have been identified in FXR-regulated gene promoter regions and they are described in the nuclear receptor database (Podvinec et al.,2002). Examination of the HSD3B2 gene promoter sequence indicated a putative FXRE at -137 to -124, with the sequence 5'-TAACCTAAAGGTCA-3', which forms an inverted repeat spaced by 2 nucleotides (IR2). The above FXRE was confirmed by deletion and mutation analysis. Deletion analysis (Fig. 7A) indicated that the putative FXR-response region lies between -166 and -101 of the HSD3B2 5'-flanking region. A previous study indicated that this region also contains a consensus NGFI-B response element (NBRE) at -131 to -124 (Bassett et al.,2004). However, further testing indicated that NGFI-B had no effect on FXR-mediated activation of HSD3B2 (data not shown). Mutation analysis of this site and the 5' half-site of IR2 (Fig. 7C) illustrated that these sites are necessary for FXR-mediated activation of HSD3B2 promoter activity. Alignment with the 1 kb 5'-sequence of the human HSD3B2 promoter showed that there is no FXRE sequence present in mouse Hsd3b1 (ortholog of human HSD3B2 gene) promoter region (data not shown).

The binding of FXR to the HSD3B2 promoter region was further confirmed by ChIP assay using nucleofected FXR over-expressing H295R cells. As shown in Fig.8, FXR was only detected in cells treated with ligand, which suggested that the affinity of FXR for HSD3B2 promoter was mediated *in vitro* by CDCA. And in accordance to the mRNA results (Fig. 3), FXR binding to HSD3B2 promoter was also increased with the synthetic ligand GW4064. Interestingly, in the case of the OSTα promoter, there was some binding of FXR to its site even without treatment with exogenous ligand, and the overall amount of binding was greater than that detected in HSD3B2 promoter (Fig. 8A). There may be two reasons for the low levels of the HSD3B2 promoter PCR product that we observed. First it could be caused by the low endogenous transcriptional activity of HSD3B2 seen in the H295R cell line and second there is only one FXRE in the HSD3B2 promoter, while the OSTα promoter contains two FXREs.

To further narrow the FXRE in the HSD3B2 promoter, we used EMSA with oligonucleotides that included the -137/-124 region of HSD3B2 promoter sequence (Fig.9) (Extra nucleotides were added to both ends of the sequence to prevent possible space obstruction cause by IRDye). Both *in-vitro* synthesized FXR and nuclear extracts from FXR over-expressing H295R cells bound to the FXRE sequence and the binding was replaced by wild type competent oligonucleotides but not by non-specific oligonucleotide sequence (CYP11B2 Ad1). This suggests that the binding of FXR to the region was specific and that the 5'-TAACCTAAAGGTCA -3' sequence in HSD3B2 promoter is a FXRE.

4. Discussion

FXR expression was initially identified in the liver, gut, adrenal gland and kidney in rodents (Forman et al.,1995a). Subsequent studies confirmed the same expression pattern in human tissues (Huber et al.,2002; Zhang et al.,2003). To date, most research has focused on FXR function in liver, and FXR has been shown to regulate a number of target genes involved in bile acid, lipid and glucose metabolism (Repa et al.,2000; Song et al.,2001; Chiang,2002;

Claudel et al.,2003; Francis et al.,2003; Pircher et al.,2003; Sirvent et al.,2004; Claudel et al., 2005; Cai and Boyer,2006b; Ma et al.,2006). In contrast to the intense interest in liver, little has been done to define the role of FXR in the adrenal gland. In this study, we have identified HSD3B2 as an FXR target gene in human adrenal gland.

FXR is expressed in both adult and fetal adrenal glands at relatively high levels (Forman et al., 1995a; Lee et al.,2006b; Houten et al.,2007). The recent study by Auwerx and colleagues showed that FXR expression in mouse adrenal glands can be stimulated by oral feeding of the agonist GW4064 (Houten et al.,2007). This provides another aspect of FXR regulation and further supports a role for FXR in the adrenal gland. In contrast to other organs that highly express FXR such as kidney, liver and intestine, the adrenal gland is however not commonly associated with any aspect of bile acid regulation, and calls for investigation of adrenal- specific functions of FXR.

The primary function of the adrenal gland is the production of steroid hormones; therefore we examined the effects of FXR on adrenal cell expression of transcripts encoding steroidogenic enzymes. This identified HSD3B2 as a potential FXR target in human adrenal cells. Further deletion and mutation experiments suggested a potential FXRE at -137 to -130 in the HSD3B2 promoter region. FXR binding to this element was confirmed by ChIP assay. When combined, these data strongly suggest that HSD3B2 is a direct FXR target gene in the human adrenal gland.

First identified from a human adrenal cDNA library (Rheaume et al.,1991), HSD3B2 encodes a protein of 371 amino acids that is primarily expressed in the adrenal, ovary, and testis (Lachance et al.,1992; Thomas et al.,2001). It catalyzes the sequential 3ß-hydroxysteroid dehydrogenation and delta-5-4 isomerization of delta-5 steroid precursors that include pregnenolone, 17-hydroxypregnenolone, DHEA, and androst-5-ene-3ß, 17ß-diol into their respective 4-ketosteroids, namely progesterone, 17-hydroxyprogesterone, androstenedione, and testosterone. Thus HSD3B2 is required for the biosynthesis of all classes of steroid hormones, including glucocorticoids, mineralocorticoids, progestins, androgens, and estrogens (Simard et al.,2005).

