Transcriptional regulation of Dehydroepiandrosterone Sulfotransferase (SULT2A1) by Estrogen-Related Receptor α (ERRα)

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Transcriptional regulation of Dehydroepiandrosterone Sulfotransferase (SULT2A1) by Estrogen-Related Receptor α (ERRα)

by

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DISSERTATION

Presented to the Faculty of the Medical School

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF MEDICINE WITH DISTINCTION IN RESEARCH

Portions of this work have been previously published in *Endocrinology*

The University of Texas Southwestern Medical Center at Dallas

April 2006

ABSTRACT

Transcriptional regulation of Dehydroepiandrosterone Sulfotransferase (SULT2A1) by Estrogen-Related Receptor α (ERRα)

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The University of Texas Southwestern Medical Center at Dallas, 2006

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The estrogen-related receptors (ERR α, β and γ) are a subfamily of orphan nuclear receptors (designated NR3B1, NR3B2 and NR3B3) that are structurally and functionally related to estrogen receptors α and β. Herein we test the hypothesis that ERRα regulates transcription of the genes encoding the enzymes involved in adrenal steroid production. Real-time RT-PCR was first used to determine the levels of ERRα mRNA in various human tissues. Adult adrenal levels of ERRα transcript were similar to that seen in heart, which is known to highly express ERRα. Expression of ERRα in the adult adrenal was then confirmed using western blotting and immunohistochemistry. To examine the effects of ERRα on steroidogenic capacity we used reporter constructs with the 5'-flanking regions of steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage (CYP11A), 3β hydroxysteroid dehydrogenase type II (HSD3B2), 17αhydroxylase, 17,20 lyase (CYP17), and DHEA sulfotransferase (SULT2A1). Co-transfection of these reporter constructs with wild-type ERRα or VP16-ERRα expression vectors demonstrated ERRα enhanced reporter activity driven by flanking DNA from CYP17 and SULT2A1. SULT2A1 promoter activity was most responsive to the ERRα and VP16-ERRα, increasing activity 2.6- and 79.5-fold respectively. ERRα effects on SULT2A1 were greater than the stimulation seen in response to steroidogenic factor 1 (SF1). Transfection of serial deletions of the 5'-flanking DNA of the SULT2A1 gene and EMSA experiments indicated the presence of three functional regulatory *cis*-elements which shared sequence similarity to binding sites for SF1. Taken together, the expression of ERRα in the adrenal and its regulation of SULT2A1 suggest an important role for this orphan receptor in the regulation of adrenal steroid production.

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PRIOR PUBLICATIONS AND PRESENTATIONS

Publications:

2005 "Transcriptional regulation of Dehydroepiandrosterone Sulfotransferase (SULT2A1) by Estrogen-Related Receptor α (ERRα)" Seely J, Amigh K, Suzuki T, Mayhew B, Sasano H, Giguere V, Laganiere J, Carr B, Rainey W. *Endocrinology* 2005 146: 3605-3613.

Presentations:

2005 "The role of Estrogen-Related Receptor α (ERRα) in the regulation of adrenal androgen production" Seminar as partial fulfillment of the requirements for the degree of Doctorate of Medicine with Distinction in Research.

2003 "Transcriptional regulation of Dehydroepiandrosterone Sulfotransferase (SULT2A1) by Estrogen-Related Receptor α (ERRα)" Poster presentation at the 2003 Endocrine Society National Conference, Philadelphia, Pennsylvania.

2003 "Transcriptional regulation of Dehydroepiandrosterone Sulfotransferase (SULT2A1) by Estrogen-Related Receptor α (ERRα)" Poster presentation and seminar at the 40th Annual UTSW Medical Student Research Forum, Dallas, Texas.

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LIST OF ABBREVIATIONS

AR	androgen receptor
CYP	cytochrome P450 enzyme
DAX1	dosage sensitive sex-reversal (DSS), adrenal hypoplasia congenita (AHC) critical region on the X-chromosome, gene 1
DES	diethylstilbestrol
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
ER	estrogen receptor
ERE	estrogen response element
ERR	estrogen-related receptor
ERRE	estrogen-related response element
GR	glucocorticoid receptor
HSD3B2	3 beta-hydroxysteroid dehydrogenase isozyme 2
MCAD	medium-chain acyl-CoA dehydrogenase
MR	mineralocorticoid receptor
OHT	hydroxytamoxifen
PAGE	polyacrylamide gel electrophoresis
PR	progesterone receptor
RT-PCR	reverse transcription polymerase chain reaction
SFRE	steroidogenic factor response element
SF1	steroidogenic factor 1
StAR protein	steroidogenic acute regulatory protein
SULT2A1	cytosolic sulfotransferase 2A1
VP16	viral protein 16

CHAPTER I

Introduction

The adrenal produces large amounts of dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS) during fetal development, which fall rapidly after birth and remain low for the first five years of life (Fig. 1). DHEAS levels then rise and peak during the second decade of post-natal life, followed by an age-dependent decline (1). The developmental and age-related changes in DHEA(S) are not paralleled by any other steroid hormone suggesting that the mechanisms regulating DHEA(S) formation are unique. In addition there are clear gender differences in the circulating levels of DHEA and DHEAS, with men having higher circulating levels than women (Fig. 1) (1). The mechanisms that cause the gender differences in circulating DHEA(S) and the drastic variation in DHEA(S) levels during development and aging remain a mystery. However recent studies have shown that the enzymes that are involved in the synthesis of DHEA-S are likely regulated differentially by a variety of transcription factors and selective nuclear hormone receptors (2-4).

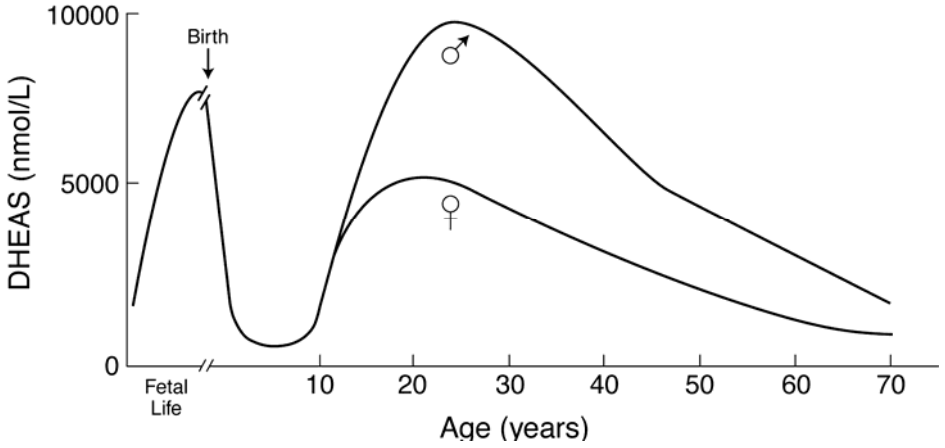


Figure 1. Variation in circulating dehydroepiandrosterone sulfate (DHEAS) levels throughout human life. During pregnancy, fetal levels of DHEAS rise progressively to reach a peak at term, and following birth there is a rapid decline. At ~5–6 years of age, DHEAS levels rise again (adrenarche), reach a peak during early adulthood, and decline thereafter. The levels of circulating DHEAS during adult life also exhibit a gender difference, with higher levels found in men than in women. Reproduced with permission from Ref. (1).

In this study I have focused on the mechanism regulating the expression of the cytosolic sulfotransferase enzyme (SULT2A1), commonly known as steroid sulfotransferase. The expression of human SULT2A1 occurs predominantly in the liver and adrenals and does not appear to be sex-regulated. The production of sulfonated steroids is very high in the adrenal such that the production of dehydroepiandrosterone sulfate (DHEA-S) is quantitatively the most abundant hormone secreted by the human adrenal. Circulating levels of DHEA-S are likewise very high in the adult adrenal due to both the high production rate and the relatively low clearance rate from the blood (5-7). SULT2A1 has been localized by immunohistochemistry to the DHEA-producing zona reticularis of the adrenal cortex where it catalyzes the conversion of DHEA to its sulfated form (Fig. 2) (1,8-10). While the enzymatic activity of SULT2A1 has been studied in some detail, little is known about the regulation of human SULT2A1 expression in any tissue.

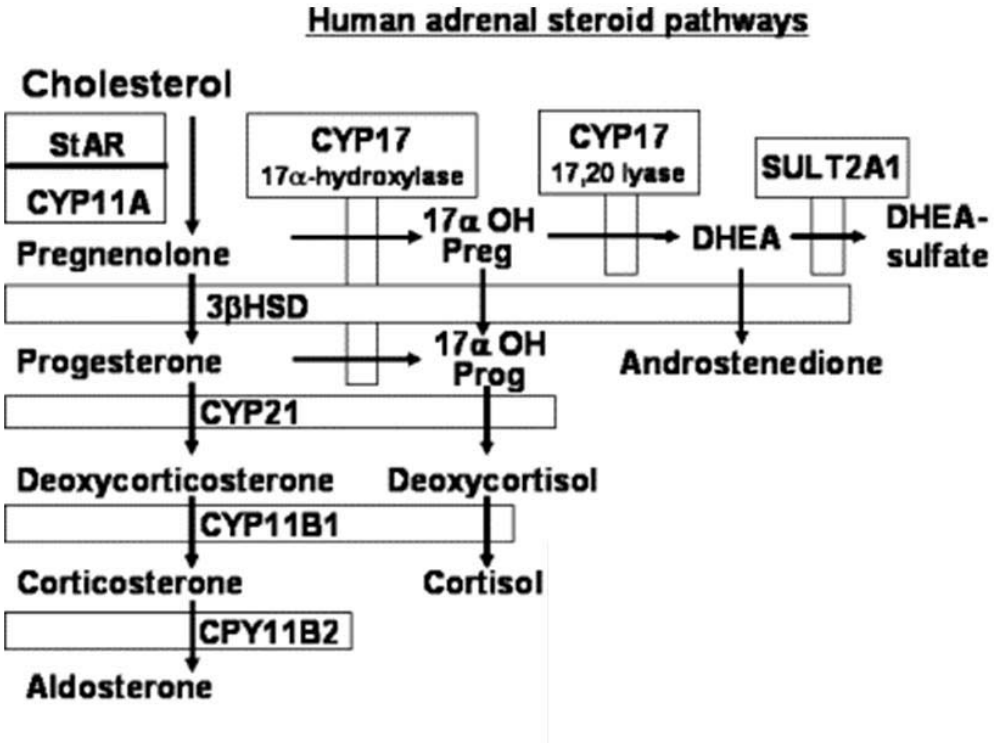


Figure 2. Steroidogenic pathway in the human adrenal gland. Steroid biosynthetic pathways illustrating the three main products of the human adrenal: aldosterone, cortisol, and DHEAS. Also included are the enzymes that synthesize these products. Reproduced with permission from Ref. (11).