One intriguing result from the FXR immunohistochemistry experiments was the observation of high nuclear expression of FXR in ZF cells, but primarily cytoplasmic localization in ZR. Although there has been no report regarding FXR trafficking between cytoplasm and nuclei, it is well established that some nuclear receptors are effective only when present in nuclei and that receptors can effectively move between the nuclei and cytoplasm (Guiochon-Mantel et al.,1994; Czar et al.,1995; DeFranco,1999; Hager et al.,2000). This suggested that FXR transcriptional activity may differ between ZF and ZR. In contrast, similar mRNA expression levels of FXR were observed in both ZF and ZR (data not shown). HSD3B2 mRNA and protein are present at high levels in zona fasciculata but almost absent from zona reticularis (Sasano, 1990; Endoh et al.,1996; Gell et al.,1998). The regulatory mechanism for the HSD3B2 expression pattern in the adrenal has not been fully elucidated. The zone-specific distribution of HSD3B2 is in part responsible for DHEA production in the reticularis and cortisol synthesis in the fasciculata (Hornsby,1995; Rainey and Nakamura,2008). Therefore, by increasing HSD3B2 expression, FXR-mediated stimulation would increase the synthesis of cortisol while decreasing DHEA/DHEA-S production, effectively changing the ratio of cortisol to DHEA-S in adrenal, leading to the fasciculata phenotype. The significance of FXR in the regulation of zonation needs further evidence, but our results suggest that FXR is in the adrenal and can regulate HSD3B2 expression.

Since FXR has been reported to work with RXR as heterodimer for its transcriptional activity, we co-transfected the FXR and RXR expression vectors together with HSD3B2 promoter

reporters, but no significant addictive effect of RXR under basal- or ligand-stimulated states was detected (data not shown). This suggests that RXR is not limiting in our transfection studies. Also, we tested NGFI-B – the nuclear receptor whose response element overlaps with that of FXR and which is known to regulate HSD3B2 (Bassett et al.,2004). NGFI-B and FXR appeared to act independently as there was no addictive effect on promoter activity. Furthermore, in agreement with Houten and colleagues (Houten et al.,2007), we did not find the classic FXR target SHP to be regulated by ligand treatment in human adrenal (data not shown).

The generation of a FXR knockout mouse model (Sinal et al.,2000) offered a very powerful tool for the FXR study, but unfortunately, due to species differences, it may not be an appropriate model for the study of mouse Hsd3b1 (the ortholog of the human HSD3B2 gene). Using ECR browser (DCODE.org, Comparative Genomics Center), we compared the -1 kb 5' promoter sequence of both human HSD3B2 and mouse Hsd3b1. There is only 74 % similarity between these two sequences. Not surprisingly, the FXRE defined for the human gene was not conserved in mouse Hsd3b1 promoter. This may explain why incubation of wild mouse adrenal glands with GW4064 did not induce Hsd3b1 mRNA expression and there was no difference in Hsd3b1 levels between wild and FXR$^{-/-}$ mice (Lee et al.,2006b). In support of this premise, transfection of the mouse Y-1 adrenal cell line with FXR expression vectors in a manner that increased FXR mRNA levels by more than 500-fold did not alter mRNA levels of Hsd3b1. Furthermore, in contrast to human adrenals, Hsd3b1 is expressed through-out the mouse adrenal cortex including zona reticularis (Schulte et al.,2007). Thus HSD3B2 is regulated differently between human and mouse adrenal glands and it appears that FXR plays a more important role in human adrenal glands than in mouse adrenals.

There are at least two potential sources of ligands available for FXR activation within the adrenal gland. First, bile acids could be transported from the plasma into adrenal cells through the OSTα / OSTβ transport system that is present the adrenal cells (Seward et al.,2003). This hypothesis is supported by the ability of FXR to increase adrenocortical cell expression of the OSTα gene. Second, adrenal-derived steroids or steroid intermediates that are found at very high levels within adrenal cells, may act to inhibit or activate FXR transcriptional activity. Indeed, an adrenal and gonad steroid metabolite was recently shown to bind and activate FXR transcriptional activity (Wang et al.,2006). A thorough screening of all adrenocortical steroid metabolites for potential FXR ligands may provide new mechanisms for regulating FXR transcriptional activity.

In summary, we established that FXR is expressed at high levels in the human adrenal gland, and that it stimulates HSD3B2 under the control of both the natural bile acid ligand CDCA and the synthetic agonist GW4064. The identification of HSD3B2 as a novel FXR target in the adrenal gland will give new direction to the study of FXR and help expand the understanding of FXR function in human tissues.