Nuclear hormone receptors are classically defined as ligand-dependent transcription factors (12). However, some nuclear receptors have been discovered that have no known endogenous ligands. These nuclear receptors are known collectively as orphan nuclear receptors. With only a few exceptions, nuclear receptors share a similar organizational pattern in their protein structure creating two specific domains called the DNA-binding domain (DBD) and the ligand-binding domain (LBD) as shown in Figure 3. The DBD mediates interaction with target DNA sequences of genes. The LBD undergoes a 3-dimensional conformational change when a ligand binds the LBD which then allows the nuclear hormone receptor to dimerize with other nuclear hormone receptors, recruit and interact with coactivators, and perform ligand-dependent transcriptional activation at target genes.

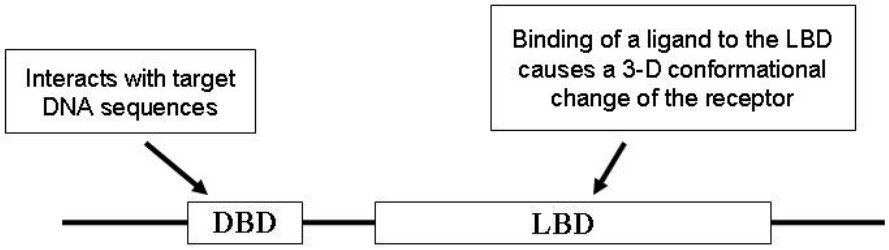


Figure 3. Schematic representation of nuclear hormone receptor structure.
DBD = DNA-binding domain. LBD = Ligand-binding domain. The resulting conformational change in the receptor structure then allows the nuclear hormone receptor to dimerize with other nuclear hormone receptors, recruit and interact with coactivators, and perform ligand-dependent transcriptional activation at target genes.

The estrogen-related receptor (ERR) family is a nuclear hormone receptor family comprised of three members: ERRα (NR3B1), ERRβ (NR3B2) and ERRγ (NR3B3) (13,14). Although all three ERR family members are closely related to the classic estrogen receptors, natural estrogens do not activate these receptors. At present, only a few synthetic compounds have been shown to bind and antagonize the effects of ERR family members--one of these is DES (diethylstilbestrol) (15-18).

DES was a widely used synthetic estrogen between the 1940's and 1970's to prevent spontaneous abortions. However, it was later discovered that people exposed in utero to DES later suffered from various gynecological problems such as malformations of the reproductive tract and vaginal cancer due to disruption of WNT signaling pathways during development. When bound to ERR family members alpha, beta, and gamma, DES inhibits transcriptional activation by interrupting associations with coactivator molecules (19).

The synthetic molecule XCT790 (thiadiazoleacrylamide 12) was discovered using high-throughput screening of 340,000 synthetic molecules for modulators of ERRα activity. XCT790 binds to ERRα and inhibits transcriptional regulation of its known target genes (18).

Among the 12 persistent organic pollutants identified by the United Nations Environment Program as requiring urgent attention are the organochloride pesticides toxaphane and chlordane. They have also been credited with some estrogen-like activity in certain studies and like XCT790, when bound to ERRα; they inhibit transcriptional regulation of known target genes (20).

Since there are no known endogenous ligands regulating ERRα activity the question remains as to whether an endogenous activating ligand actually exists for this receptor family. Recent crystallographic studies suggest the pocket formed at the LBD is only large enough to accommodate 4-5 carbon molecules and therefore would only accommodate half of the estradiol molecule (21). These studies also suggest that in the absence of ligand, ERRs assume the conformation of ligand-activated nuclear receptors (22). Interestingly, the presence of a phenylalanine residue at in the LBD pocket is apparently essential for the constitutive activity of ERRα which is abolished when this phenylalanine is mutated (21). It is thus possible that ERRs are constitutively active orphan nuclear receptors regulated mainly in an antagonistic manner. This argument was further supported in recent studies that showed that while homodimerization might enhance transcriptional activity of ERRα and ERRγ respectively, when the two form heterodimers (ERRα-ERRγ dimer) the transcriptional activity of both is inhibited (23).

There have also been studies showing the ability of ERRα to form heterodimers with ERα *in vitro* suggesting yet another possible regulator of ERRα transcriptional activity through heterodimer formation. However, no *in vivo* studies have been able to support this evidence (24).

Of the three isoforms of the estrogen-related receptors, ERRα is the most widely expressed in adult tissues (13,25). ERRα is expressed in both fetal and adult tissues with high levels of expression noted in the heart and kidney (12,26-29). However, relatively little is known concerning its regulation or gene targets in development or adulthood.

Tissues in which ERRα is highly expressed include cardiac muscle, adipose, skeletal muscle, brain tissue, breast tissue, kidney, and bone (13). In cardiac muscle, adipose, skeletal muscle and kidney, ERRα acts as a regulator of fatty acid metabolism. It was shown by Sladek et al. in 1997 that ERRα regulates transcription of the human medium-chain acyl coenzyme A dehydrogenase gene (MCAD). MCAD is a key enzyme involved in the mitochondrial beta-oxidation of fatty acids and is one of three nuclearly encoded proteins mediating the initial step in mitochondrial β-oxidation of fatty acids. Modulation of MCAD gene expression is an important control of the rate of tissue fatty acid β-oxidation and is tightly regulated by tissue energy demands during organ development as well as by energy substrate supply. The highest MCAD levels are found in organs that utilize lipids as a source of cellular energy, such as the adipose, cardiac muscle, skeletal muscle, and kidneys. In humans, MCAD deficiency may cause childhood nonketotic hypoglycemia, coma, or sudden death, often in association with prolonged fasting or intercurrent illness (25).

ERRα knockout mice (ERRα -/-) fed high-fat diets gained 10% less weight than their wild-type counterparts (30). Although these ERR-deficient mice weighed less compared to their wild-type littermates, histological analysis of adult tissues failed to show morphological abnormalities. No statistically significant changes in eating behavior or energy expenditure between the wild-type and ERR-null littermates were detected, and the mice were viable, fertile and have a normal life span. One possible explanation for these results is that the increase in energy expenditure is small and significant only over a long period of time. It has also been shown that MCAD is

upregulated in the adipose of the ERRα -/- mice. Thus, ERRα likely acts a repressor of MCAD expression thereby decreasing the rate of fatty acid metabolism (30).

In bone, ERRα mRNA expression in bones is associated with new bone formation (26). ERRα has been shown to be present in the ossification zones of the mouse embryo and regulates the synthesis of osteopontin (a protein expressed by osteoblasts and released in the bone matrix which is a marker of the late stages of osteoblastic differentiation). Overexpression of ERRα in three different osteoblast-like cell lines results in an elevation of the amount of osteopontin-corresponding mRNA. Thus, osteopontin is a target gene for ERRα, pointing to the role of the ERRα in osteoblast differentiation (31). When expression of ERRα is blocked by antisense oligonucleotides in either proliferating or differentiating bone cell cultures there is resultant inhibition of cell growth and inhibition of differentiation (32). On the other hand, ERRα overexpression in bone cells lines increased differentiation and maturation of progenitors to mature bone-forming cells (32).

Although the role of ERRα in breast tissue is not completely understood, it is considered to be a poor prognostic indicator when present in breast cancer tissue (19). It has been shown that ERRα regulates the expression of the pS2 (TFF1) gene which is a known breast cancer prognostic marker (19). pS2 gene expression is “estrogen-inducible” meaning that estrogen bound to the ER, acting at a known ERE, induces transcription of the pS2 gene. However, Lu et al also demonstrated the presence of an ERRE in the pS2 gene promoter that is required for the gene’s expression to be estrogen-inducible (19). This is one of several instances where there is functional “cross-talk” (significant ER – ERR crosstalk) between the estrogen receptor and ERRα.

In vitro studies have demonstrated the mRNA expression of ERRα in breast cancer cell lines (19) and in carcinoma tissues (33). This increased mRNA expression correlated with ER- or PR- tumors thus it correlated with a poor prognosis. However, Liu et al demonstrated that estrogens stimulate ERRα expression in human breast cell lines suggesting ERRα is a downstream target of ERα (34). It has also been reported that ERRα induces expression of the enzyme aromatase (which converts testosterone

to estradiol) in breast cancer fibroblasts (35). Still, much more research is needed to develop a clear concept of the role ERRα plays in normal and diseased breast tissue.

As mentioned earlier, the ERR family of nuclear hormone receptors are closely related to the estrogen receptors (ER). This relationship is reflected most in the sequence similarity of the ERα and ERRα DNA-binding domains (69% sequence homology). Although ERRα displays this sequence similarity to estrogen receptors, its transcriptional activity is not limited by recognition of estrogen response elements (ERE). While ERRα can bind EREs as homodimers or heterodimers (19,36), it can also bind extended nuclear receptor half-sites (i.e. TnAGGTCA), also known as estrogen-related receptor response elements (ERRE) as monomers, homodimers or heterodimers (24,25,36,37) and have been shown to both enhance and repress gene transcription (19,20,25,26,31,37-41). Another orphan receptor, steroidogenic factor 1 (SF1; NR4A1) also regulates transcription through nuclear receptor half-sites and has been shown to be critical for development of adrenals and gonads and also plays a major role in adrenal steroidogenesis (42-48). Adrenal steroidogenesis occurs in the cortex which is divided into 3 zones, the outer glomerulosa, which produces aldosterone when stimulated to do so by angiotensin II; the fasciculata which produces cortisol upon activation by adrenal corticotropic hormone; and the innermost reticularis which produces large amounts of dehydroepiandrosterone sulfate. Extensive studies of SF-1 show that it regulates several of the proteins and enzymes required for steroidogenesis in the human adrenal glands including steroidogenic acute regulatory protein (StAR) protein, CYP17, HSD3B2, CYP21, and CYP11B1. (49-52).

The ability of SF1 and ERRα to regulate transcription through similar *cis*-elements and their co-expression within adrenocortical cells led us to define the role of ERRα in the regulation of steroidogenic enzyme gene transcription. Herein, we demonstrate that ERRα is expressed in the human adrenal gland and that it acts to increase expression of 17α-hydroxylase (CYP17) and DHEA sulfotransferase (SULT2A1). In addition, the effect of ERRα on transcription of the genes encoding steroidogenic enzymes was unique from that seen for SF1.

CHAPTER II

Experimental Procedures

RNA extraction, cDNA synthesis and real-time PCR: Normal human adult adrenals were obtained through the Cooperative Human Tissue Network (Philadelphia, PA) and normal human adult testis and liver total RNA were obtained from the Clontech master panel II (Cat# K4008-1, Clontech, Palo Alto, CA). Human ovaries and placenta as well as whole fetal brain, heart, and kidney were obtained from Parkland Memorial Hospital (Dallas, TX) and were determined to be normal. 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 as previously described (53). Purity and integrity of RNA was checked spectroscopically and by gel electrophoresis prior to use. Two µg of DNase treated total RNA was reverse transcribed in a final volume of 50 µl using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) and stored at –20°C.

Primers for the amplification were based on published sequences for human ERRα and SULT2A1. The following primer sequences used were: ERRα (NM_004451) Forward: 5'-CACCATCAGCTGGGCCAAGAG-3' (exon 5), and Reverse: 5'- GGTCAGACAGCGACAGCGATG (exon 6) which produced a 55 bp fragment; and SULT2A1 (NM_003167) Forward: 5'-TCGTGATAAGGGATGAAGATGTAATAA-3' (exon_1) and Reverse: 5'-TGCATCAGGCAGAGAATCTCA-3' (exon 2) which produced an 83 bp fragment. PCR reactions were performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) in a total volume of 30 µl reaction mixture following the manufacturer's recommendations using the SYBR Green Universal PCR Master Mix (Applied Biosystems), 0.1 µM of each primer, and 5 µl of each first-strand cDNA sample. A dissociation protocol was performed at the completion of each experiment to verify that a single specific PCR product was amplified. Standard curves were prepared using the human ERRα expression vector. No-template controls contained water in place of first-strand cDNA. Each sample was normalized on the basis of its 18S ribosomal RNA content. The 18S quantification was

performed using a TaqMan Ribosomal RNA Reagent kit (Applied Biosystems) following the manufacturer's recommendations.

Protein immunoblotting analysis: Cultured H295R adrenocortical cells and adult adrenal and liver samples were used to prepare nuclear extract following the protocol described previously (54). Polyacrylamide gel electrophoresis (PAGE) was carried out on the samples using a precast Novex gel electrophoresis system with 4–12% bis-Tris NuPage gels (Invitrogen, Carlsbad, CA). Proteins were electrophoretically transferred onto polyvinylidene difluoride membranes by wet transfer for 1 h at 30 V. After transfer the membranes were incubated overnight at 4C with rabbit ERRα specific antibody (1:1000 dilution) designed against the N-terminal region of ERRα (Cat# AB16363, Abcam, Cambridge, MA). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, and immunoreactive bands were visualized using enhanced chemiluminescence western blotting detection reagents from Amersham Pharmacia Biotech (Piscataway, NJ). Lamin B (Cat# SC-6216, Santa Cruz, Santa Cruz, CA) was used as a loading control for nuclear extracts.

Cell culture and transfection assay: Transfection assays were performed using CV-1 cells which were derived from the kidney of the African green monkey (ATCC #CCL-70) (55) because of their significantly lower levels of ERRα expression than adrenal cells (data not shown). CV-1 cells were cultured in Dulbecco's modified Eagle's/Ham F12 (DMEM/F12) medium (Invitrogen) supplemented with 5% NuSerum (Collaborative Biom, Bedford, MA) and antibiotics. For transfection experiments Fugene 6 (Roche, Indianapolis, IN) was used to transfect 1 μg of reporter plasmid and 0.3 μg of expression vectors. To assure constant amounts of DNA per well for each transfection, pCMX empty vector was used. The cells were co-transfected with 50 ng/well of β-galactosidase plasmid (Promega, Madison, WI) to normalize luciferase activity. Cells were harvested 22-24 hours after recovery and assayed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI).

Preparation of reporter constructs and expression vectors: The 5' flanking DNA from the human genes for StAR, CYP11A1, HSD3B2, CYP11B1, CYP11B2, CYP17, and SULT2A1 were inserted upstream of the firefly luciferase gene in the reporter vector pGL3-basic (Promega). SULT2A1 deletion constructs were described previously

(56). Empty pGL3-Basic served as the control vector to measure basal activity in all transfections. The human ERRα and VP16-ERRα were described previously (16). The coding regions of human SF1 and DAX were inserted into the eukaryotic expression vector pcDNA 3.1 zeo (Invitrogen) (50). Mutations to putative ERRα binding sites in the SULT2A1 promoter were created using the QuikChange XL Site Directed Mutagenesis kit (Stratagene, La Jolla, CA) following manufacturer's recommendations. Primer sets used for the mutations have been previously described (56).

Immunohistochemistry

Immunohistochemical analysis was performed employing the streptavidin-biotin amplification method using a Histofine Kit (Nichirei, Tokyo, Japan). Antibodies used include SULT2A1 (rabbit polyclonal) (8), SF1 (rabbit polyclonal) (kindly provided by Dr. K. Morohashi (National Institute for Basic Biology, Okazaki, Japan) (57), and ERRα (mouse monoclonal; 2ZH5844H) purchased from Perseus Proteomics (Tokyo, Japan) (58). Antigen retrieval for immunostaining of SF1 and ERRα was performed by heating the slides in an autoclave at 121 C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). The dilutions of the primary antibodies used in this study were 1:1000. 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), and counterstained with hematoxylin. For negative controls (data not shown) normal rabbit or mouse IgG was used instead of primary antibodies, and no specific immunoreactivity was detected in these sections. Histological identification of three zones of the human adrenal cortex was based on previously published criteria (9).

EMSA

Nuclear extracts from human adult adrenal tissue were prepared as described above. EMSA assays were performed as previously described (54) using the oligonucleotides for cis-elements in the SULT2A1 gene as were described previously (56).

Data analysis and statistical methods

Data from at least three experiments run in triplicate, for a total of nine independent observations for each condition, were pooled and analyzed using single-factor ANOVA with Student-Newman-Keuls multiple comparison method, using SigmaStat version 3.0 (SPSS, Chicago, IL). Significance was accepted at the 0-0.05 level of probability.

CHAPTER III

Results

Human adrenal tissue expresses ERRα mRNA and protein

In order to demonstrate expression of ERRα in the human adult adrenal gland and to characterize the expression pattern of ERRα in steroidogenic tissues, quantitative real-time RT-PCR was performed using mRNA isolated from human adult adrenal, ovary, testis, placenta and fetal adrenal as well as control tissues (fetal brain, heart, and kidney) reported to highly express ERRα (13,25) (Fig. 4). ERRα transcripts in the fetal adrenal (0.61 attmol/μg18S) and placenta (0.60 attmol/μg18S) were comparable to the levels detected in the testis (0.50 attmol/μg18S), brain (0.49 attmol/μg18S) and kidney (0.60 attmol/μg18S) tissue samples. Compared to other tissues, the expression of ERRα mRNA in the liver was low (0.205 attmol/μg18S). This observation is in agreement with the levels of ERRα mRNA detected in previously published northern analysis data (13,28).

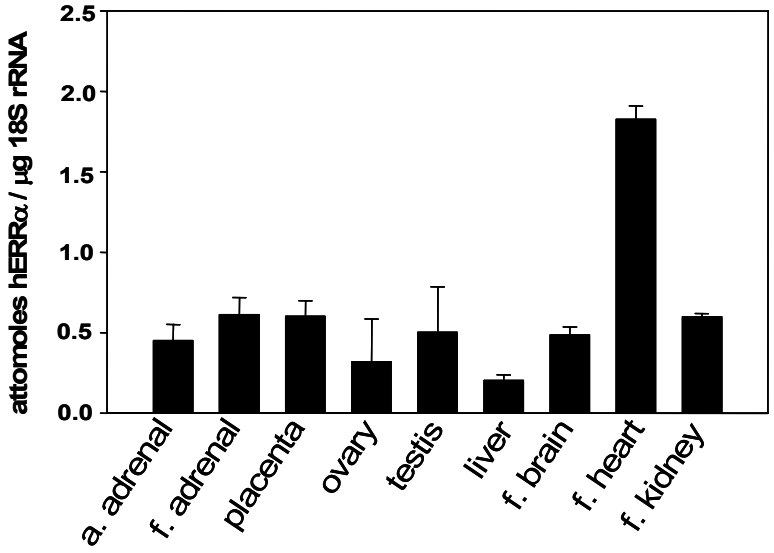


Figure 4. Quantification of ERRα transcript levels in human steroidogenic tissues. Real-time RT-PCR was performed to quantify the level of ERRα mRNA in adult adrenal (a. adrenal), fetal adrenal (f. adrenal), placenta, ovary, testis, liver, fetal brain (f. brain), fetal heart (f. heart) and fetal kidney (f. kidney) as described in Materials and Methods. Data represent the mean ±SEM of at least three independent DNase-treated RNA samples and are expressed in attomoles of mRNA per μg of 18S ribosomal RNA.

Once the presence of ERRα mRNA in the human adrenal was demonstrated, western analyses were performed to confirm the presence of the ERRα protein (Fig. 5). Incubation with ERRα-specific antibody revealed the presence of ERRα protein in nuclear extracts of human adult adrenal glands as well as in the H295R adrenocortical cell line. No detectable level of ERRα protein was found in liver nuclear extract, which correlates with the real-time RT-PCR data and with previous studies indicating low levels of ERRα expression in liver (13,28).

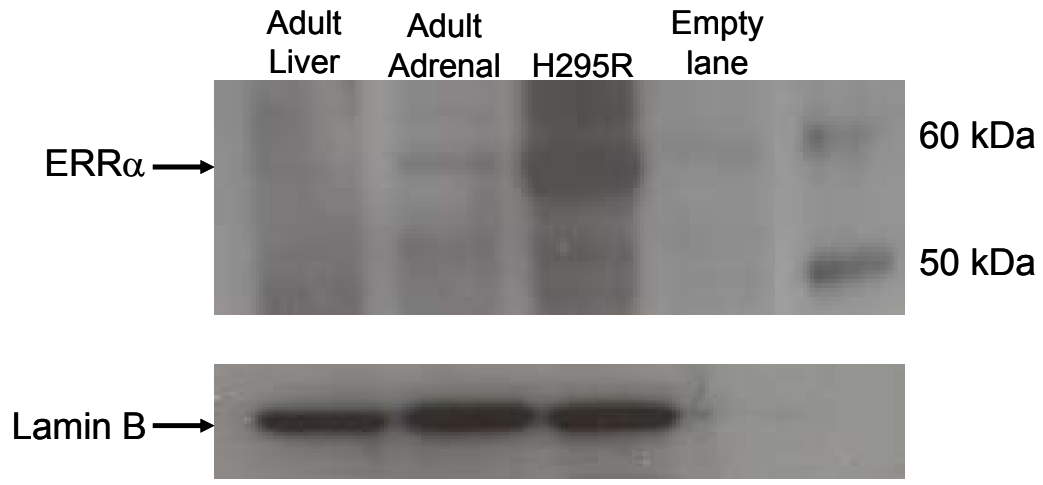


Figure 5. Western analysis of the human ERRα protein. Fifteen micrograms of nuclear extract protein from cultured H295R cells, human adult adrenal, and human liver were separated on a 4-12% Bis-Tris NuPage gel (Invitrogen) and blotted onto a polyvinylidene difluoride membrane as described in Materials and Methods. Lamin B was used as a loading control.