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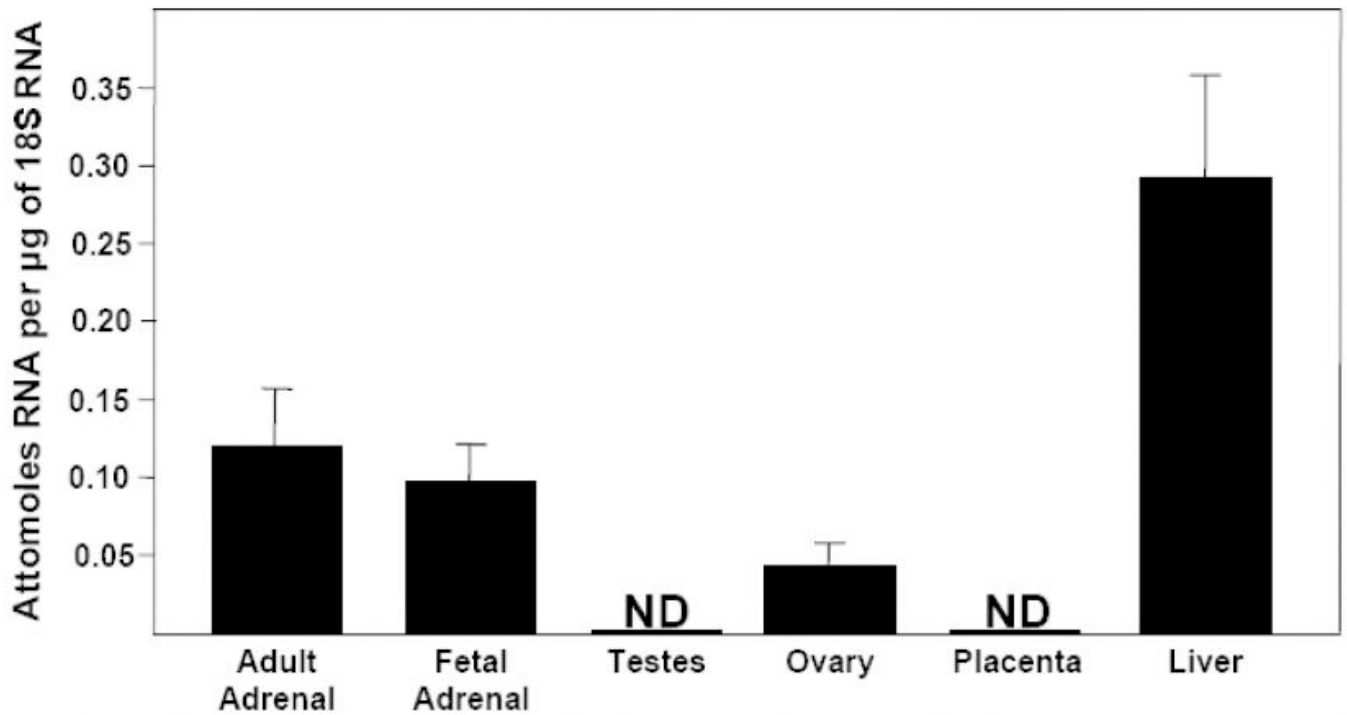
Fig.1.
FXR transcript levels in human steroidogenic tissues. Quantitative PCR was used to quantify FXR mRNA levels in adult and fetal adrenal, testes, ovary, placenta, and liver as described in Materials and Methods. Data represent the mean ±SEM of at least 5 separate DNase-treated RNA samples and are expressed in attomoles of mRNA per μg of 18S ribosomal RNA. (ND: not detectable)
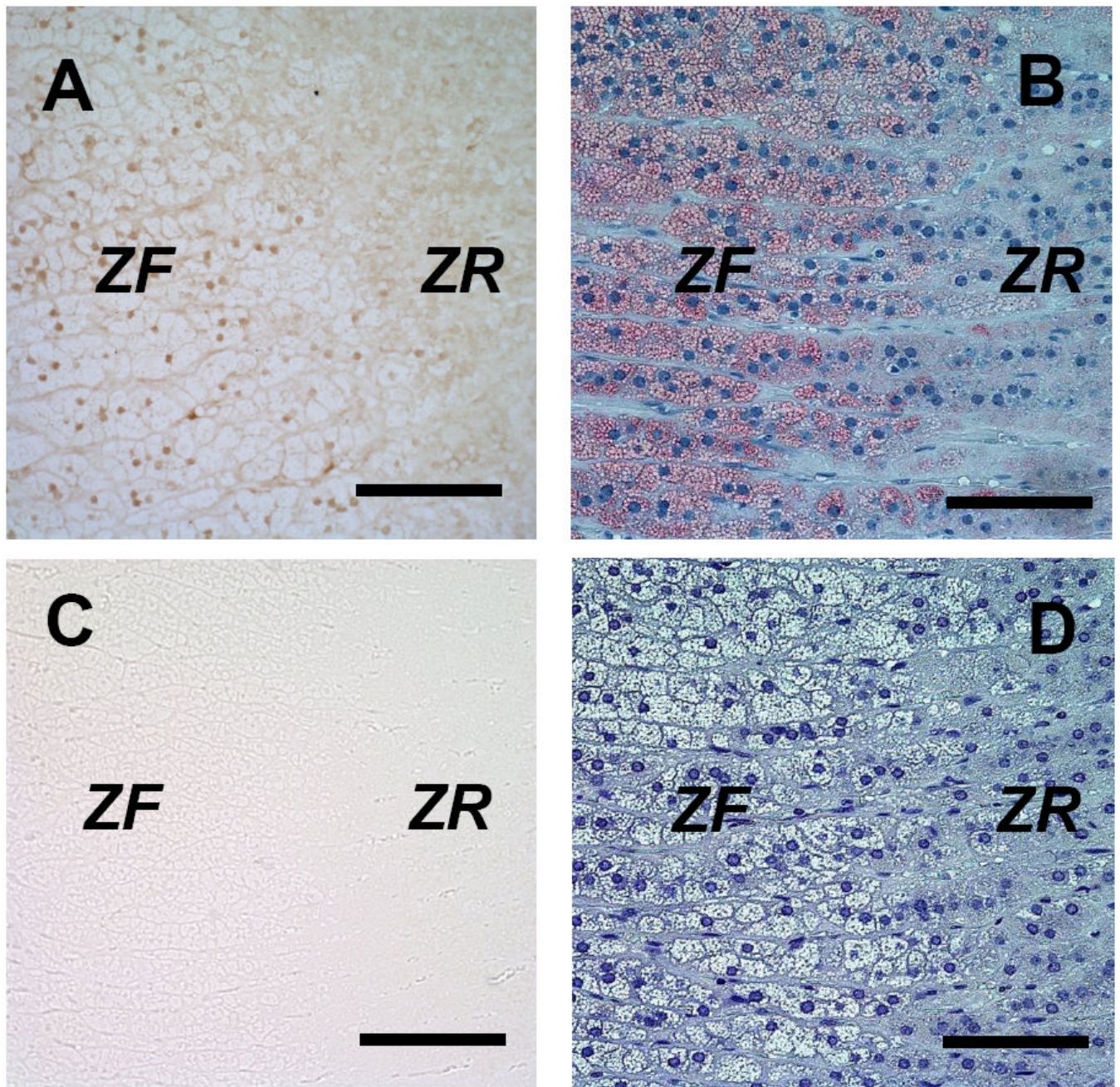
Fig.2.
Immunohistochemical staining for FXR and HSD3B2 in representative adult human adrenal sections. Immunohistochemical localization of