Finally immunohistochemical staining of adrenal sections was performed to localize the expression of ERRα within the human adrenal gland (Fig. 6). ERRα immunoreactivity was detected in nuclei of cortical cells of the entire adrenal cortex, including zona glomerulosa (Fig. 6A), fasciculata, and reticularis (Fig. 6B). No ERRα was detected in the cells of the adrenal capsule or inner medullary cells. No staining was observed in the absence of ERRα antibody.

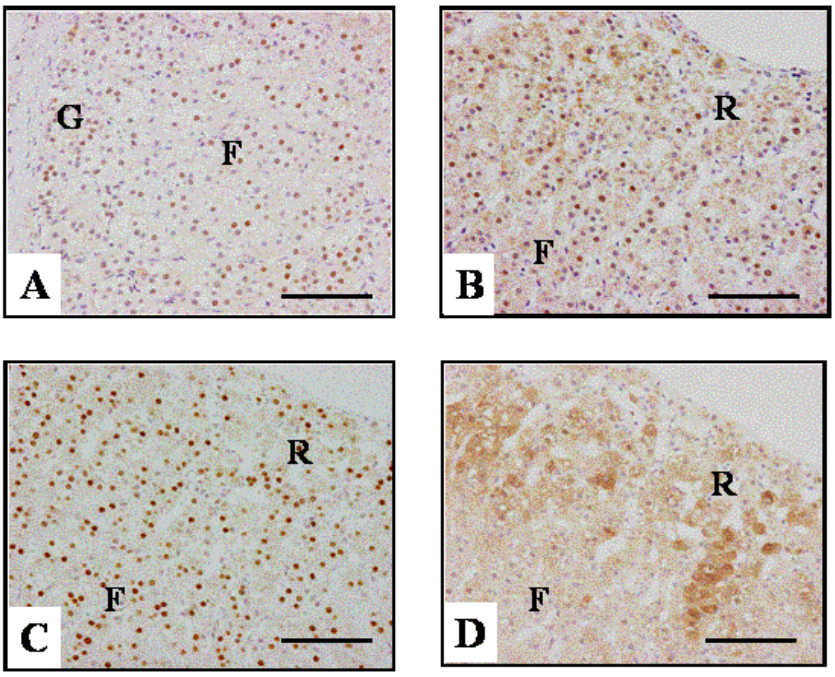


Figure 6. Immunohistochemical analysis of ERRα (Panel A, B), SF1 (Panel C), and SULT2A1 (Panel D) in human adult adrenal gland. ERRα immunoreactivity was detected in the nucleus of cortical cells in zonae glomerulosa (Panel A), fasciculata, and reticularis (Panel B). SF1 immunoreactivity was detected in the nucleus of cortical cells in all these three zones (Panel C), while SULT2A1 immunoreactivity was detected in the cytoplasm of cortical cells in zona reticularis (Panel D), as were reported previously (3,9). Panels B-D were demonstrated as same tissue field in the serial sections. Bar = 100 μm, respectively. G = glomerulosa, F = fasciculata, and R = reticularis.

ERRα activates the transcription of SULT2A1 and CYP17

To better determine the role of ERRα in the regulation of adrenal steroidogenesis, we examined its effects on the transcription of the genes encoding the enzymes involved in steroid hormone biosynthesis and the StAR protein. The promoter constructs tested include CYP11A1, StAR, HSD3B2, CYP17, CYP11B1, CYP11B2, and SULT2A1. To minimize competition with endogenously expressed ERRα, CV-1 cells were used for transfection studies because these cells express low levels of ERRα (data not shown). Of all constructs tested, only CYP17 (1.5-fold) and SULT2A1 (2.6-fold) showed transcriptional activation when co-transfected with 0.1 μg ERRα (Fig. 7A). We also examined the effects of a VP-16 / ERRα chimera, which contains a VP16 insert directly 5' to the ERRα coding sequence. This VP16 insert is a portion of the viral

protein 16 promoter region isolated from the herpes simplex virus, and when placed upstream to the ERRα coding sequence, it mimics a ligand-activated transcriptional response. Again only the CYP17 (12.1-fold) and SULT2A1 (79.5-fold) reporter constructs exhibited a marked responsiveness to the VP16 ERRα chimera (Fig. 7B).

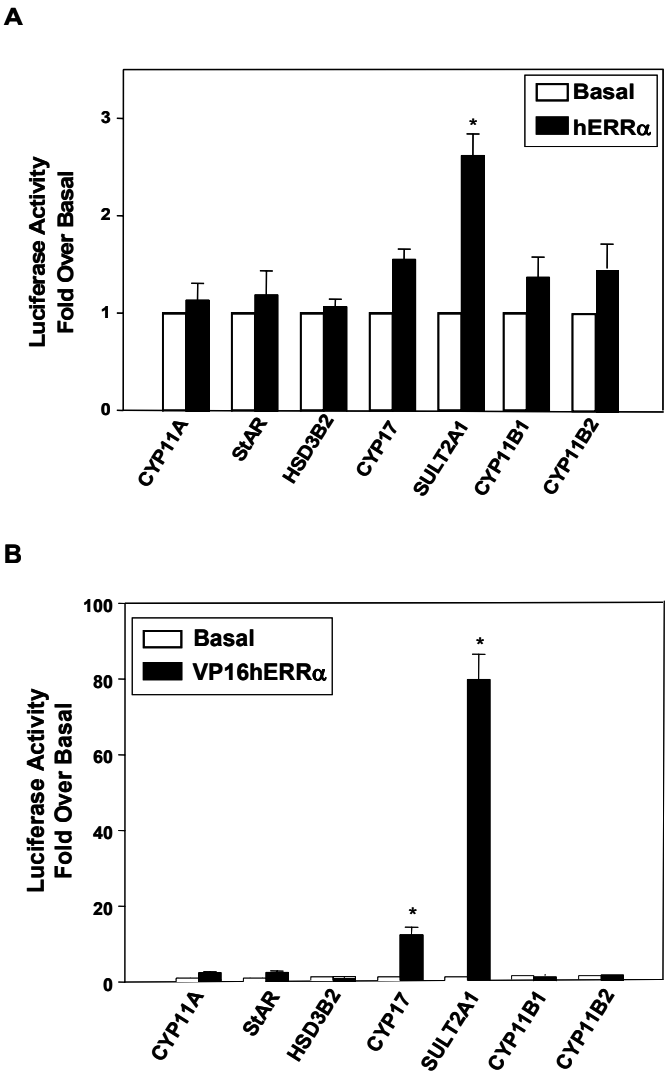


Figure 7. Effects of ERRα (panel A) and VP16ERRα (panel B) expression constructs on steroidogenic promoters. CV-1 cells were transfected with luciferase promoter contructs containing CYP11A1, StAR, HSD3B2, CYP17 or SULT2A1 (1 µg/well). Cells were co-transfected with expression plasmids containing the coding sequence of ERRα or VP16hERRα at a concentration of 0.1 µg/well. At twenty-four hours post-transfection the cells were lysed and assayed for luciferase. Data was normalized to co-transfected β-galactosidase expression vector and results are expressed as fold increase over basal activity. Results are presented as mean ± SEM of data from at least three independent experiments performed in triplicate. *Asterisk* indicates ERRα or VP16ERRα activation of the SULT2A1 promoter construct to be significant (P ≤0.001).

ERRα was then co-transfected into CV-1 cells with the SULT2A1 reporter construct and increasing concentrations of expression vectors encoding ERRα or VP16ERRα (Fig. 8). The SULT2A1 promoter was activated by ERRα and VP16ERRα in a concentration-dependent manner with activity increasing to 10.2- and 223.4-fold above basal levels, respectively, when cells were co-transfected with 1.0 μg/well of expression plasmid.

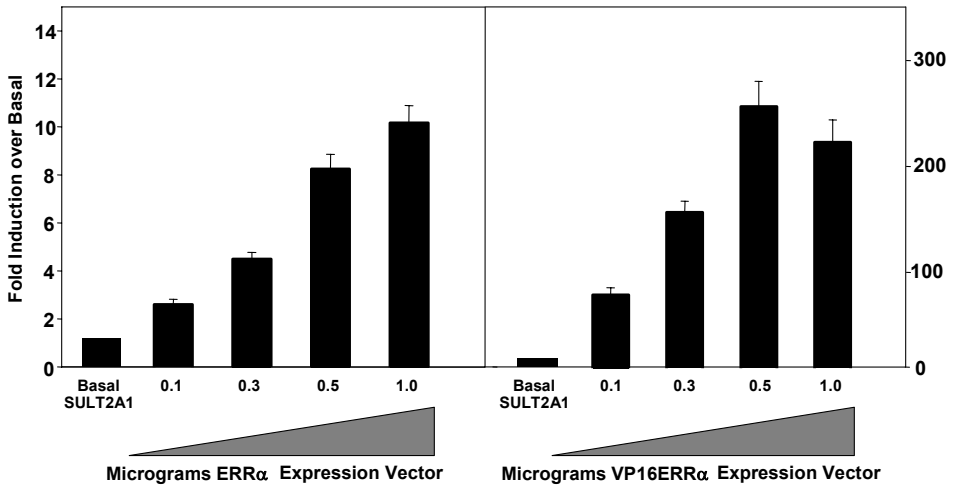
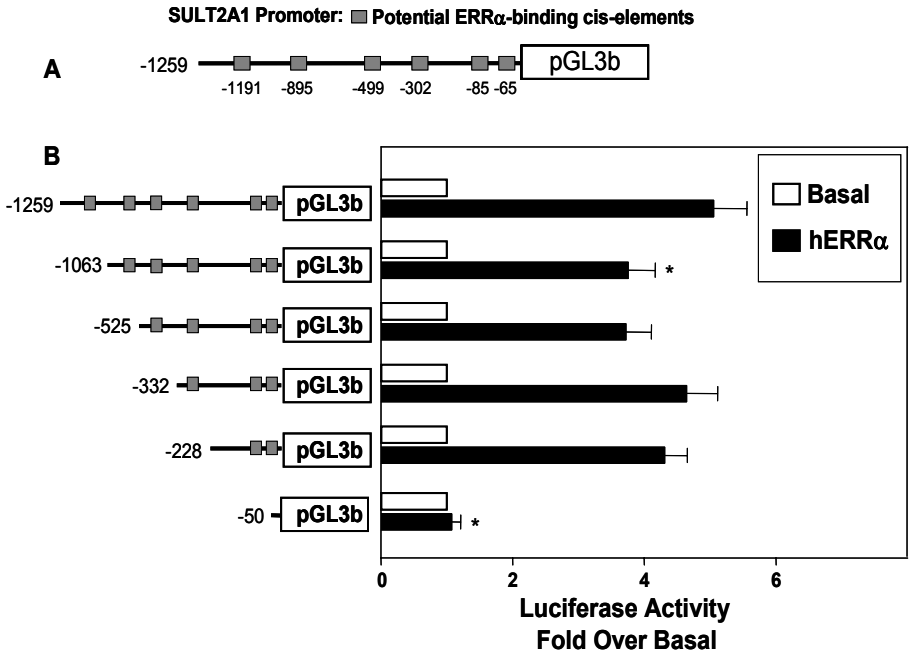