FXR (brown) was detected in the nuclei of zona fasciculata and cytoplasm of zona reticularis cells (panel A), and HSD3B2 was expressed in the cytoplasm (red) in human adrenals zona fasciculata (panel B). Negative control, in which primary antibody was not included, was also performed, and no specific immunoreactivity was detected in the tissue section with DAB staining (panel C) or FastRed staining (panel D). Hemotoxin nuclear staining was performed with FastRed staining (panel B and D).
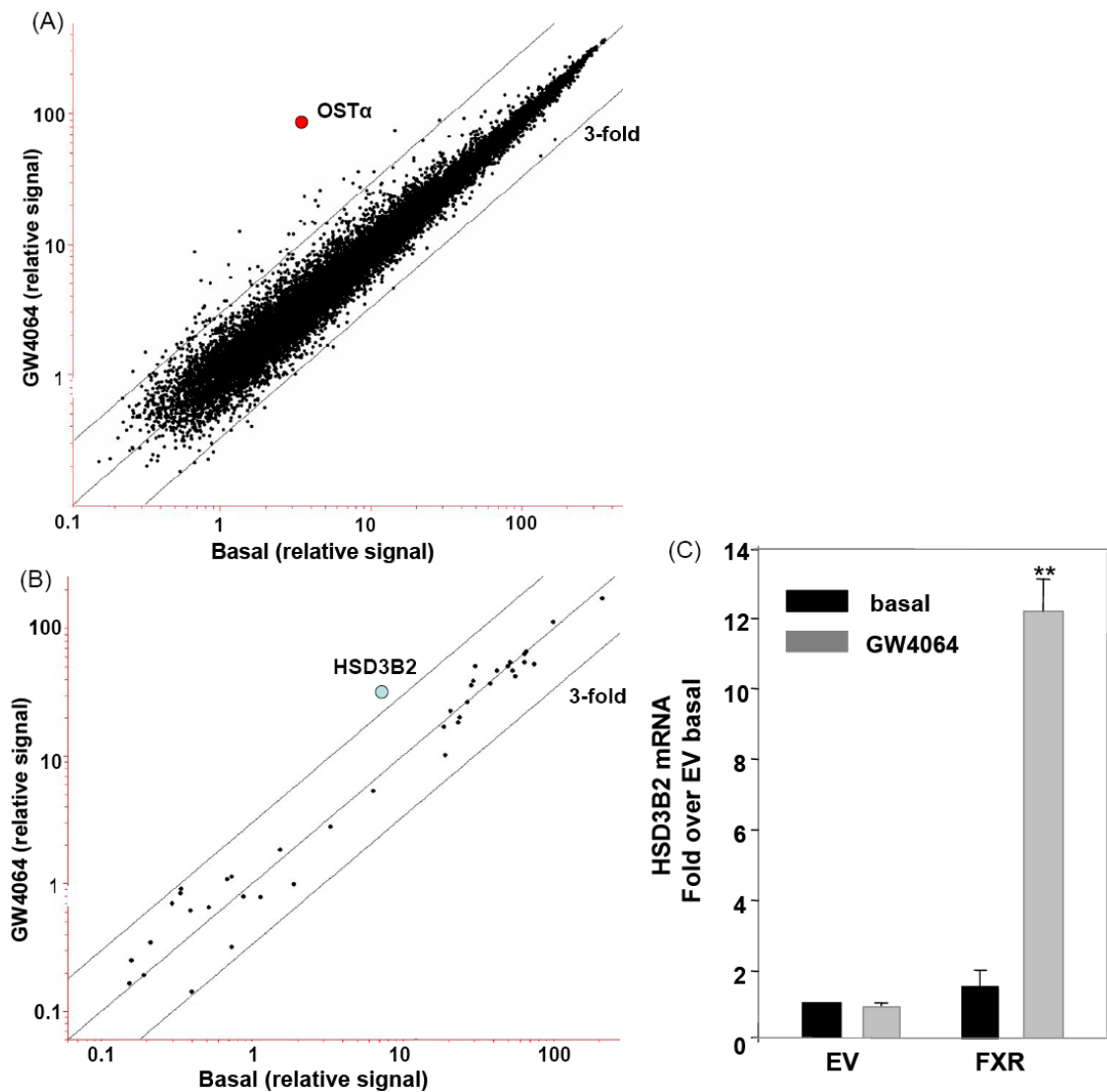
Fig.3.
FXR response genes in H295R cells. Panel A. Microarray of the whole transcriptome of FXR transfected H295R cells. H295R