Figure 8. Concentration-dependent effects of ERRα and VP16ERRα on SULT2A1 reporter gene activity. CV-1 cells were transfected with luciferase reporter constructs containing the SULT2A1 promoter construct at a concentration of 1 μg/well. Cells were co-transfected with the indicated amount of ERRα or VP16hERRα expression plasmid or the empty expression plasmid pCMX. Cells were lysed and assayed for luciferase activity after 24 h. Data was normalized to co-transfected β-galactosidase expression vector and results are expressed as fold increase over basal activity. Results represent the mean ±SEM of data from at least three independent experiments each performed in triplicate. All values are significantly higher than basal (P≤0.001).

Identification of ERRα Response Elements (ERREs) by deletion and mutation analyses

The SULT2A1 5'-flanking DNA contains several putative nuclear receptor half sites (Fig. 9A) that match or closely resemble the consensus binding site TCAAGGTCA for ERRα. To determine if these nuclear receptor half-sites were potential ERREs, a series of deletion constructs were created containing progressively shorter fragments of SULT2A1 5'-flanking DNA. These deletion constructs were co-transfected into CV-1 cells with empty pCMX expression plasmid or pCMX containing the ERRα (Fig. 9B) or

VP16ERRα (Fig. 9C) coding sequences. VP16ERRα responsiveness was reduced by approximately 50% of that seen with the wild type promoter when the -1063 promoter deletion construct was co-transfected. Reporter activity further decreased following transfection with the -50 deletion fragment of the SULT2A1 to a level similar to that observed with pGL3Basic vector. The pattern of effects of the various SULT2A1 promoter deletions on reporter activity was similar for both wild-type ERRα and VP16ERRα. The combination of sequence and deletion analyses indicates three potential ERRα *cis*-elements: -1191, -85, and -65.



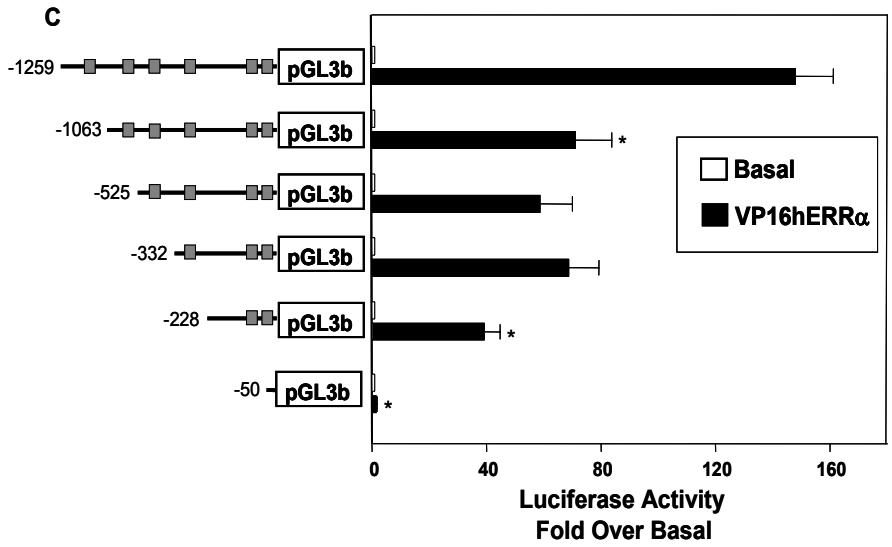


Figure 9. The roles of ERRα binding *cis*-elements in the regulation of SULT2A1 transcription. Panel A. Schematic representation of SULT2A1 promoter with potential ERRα binding sites. *Gray boxes* represent potential *cis*-binding sites and the *numbers* below represent the base pair at which the site begins based on the translational start site. Panels B and C. CV-1 cells were transfected with ERRα (Panel B) or VP16hERRα (Panel C) expression plasmids (0.3 µg/well) and pGL3Basic reporter constructs containing progressively smaller amounts of SULT2A1 5'-flanking DNA (1 µg/well) as indicated. Cells were lysed and assayed for luciferase 24h post transfection. Data were normalized to co-transfected β-galactosidase activity and fold induction was calculated relative to the basal promoter control. Results represent the mean ± SEM of data from at least three independent experiments performed in triplicate. *Asterisk* indicates ERRα or VP16ERRα activation of the SULT2A1 promoter construct to be significantly lower than the next largest promoter deletion construct ($P \leq 0.018$).

To determine the relative importance of each putative ERRα *cis*-element, all three sites were individually mutated in the context of the full-length (-1259bp) SULT2A1 gene promoter (Fig. 10A). Reporter activity of the wild-type and mutated reporter constructs were examined when co-transfected with either ERRα (Fig. 10B) or VP16ERRα (Fig. 10C) expression vectors. Mutation of the -1191 site lowered VP16ERRα responsiveness to approximately 40% of wild type. After mutation of the -85 site, VP16ERRα responsiveness is lowered to 5% of wild type. Finally, VP16ERRα responsiveness of the SULT2A1 gene promoter is lowered to 16% of wild type after mutation of the possible ERRα response element at -65. The effect of mutation of these cis-elements was similar for both wild-type ERRα and VP16-ERRα with regard to the fold induction of reporter activity.

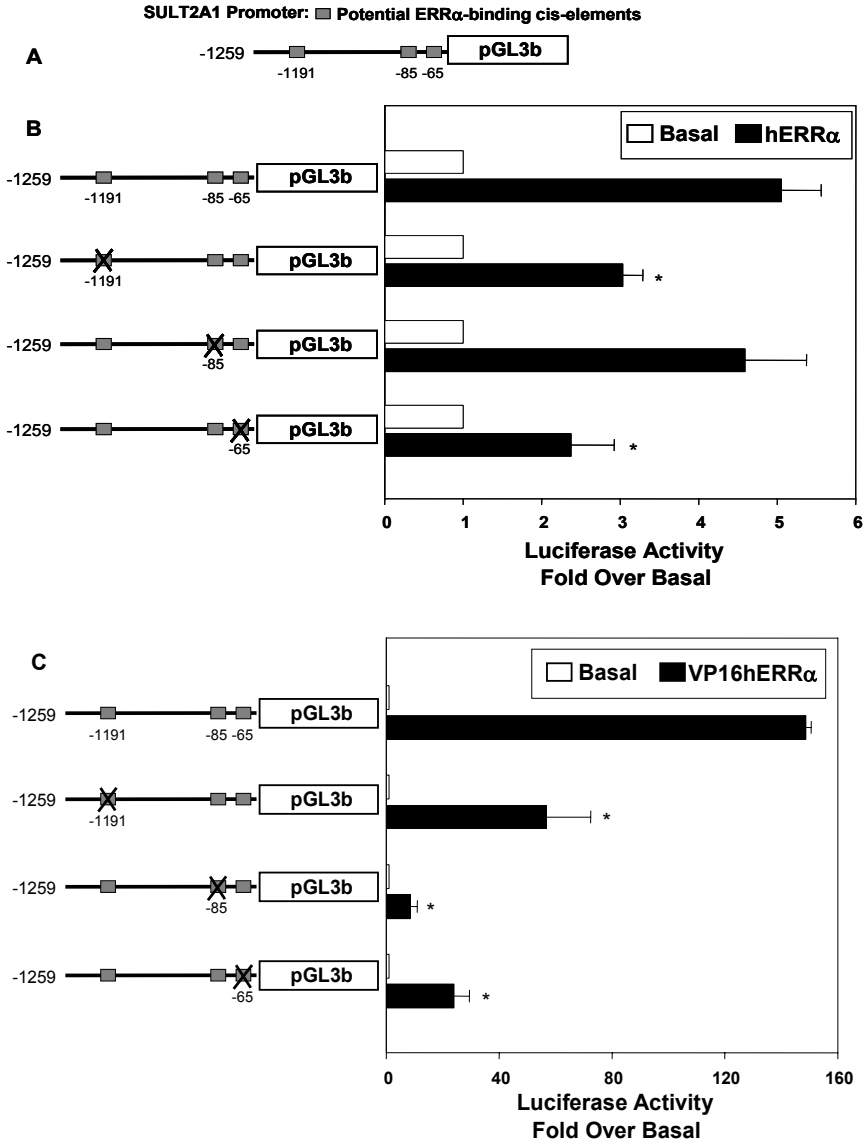


Figure 10. Mutation of putative ERRα binding sites in the SULT2A1 promoter. Panel A. Schematic representation of SULT2A1 promoter with potential ERRα binding sites indicated by *gray boxes*. Panels B and C. CV-1 cells were transfected with ERRα (Panel B) or VP16hERRα (Panel C) expression plasmids (0.3 μg/well) and pGL3Basic reporter constructs containing the variations of the SULT2A1 promoter containing site-specific mutations (1 μg/well) as indicated. Cells were lysed and assayed for luciferase 24h post transfection. Data were normalized to co-transfected β-galactosidase activity and fold induction was calculated relative to the basal promoter control. Results represent the mean ± SEM of data from at least three independent experiments performed in triplicate *Asterisk* indicates ERRα or VP16ERRα activation of the SULT2A1 promoter construct to be significantly lower than that of the non-mutated (-1259) SULT2A1 clone ($P < 0.001$).

ERRα binding to putative ERRα cis-elements of the SULT2A1 promoter

To determine whether ERRα interacts directly with either the -85 or -65 *cis*-element of the SULT2A1 promoter, ^{32}P-labeled oligonucleotides containing sequence to

correspond with these specific elements were prepared and used in EMSA analysis (Fig. 11). Both elements bound proteins in nuclear extracts from adult adrenal tissue although the -85 site bound with greater efficiency than the -65 site. Binding could be completely blocked by addition of non-labeled oligonucleotide (data not shown). Complex formation was partially retarded causing a shift when antibody targeting N-amino terminus of ERRα was added to the reaction mixture in a dose-dependent fashion.