adrenocortical cells were transfected with FXR expression vector and treated with/without GW4064 (1 μM) for 24 h, followed by RNA isolation and microarray analysis. Data is shown as a scatter plot that compares basal and GW4060 treatment. Panel B. Microarray data comparing only 42 steroidogenic enzyme genes between basal and GW4064 treated samples. Panel C. FXR effects on HSD3B2 mRNA as determined by qPCR. Results represent the mean ± SD of data from at least three independent experiments, each performed in triplicate. Statistics were calculated by 1-way ANOVA followed by Dunnett's method, ** $P < 0.01$.
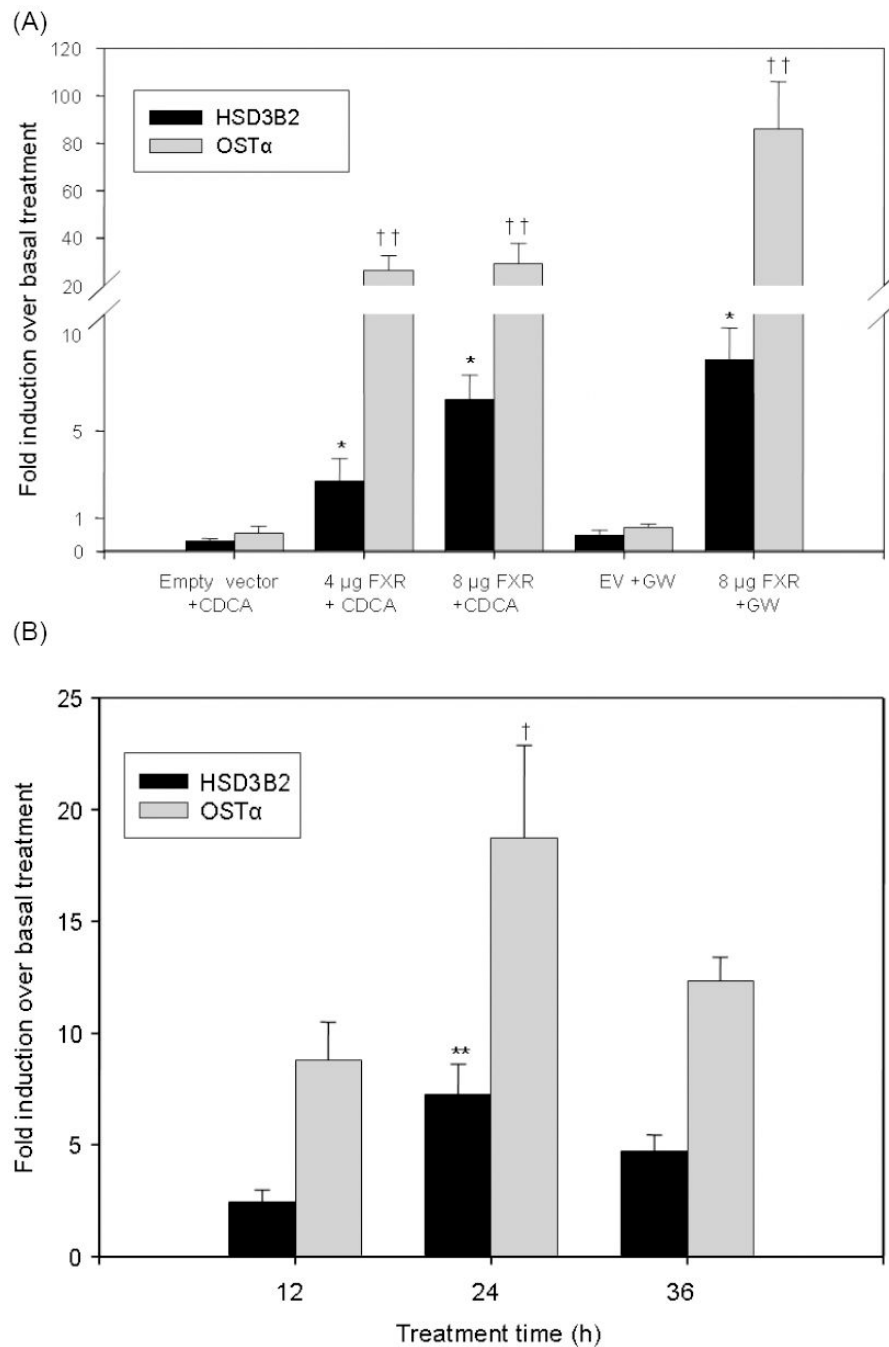
(A)

(B)

Fig.4.
FXR effects on endogenous HSD3B2 and OSTα levels. Panel A. Concentration-dependent effects of FXR on HSD3B2 and OSTα expression levels. H295R adrenocortical cells were transfected with empty vectors or indicated doses of FXR expression vector, and treated with CDCA (10 μM) for 24 h. Quantitative PCR data were normalized to 18S ribosomal RNA and are shown as the fold induction over basal (no ligand treatment). Results represent the mean ± SD of data from at least three independent experiments each performed in triplicate. Statistics were calculated by 1-way ANOVA followed by Dunnett's method, * $P<0.05$, compared to empty vector HSD3B2; †† $P<0.001$, compared to OSTα with empty vector transfection. Panel B. Time course of CDCA effects on FXR-mediated induction of endogenous HSD3B2 and

OSTα expression. H295R adrenocortical cells were transfected with FXR and treated with CDCA for the indicated time. Results represent the mean ± SD of data from at least three independent experiments. Statistics were calculated by 1-way ANOVA followed by Dunnett's method, ** $P<0.01$, compared to 12 h HSD3B2 level; † $P<0.05$, compared to 12 h OSTα level.
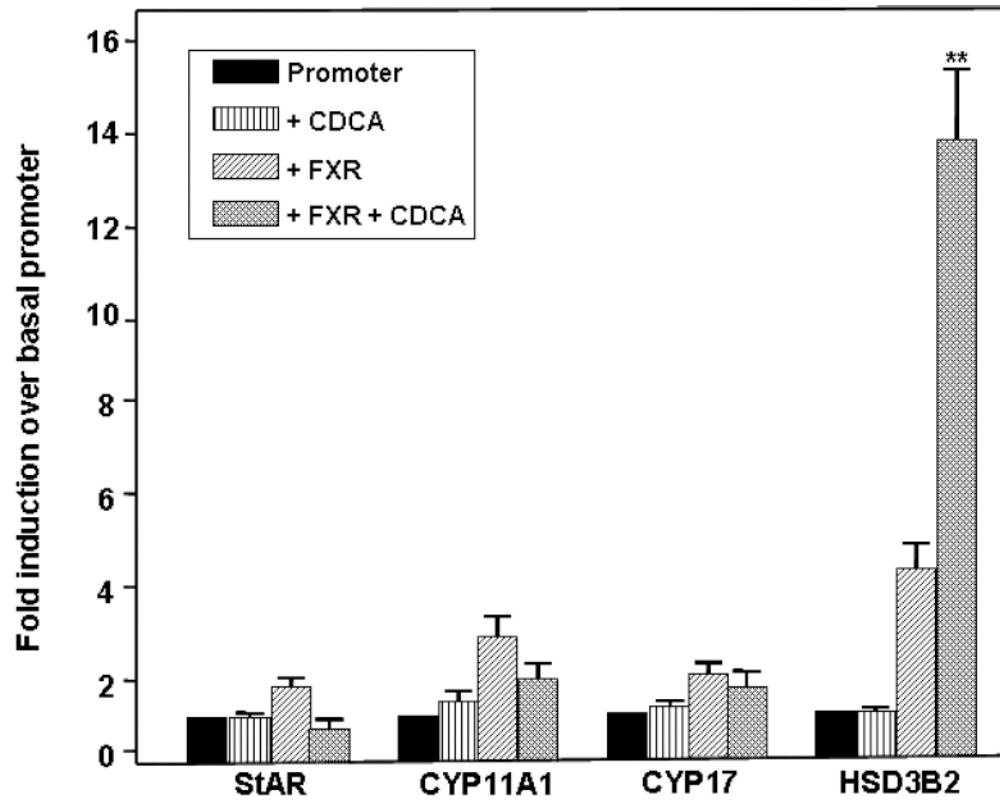
Fig.5.
Effects of FXR on steroidogenic enzyme promoters in H295R adrenocortical cells. Cells were transfected with indicated luciferase promoter constructs (1 μg/well) with or without FXR (0.1 μg/well), and treated with or without FXR ligand CDCA (10 μM). Data were normalized to co-transfected β-gal vector and results are expressed as fold induction over basal promoter. Data represent the mean ± SEM of data from at least three independent experiments performed in triplicate. Statistics were calculated by 1-way ANOVA followed by Dunnett's method, ** P<0.01. (StAR: Steroidogenic Acute Regulatory protein; CYP11A1: cholesterol side-chain cleavage; CYP17: 17α-hydroxylase)