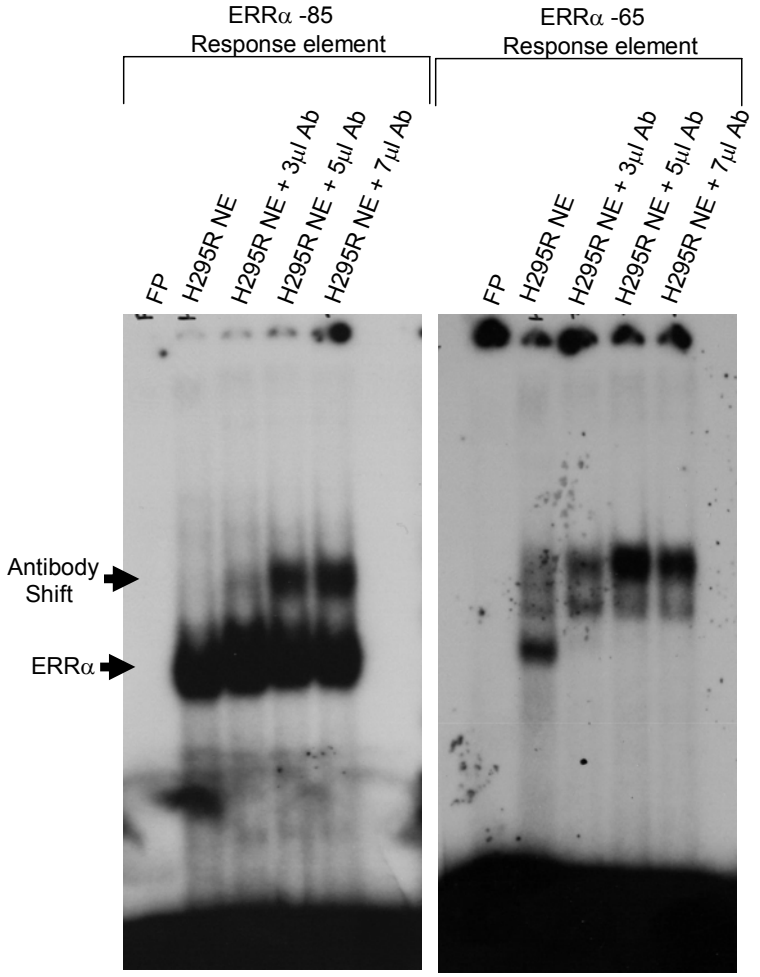


Figure 11. EMSA of ERRα -85 and -65 *cis*-elements. EMSA was performed using ^{32}P-labeled oligonucleotide probes containing the -85 or -65 consensus sequences of SULT2A1. Radiolabeled probe alone corresponds to FP (free probe), and H295R adrenocortical cell nuclear extract corresponds to H295R NE. Bands corresponding to probe/ERRα (as well as SF-1) complexes are indicated. The remaining lanes correspond to H295R NE incubated with increasing levels of antibody targeted to the amino terminus of the ERRα protein. Supershift bands corresponding to probe/ERRα/ antibody complexes are indicated.

Effects of SF1 on the regulation of the SULT2A1 gene promoter by ERRα

It is known that SF1 enhances transcription of the genes encoding steroid-metabolizing enzymes through its action at nuclear receptor half-sites and we have previously shown that SF1 regulates SULT2A1 (3,56). Our current data suggest that ERRα also enhances transcription of a steroid-metabolizing enzyme gene, SULT2A1, via binding of nuclear receptor half-sites. Therefore, we next examined the combined effects of SF1 and ERRα on the SULT2A1 reporter construct.

CV-1 cells were co-transfected with the luciferase reporter construct for SULT2A1 and expression plasmids containing the coding sequences for ERRα, SF1 or a combination of both ERRα and SF1 (Fig. 12). The level of SF1 (0.1 μg/well) used in the transfection was optimized prior to testing and enhanced transcription of the SULT2A1 promoter approximately 4-fold over basal while ERRα (0.3μg/well) enhanced transcription 5.1-fold over basal. When SF1 and ERRα were co-transfected SULT2A1 transcription was enhanced 4.4-fold over basal, indicating that these factors likely exert their effects on SULT2A1 transcription independently—perhaps using the same *cis*-elements.

Effects of DAX1 on ERRα activation of SULT2A1 transcription

DAX1 (NR0B1) is another orphan nuclear receptor that is a known inhibitor of SF1 activity (60,61). As SF1 and ERRα utilize some of the same nuclear receptor half sites, the question arises whether DAX1 can also inhibit the effects of ERRα on the SULT2A1 promoter. CV-1 cells were co-transfected with the luciferase reporter constructs for SULT2A1 and expression plasmids containing the coding sequences for ERRα, SF1 or both in combination with the expression plasmid for DAX1 (Fig. 12). Transfection of both ERRα and SF1 enhance SULT2A1 reporter gene transcriptional activation more than 4.4-fold over basal activity. Addition of DAX1 completely ablated the ability of both ERRα and SF1 to enhance SULT2A1 transcription reducing activity to that observed with pGL3Basic vector.

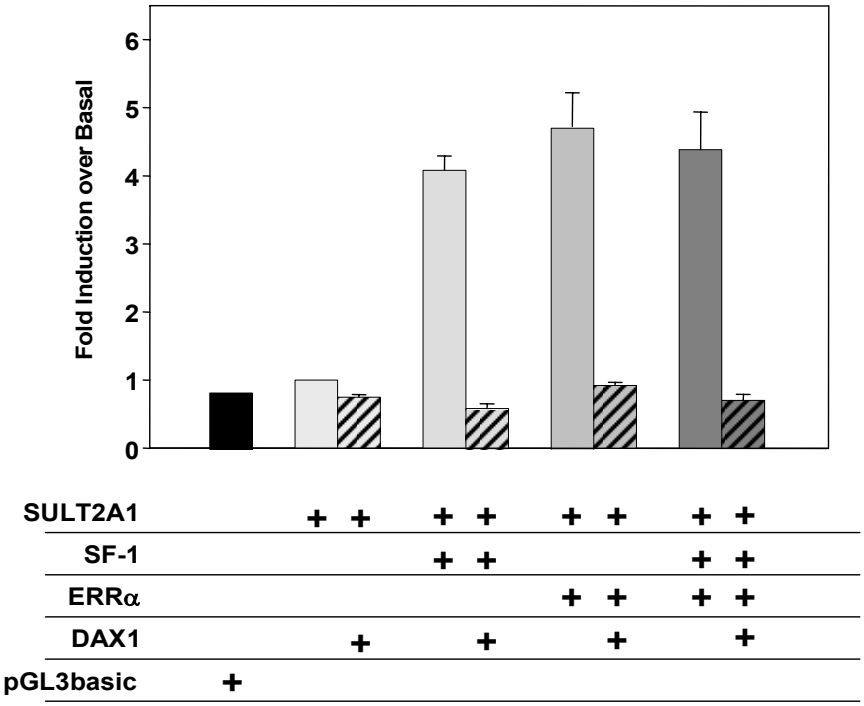


Figure 12. **Comparison of ERRα and SF1 enhanced SULT2A1 transcription and the effects of DAX1.** CV-1 cells were transfected with luciferase reporter constructs for SULT2A1 (1 µg/well). Cells were co-transfected with expression plasmids containing the coding sequences for ERRα or SF1 alone or in combination with the expression plasmid for DAX1 at concentrations of 0.1 µg/well. After 24 hours the cells were lysed and assayed for luciferase activity. Data were normalized to co-transfected β-galactosidase expression vector and results are expressed as a fold induction over the basal reporter activity. Results represent the mean ± SEM of data from at least three independent experiments each performed in triplicate.

CHAPTER IV

Conclusions and Recommendations

ERRα IS SIGNIFICANTLY EXPRESSED IN HUMAN ADRENOCORTICAL CELLS

Conclusions

ERRα is widely expressed in adult tissues, exhibits a broad range of target genes, and previous reports have shown expression in the mouse adrenal gland (13). Herein we demonstrated that ERRα is also present in the human adrenal gland. Both real-time RT-PCR and western blot analysis confirm the presence of ERRα in the human adult adrenal. Furthermore, immunohistochemical staining localizes ERRα to the nuclei of the adrenocortical cells across all three cortical zones with the highest expression being in the zona reticularis.

Recommendations

According to the real-time RT-PCR data for all tissues analyzed, ERRα is also significantly expressed in the fetal adrenal and placenta. Future studies are needed to investigate the role of ERRα in these developmental tissues. It is known that DHEAS plasma levels are relatively high during fetal life. Thus, with the data presented here it is a possibility that the ERRα expressed in the fetal adrenal might play a role in the fetal production of DHEAS. However, in the placenta the role of ERRα is currently not clear. Previous studies have shown that ERRα, ERRβ, and ERRγ are all significantly expressed in the placental tissue during fetal development. ERRβ is thought to play an important role in early placentation (59). However, the roles of ERRα and ERRγ in the placenta remain unknown.

ERRα IS A REGULATOR OF SULT2A1 GENE TRANSCRIPTION

Conclusions

Transfection analyses indicate that steroid sulfotransferase, encoded by the SULT2A1 gene, was the steroid metabolizing gene most responsive to co-transfection with ERRα, while CYP17 also showed a moderate response. Analysis of the SULT2A1 5'-flanking region revealed six possible nuclear receptor half-sites for ERRα action (-1191, -895, -499, -302, -85, -65 sites). However, deletion analysis of the flanking region indicated that when interacting with the ERRα construct, the -85 and -65 sites played significant roles in the activation of the SULT2A1 gene transcription. Use of the VP16ERRα chimera further indicated that the -1191 site on the SULT2A1 promoter was significantly involved in gene transcription. Mutational manipulation of the individual *cis*-elements confirmed that maximal ERRα enhancement of SULT2A1 reporter gene activity relied on all three sites but particularly the -85 and -65 sites. Further investigation via EMSA confirmed a direct interaction of the ERRα protein with the -85 and -65 sites *in vitro*.

Recommendations

Although the -85 and -65 sites appear to play the major role in the enhancement of SULT2A1 reporter gene activity, additional studies are needed to better characterize the role of the -1191 site. There is also no known SF1 activity at the -1191 site, therefore it may play an important role in facilitating ERRα activation of the gene promoter versus SF1 activation of the same promoter when both nuclear hormone receptors are co-expressed endogenously.