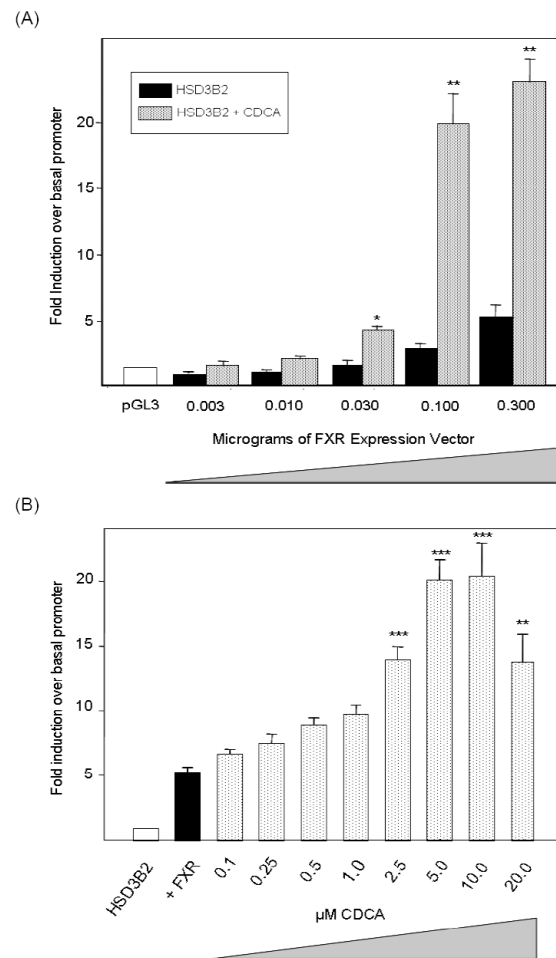
Fig.6.
FXR regulation of HSD3B2 reporter gene activity. Panel A. Concentration-dependent effects of FXR on HSD3B2 reporter gene activity. H295R cells were co-transfected with HSD3B2 promoter constructs (1 μg/well) and indicated amount of the FXR expression plasmid or the empty expression plasmid. Luciferase activity was tested after treatment with/ without CDCA (10 μM) for 24 h. Panel B. Concentration-dependent effects of CDCA on FXR-mediated induction of HSD3B2 reporter gene activity. H295R adrenocortical cells were co-transfected with HSD3B2 reporter construct and FXR expression vector in the presence of increasing amounts of CDCA for 24 h. Data were normalized to co-transfected β-gal vector and data are shown as the fold induction over the basal reporter. Results represent the mean ± SEM of data from at least three independent experiments each performed in triplicate. Statistical significance was analyzed by a paired t-test, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.
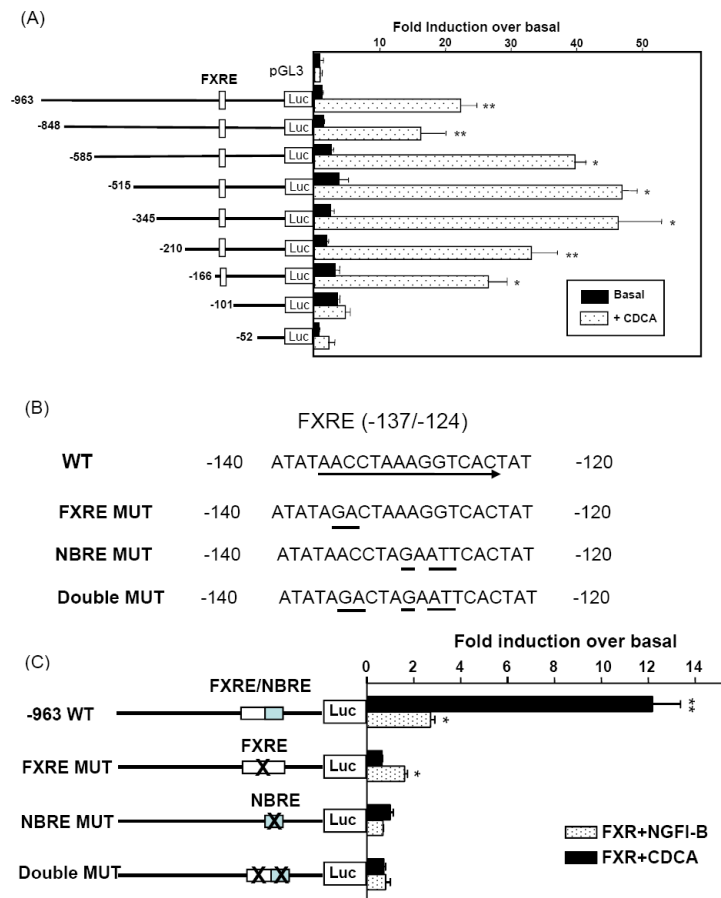
Fig.7.
A FXRE was necessary for FXR activation of HSD3B2 promoter activity. Panel A. Deletion analysis was used to localize the FXRE within the human HSD3B2 promoter. H295R adrenocortical cells were co-transfected with HSD3B2 reporter constructs (1 μg/well) and FXR expression plasmid (0.1 μg /well) in the presence of CDCA (10 μM) for 24 h. Cells were then lysed and assayed for luciferase activity. Data were normalized to co-transfected β-gal vector and data are shown as fold-induction over the basal pGL3 reporter activity. Results represent the mean ± SD of data from at least three independent experiments, each performed in triplicate. Statistics were calculated by paired