Also, it is known that plasma levels of DHEAS are relatively high during fetal life, yet levels decrease significantly during early childhood prior to climbing again in early adulthood. Our data indicates that ERRα is significantly expressed in both fetal and adult adrenal tissues during the peak times of adrenal androgen production. However, no studies have been done to determine ERRα levels in the adrenal glands after birth and during childhood when DHEAS levels are substantially lower.

Also, CYP17 is a required enzyme in the pathway of DHEAS production in the adrenal cortex. Our data indicates that ERRα significantly enhances transcription of the CYP17 reporter gene construct. Therefore, future studies are needed to investigate the possible role of ERRα in the regulation of CYP17 enzyme expression.

ERRα AND SF1 REGULATE SULT2A1 GENE EXPRESSION AT THE SAME NUCLEAR RECEPTOR EXTENDED HALF-SITES

Conclusion

Since most of the ERRα-related enhancement of SULT2A1 gene activity could be localized to the -85 and -65 sites of the gene promoter, and SF1 has previously been shown to act at the same sites, the question arises as to whether SF1 and ERRα act collectively at these sites or in competition with each other. Optimized levels of SF1 (0.1 µg/well) enhanced transcription of the SULT2A1 promoter approximately 4-fold over basal while optimized levels of ERRα (0.3µg/well) enhanced transcription 5.1-fold over basal. When SF1 and ERRα were co-transfected SULT2A1 transcription was enhanced 4.4-fold over basal, indicating that these factors likely exert their effects on SULT2A1 transcription independently—perhaps using the same -85 and -65 *cis*-elements in a competitive fashion.

Recommendations

Future studies that focus on the direct or indirect interaction between ERRα and SF1 in the regulation of SULT2A1 gene transcription are needed. From our results it appears that the two nuclear hormone receptors compete for the same *cis*-elements on the SULT2A1 gene promoter, yet the mechanism of competition is not well understood. Determining what factors favor binding of ERRα to the *cis*-elements versus binding of SF1 to the same sites will provide new insight not only into the regulatory properties of ERRα, but also into those of SF1 which plays such a vital role in multiple steroidogenic enzyme production pathways.

DAX1 IS A POTENT INHIBITOR OF ERRα ACTIVATION OF THE SULT2A1 GENE TRANSCRIPTION

Conclusion

The addition of DAX1 to transfection experiments in CV-1 cell models completely ablated the ability of both ERRα and SF1 to enhance SULT2A1 transcription reducing activity to that observed with pGL3Basic vector. Thus we conclude that both SF1 and ERRα are inhibited by DAX-1, a nuclear hormone receptor previously shown to interact with SF1 and block its transcriptional activity through the recruitment of co-repressors (60).

Recommendations

The use of the same *cis*-elements by ERRα and SF1 in the regulation of SULT2A1 gene transcription suggests a parallel purpose for the two nuclear hormone receptors. Also, the fact that DAX1 represses transcriptional activity of both ERRα and SF1 suggests there may be significant overlap in the mechanisms regulating the activity of SF1 and ERRα. However, our results (Fig. 7) demonstrate that the effects of ERRα in the steroidogenic pathway are not the same as is seen for SF1 suggesting that the role of these two transcription factors in the adrenal is distinct (3,50). Thus, future studies are needed to better understand the similarities and differences in ERRα and SF1 regulation of the SULT2A1 gene transcription and why two similarly regulated nuclear hormone receptors were developed to regulate the production of steroid sulfotransferase SULT2A1 in the human adrenal.

ACKNOWLEDGEMENTS

William E. Rainey PhD for his incredible mentorship not only as a scientist, but also as a husband, father, and friend.

Bruce Carr MD for his consistent guidance and for serving on my Distinction in Research committee.

Lisa Halvorson MD for serving on my Distinction in Research committee.

Karla Saner-Amigh PhD for her major role in the publication of this work and for her patience and kindness.

Bobby Mayhew for her daily guidance, training, and troubleshooting in every aspect of this work.

Mary Bassett PhD for sharing her expertise in multiple protocols and for her eagerness to help in any way.

Vincent Giguere PhD for his brainstorming and guidance and for generously providing the Estrogen-related Receptor expression vectors and antibodies.

Hiro Sasano PhD and T. Suzuki PhD for providing the excellent immunohistochemisty.

Michael McPhaul MD for his dedication to promoting medical student research and for his patience and advising.

Support for this study was provided by grants from the National Institutes of Health grants T32-HD07190 (BRC), HD11149 (WER), DK069950 (WER) and DK43140 (WER). Support was also provided by an operating grant from the Canadian Institutes for Health Research (VG) and a United States Army Breast Cancer Research Program Predoctoral Traineeship Award (JL). Special thanks also to the UT Southwestern Medical Student Research Program Fellowship (JS).

BIBLIOGRAPHY

1. **Rainey WE, Carr BR, Sasano H, Suzuki T, Mason JI** 2002 Dissecting human adrenal androgen production. Trends Endocrinol Metab 13:234-239
2. **Parker LN, Odell WD** 1980 Control of adrenal androgen secretion. Endocr Rev 1:392-410
3. **Jimenez P, Saner K, Mayhew B, Rainey WE** 2003 GATA-6 is expressed in the human adrenal and regulates transcription of genes required for adrenal androgen biosynthesis. Endocrinology 144:4285-4288
4. **Saner KJ, Suzuki T, Sasano H, Pizzey J, Ho C, Strauss JF 3rd, Carr BR, Rainey WE** 2005 Steroid sulfotransferase 2A1 gene transcription is regulated by steroidogenic factor 1 and GATA-6 in the human adrenal. Mol Endocrinol. Jan;19(1):184-97.
5. **Carr BR, Simpson ER** 1981 Lipoprotein utilization and cholesterol synthesis by the human fetal adrenal gland. Endocr Rev 2:306-326
6. **Ryan KJ** 1959 Biological aromatization of steroids. J Biol Chem 234:268
7. **Zbella EA, Ilekis J, Scommegna A, Benveniste R** 1986 Competitive studies with dehydroepiandrosterone sulfate and 16 alpha-hydroxydehydroepiandrosterone sulfate in cultured human choriocarcinoma JEG-3 cells: effect on estrone, 17 beta-estradiol, and estriol secretion. Journal of Clinical Endocrinology Metabolism 63:751-757
8. **Sasano H, Sato F, Shizawa S, Nagura H, Coughtrie MW** 1995 Immunolocalization of dehydroepiandrosterone sulfotransferase in normal and pathologic human adrenal gland. Mod Pathol 8:891-896
9. **Kennerson AR, McDonald DA, Adams JB** 1983 Dehydroepiandrosterone sulfotransferase localization in human adrenal glands: a light and electron microscopic study. Journal of Clinical Endocrinology Metabolism 56:786-790
10. **Wang W, Yang L, Suwa T, Casson PR, Hornsby PJ** 2001 Differentially expressed genes in zona reticularis cells of the human adrenal cortex. Mol Cell Endocrinol 173:127-134
11. **Rainey WE, Saner K, Schimmer BP** 2004 Adrenocortical cell lines. Mol Cell Endocrinol. Dec 30;228(1-2):23-38.
12. **Desvergne B, Michalik L, Wahli W** 2006 Transcriptional regulation of metabolism. Physiol Rev. Apr;86(2):465-514
13. **Giguere V, Yang N, Segui P, Evans RM** 1988 Identification of a new class of steroid hormone receptors. Nature 331:91-94
14. **Hong H, Yang L, Stallcup MR** 1999 Hormone-independent transcriptional activation and coactivator binding by novel orphan nuclear receptor ERR3. J Biol Chem 274:22618-22626
15. **Tremblay GB, Bergeron D, Giguere V** 2001 4-Hydroxytamoxifen is an isoform-specific inhibitor of orphan estrogen-receptor-related (ERR) nuclear receptors beta and gamma. Endocrinology 142:4572-4575
16. **Tremblay GB, Kunath T, Bergeron D, Lapointe L, Champigny C, Bader JA, Rossant J, Giguere V** 2001 Diethylstilbestrol regulates trophoblast stem cell differentiation as a ligand of orphan nuclear receptor ERR beta. Genes Dev 15:833-838
17. **Coward P, Lee D, Hull MV, Lehmann JM** 2001 4-Hydroxytamoxifen binds to and deactivates the estrogen-related receptor gamma. Proc Natl Acad Sci U S A 98:8880-8884
18. **Willy PJ, Murray IR, Qian J, Busch BB, Stevens WC, Jr., Martin R, Mohan R, Zhou S, Ordentlich P, Wei P, Sapp DW, Horlick RA, Heyman RA, Schulman IG** 2004 Regulation of PPARgamma coactivator 1alpha (PGC-1alpha) signaling by an estrogen-related receptor alpha (ERRalpha) ligand. Proc Natl Acad Sci U S A 101:8912-8917
19. **Lu D, Kiriyama Y, Lee KY, Giguere V** 2001 Transcriptional regulation of the estrogen-inducible pS2 breast cancer marker gene by the ERR family of orphan nuclear receptors. Cancer Res 61:6755-6761
20. **Yang C, Chen S** 1999 Two organochlorine pesticides, toxaphene and chlordane, are antagonists for estrogen-related receptor alpha-1 orphan receptor. Cancer Res 59:4519-4524