t-test, * $P<0.05$, ** $P<0.01$. Panel B. Illustration of potential FXRE in HSD3B2 promoter region, and mutated sites in the FXRE and NBRE. Panel C. Effects of CDCA or NGFI-B on mutated HSD3B2 promoter in H295R cells. Cells were transfected with HSD3B2 promoter constructs and FXR expression plasmid with or without CDCA or NGFI-B (0.1 μg/ well). Data were normalized to co-transfected β-gal vector and results are expressed as fold induction over basal promoter. Results were presented as mean ± SD of data from at least three independent experiments performed in triplicate. Statistics were calculated by paired t-test, * $P<0.05$, ** $P<0.01$.
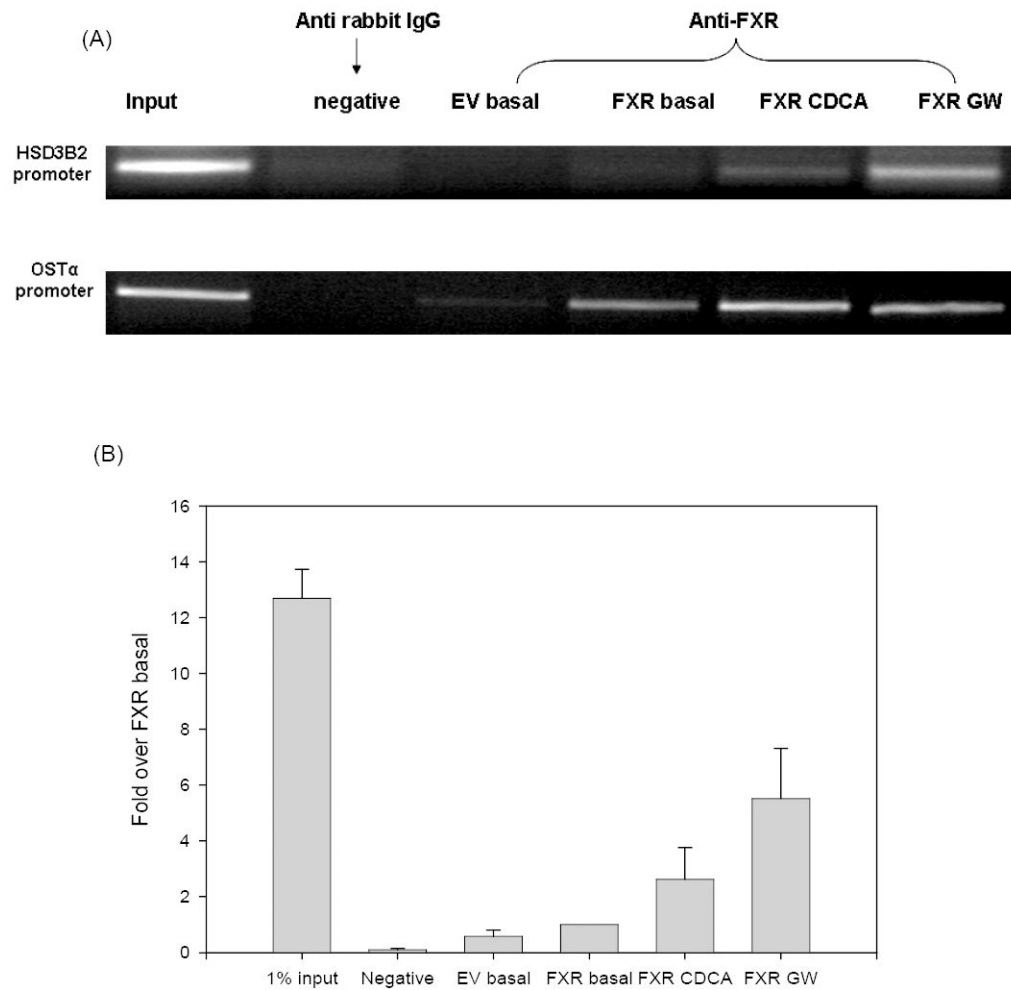
Fig.8.
Chromatin immunoprecipitation (ChIP) assay demonstration of FXR binding to the HSD3B2 promoter. ChIP was performed as described in the Materials and methods, where indicated cells were treated with CDCA (10 μM) for 24 h. Panel A: Gel electrophoresis of the PCR products amplified from HSD3B2 and OSTα promoter regions. Each is representative of three independent experiments. Panel B: a quantification of fold-change by comparing the signal strength of the samples compared to that of the FXR basal treatment. Results were presented as mean ± SD of data from three independent experiments.
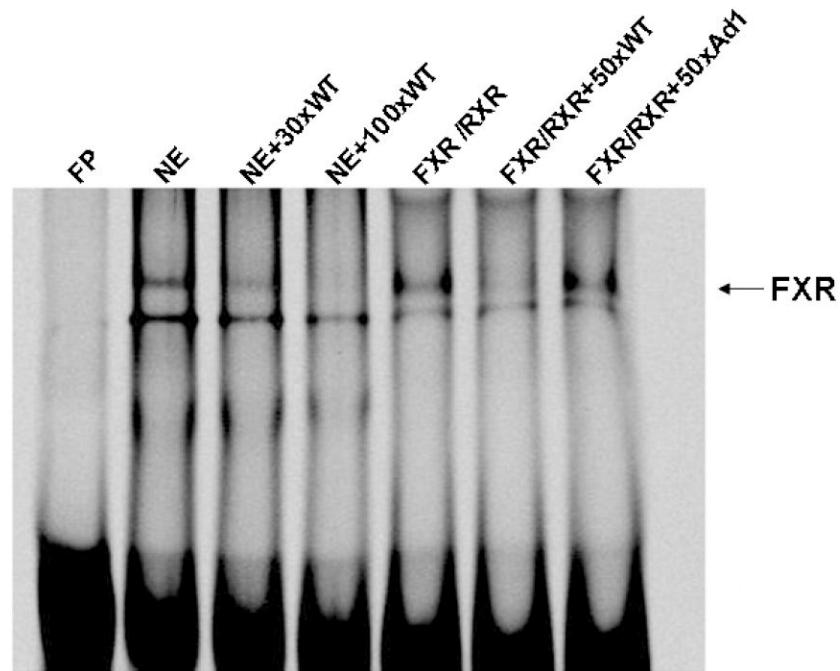
Fig.9.
EMSA for FXR binding to HSD3B2 to the FXRE. EMSA was performed using the IRDye 700
tagged oligonucleotide probe corresponding to the putative HSD3B2 FXRE (−137/-124).
Samples containing labeled probe alone (FP), probe incubated with FXR over-expressing
H295R nuclear extract (NE; 1 μg) or *in vitro* prepared FXR/RXR (2:1) mix (FXR/RXR; 3 μl)
are shown. Non-labeled self-competitor DNA (WT) or non-specific Ad1 oligonucleotide (Ad1)
was added to reaction mixtures to define specific protein/ DNA interactions.