21. **Kallen J, Schlaeppi JM, Bitsch F, Filipuzzi I, Schilb A, Riou V, Graham A, Strauss A, Geiser M, Fournier B** 2004 Evidence for ligand-independent transcriptional activation of the human estrogen-related receptor alpha (ERRalpha): crystal structure of ERRalpha ligand binding domain in complex with peroxisome proliferator-activated receptor coactivator-1alpha. J Biol Chem. Nov 19;279(47):49330-7
22. **Greschik H, Wurtz JM, Sanglier S, Bourguet W, van Dorsselaer A, Moras D, Renaud JP** 2002 Structural and functional evidence for ligand-independent transcriptional activation by the estrogen-related receptor 3. Mol Cell 9:303-313
23. **Huppunen J, Aarnisalo P** 2004 Dimerization modulates the activity of the orphan nuclear receptor ERRgamma. Biochem Biophys Res Commun. Feb 20;314(4):964-70
24. **Yang N, Shigeta H, Shi H, Teng CT** 1996 Estrogen-related receptor, herr1, modulates estrogen receptor-mediated response of human lactoferrin gene promoter. J Biological Chem 271:5795-5804
25. **Sladek R, Bader JA, Giguere V** 1997 The orphan nuclear receptor estrogen-related receptor alpha is a transcriptional regulator of the human medium-chain acyl coenzyme A dehydrogenase gene. Mol Cell Biol 17:5400-5409
26. **Bonnelye E, Vanacker JM, Dittmar T, Begue A, Desbiens X, Denhardt DT, Aubin JE, Laudet V, Fournier B** 1997 The ERR-1 orphan receptor is a transcriptional activator expressed during bone development. Mol Endocrinol 11:905-916
27. **Bonnelye E, Vanacker JM, Spruyt N, Alric S, Fournier B, Desbiens X, Laudet V** 1997 Expression of the estrogen-related receptor 1 (ERR-1) orphan receptor during mouse development. Mech Dev 65:71-85
28. **Shi H, Shigeta H, Yang N, Fu K, O'Brian G, Teng CT** 1997 Human estrogen receptor-like 1 (ESRL1) gene: genomic organization, chromosomal localization, and promoter characterization. Genomics 44:52-60
29. **Shigeta H, Zuo W, Yang N, DiAugustine R, Teng CT** 1997 The mouse estrogen receptor-related orphan receptor alpha 1: molecular cloning and estrogen responsiveness. J Mol Endocrinol 19:299-309
30. **Luo J, Sladek R, Carrier J, Bader JA, Richard D, Giguere V** 2003 Reduced fat mass in mice lacking orphan nuclear receptor estrogen-related receptor alpha. *Mol Cell Biol. Nov;23(22): 7947-56*
31. **Vanacker JM, Bonnelye E, Delmarre C, Laudet V** 1998 Activation of the thyroid hormone receptor alpha gene promoter by the orphan nuclear receptor ERR alpha. Oncogene 17:2429-2435
32. **Bonnelye E, Merdad L, Kung V, Aubin JE** 2001 The orphan nuclear estrogen receptor-related receptor alpha (ERRalpha) is expressed throughout osteoblast differentiation and regulates bone formation in vitro. J Cell Biol. May 28;153(5):971-84
33. **Ariazi EA, Clark GM, Mertz JE** 2002 Estrogen-related receptor alpha and estrogen-related receptor gamma associate with unfavorable and favorable biomarkers, respectively, in human breast cancer. Cancer Res. Nov 15;62(22):6510-8
34. **Liu D, Zhang Z, Gladwell W, Teng CT** 2003 Estrogen stimulates estrogen-related receptor alpha gene expression through conserved hormone response elements. Endocrinology. Nov;144(11):4894-904
35. **Yang C, Zhou D, Chen S** 1998 Modulation of aromatase expression in the breast tissue by ERR alpha-1 orphan receptor. Cancer Res 58:5695-5700
36. **Johnston SD, Liu X, Zuo F, Eisenbraun TL, Wiley SR, Kraus RJ, Mertz JE** 1997 Estrogen-related receptor alpha 1 functionally binds as a monomer to extended half-site sequences including ones contained within estrogen-response elements. Mol Endocrinol 11:342-352
37. **Vanacker JM, Bonnelye E, Chopin-Delannoy S, Delmarre C, Cavailles V, Laudet V** 1999 Transcriptional activities of the orphan nuclear receptor ERR alpha (estrogen receptor-related receptor-alpha). Mol Endocrinol 13:764-773
38. **Trapp T, Holsboer F** 1996 Nuclear orphan receptor as a repressor of glucocorticoid receptor transcriptional activity. J Biol Chem 271:9879-9882
39. **Wiley SR, Kraus RJ, Zuo F, Murray EE, Loritz K, Mertz JE** 1993 SV40 early-to-late switch involves titration of cellular transcriptional repressors. Genes Dev 7:2206-2219
40. **Xie W, Hong H, Yang NN, Lin RJ, Simon CM, Stallcup MR, Evans RM** 1999 Constitutive activation of transcription and binding of coactivator by estrogen-related receptors 1 and 2. Mol Endocrinol 13:2151-2162

41. **Zhang Z, Teng CT** 2000 Estrogen receptor-related receptor alpha 1 interacts with coactivator and constitutively activates the estrogen response elements of the human lactoferrin gene. J Biol Chem 275:20837-20846
42. **Luo X, Ikeda Y, Parker KL** 1994 A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell 77:481-490
43. **Sadovsky Y, Crawford PA, Woodson KG, Polish JA, Clements MA, Tourtellotte LM, Simburger K, Milbrandt J.** 1995 Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express p450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. Proc Natl Acad Sci U S A 92:10939-10943
44. **Parker KL, Rice DA, Lala DS, Ikeda Y, Luo X, Wong M, Bakke M, Zhao L, Frigeri C, Hanley NA, Stallings N, Schimmer BP** 2002 Steroidogenic factor 1: an essential mediator of endocrine development. Recent Prog Horm Res 57:19-36
45. **Ozisik G, Achermann JC, Meeks JJ, Jameson JL** 2003 SF1 in the development of the adrenal gland and gonads. Horm Res 59 Suppl 1:94-98
46. **Lala DS, Rice DA, Parker KL** 1992 Steroidogenic factor 1, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor 1. Mol Endocrinol 6:1249-1258
47. **Luo X, Ikeda Y, Schlosser DA, Parker KL** 1995 Steroidogenic factor 1 is the essential transcript of the mouse ftz-f1 gene. Mol Endocrinol 9:1233-1239
48. **Wilson TE, Fahrner TJ, Milbrandt J** 1993 The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. Mol Cell Biol 13:5794-5804
49. **Bassett MH, Zhang Y, Clyne C, White PC, Rainey WE** 2002 Differential regulation of aldosterone synthase and 11beta-hydroxylase transcription by steroidogenic factor-1. J Mol Endocrinol 28:125-135
50. **Hanley NA, Rainey WE, Wilson DI, Ball SG, Parker KL** 2001 Expression profiles of SF-1, DAX1, and CYP17 in the human fetal adrenal gland: potential interactions in gene regulation. Mol Endocrinol 15:57-68
51. **Leers-Sucheta S, Morohashi K, Mason JI, Melner MH** 1997 Synergistic activation of the human type II 3ß-hydroxysteroid dehydrogenase/delta 5 - delta 4 isomerase promoter by the transcription factor steroidogenic factor-1/adrenal 4-binding protein and phorbol ester. J Biol Chem 272:7960-7967
52. **Sugawara T, Holt JA, Kiriakidou M, Strauss JF 3rd** 1996 Steroidogenic factor 1-dependent promoter activity of the human steroidogenic acute regulatory protein (StAR) gene. Biochemistry 35:9052-9059
53. **Freije WA, Pezzi V, Arici A, Carr BR, Rainey WE** 1997 Expression of 11 beta-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) in the human fetal adrenal. J Soc Gynecol Investig 4:305-309
54. **Wang XL, Bassett M, Zhang Y, Yin S, Clyne C, White PC, Rainey WE** 2000 Transcriptional regulation of human 11beta-hydroxylase (hCYP11B1). Endocrinology 141:3587-3594
55. **Jenson FC, Girardi AJ, Gilden RV, Koprowski H** 1964 Infection of human and simian tissue cultures with rous sarcoma virus. Proc Natl Acad Sci U S A 52:53-59
56. **Saner KJ, Suzuki T, Sasano H, Pizzey J, Ho C, Strauss JF, III, Carr BR, Rainey WE** 2004 Steroid sulfotransferase (SULT2A1) gene transcription is regulated by steroidogenic factor 1 (SF1) and GATA-6 in the human adrenal. Mol Endocrinol. 2005 Jan;19(1):184-97
57. **Sasano H, Shizawa S, Suzuki T, Takayama K, Fukaya T, Morohashi K, Nagura H** 1995 Ad4BP in the human adrenal cortex and its disorders. Journal of Clinical Endocrinology Metabolism 80:2378-2380
58. **Suzuki T, Miki Y, Moriya T, Shimada N, Ishida T, Hirakawa H, Ohuchi N, Sasano H** 2004 Estrogen-related receptor alpha in human breast carcinoma as a potent prognostic factor. Cancer Res 64:4670-4676
59. **Luo J, Sladek R, Bader JA, Matthyssen A, Rossant J, Giguere V** 1997 Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERR-beta. Nature Aug 21;388(6644):778-82
60. **Crawford PA, Dorn C, Sadovsky Y, Milbrandt J** 1998 Nuclear receptor DAX-1 recruits nuclear receptor corepressor N-CoR to steroidogenic factor 1. Mol Cell Biol 18:2949-2956
61. **Lalli E, Sassone-Corsi P** 2003 DAX-1, an unusual orphan receptor at the crossroads of steroidogenic function and sexual differentiation. Mol Endocrinol 17:1445-1453

VITAE

Jeremiah (Jeremy) Brent Seely was born in Blooming Grove, Texas on May 12, 1979 as the second of three children to Howard Dean and Beverly Kay Seely. After graduating from Blooming Grove High School in 1997, he entered Angelo State University in San Angelo, Texas where he competed as an athlete on the University's Track & Field team while pursuing a pre-medical academic degree plan. During his final two years of undergraduate study he completed pilot investigations into amino acid metabolism of yeast using nuclear magnetic resonance sponsored by the University's Biochemsitry Department with additional help from the Welch Research Foundation in the form of a grant. Jeremy graduated Magna Cum Laude with a Bachelor of Science in Biochemistry in May of 2001. After marrying one of his former Track & Field teammates, Brooke McCasland, in June of 2001, Jeremy took a full-time position as a lab technician and research assistant at the University of Texas Southwestern Medical Center in Dallas in the laboratory of William E. Rainey PhD in the Reproductive Endocrinology Division. Under Dr. Rainey's guidance and continuous training under lab manager Bobby Mayhew, Jeremy became a secondary author on two lab publishings and was able to begin work on his own project studying the Estrogen-related Receptors. In August of 2002, Jeremy entered medical school at the University of Texas Southwestern Medical School and continued pursuing his studies of the Estrogen-related Receptor now focused on the role of the Estrogen-related Receptor alpha in adrenal steroidogenesis as part of the Medical Student Research Program for the next three years. In September of 2004, Jeremy and his wife Brooke welcomed their daughter Kathryn to the world. Jeremy will complete his Doctorate of Medicine with as Distinction in Research in June of 2006 and will pursue a career in family medicine.

Prior publications:

2005 "The NGFI-B family of transcription factors regulates expression of 3 beta-hydroxysteroid dehydrogenase type 2 in the human ovary" Havelock J, Smith A, Seely J, Dooley C, Rodgers R, Rainey W, Carr B. *Mol Hum Reprod.* 2005 Feb; 11(2): 79-85.

2002 "Liver receptor homologue-1 is expressed in human steroidogenic tissues and activates transcription of genes encoding steroidogenic enzymes" Sirianni R, Seely J, Attia G, Stocco D, Carr B, Pezzi V, Rainey W. *J Endocrinology* 2002 Sep; 174(3): R13-7.

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