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Differential Gene Expression in the Adrenals of Normal and Anencephalic Fetuses and Studies Focused on the Fras-1-Related Extracellular Matrix Protein (*FREM2*) Gene

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Abstract

Precis: Many genes are differentially expressed in normal compared to anencephalic human fetal adrenals (HFAs), especially the Fras-1-related extracellular matrix protein (*FREM2*) gene. *FREM2* expression appears to be regulated by adrenocorticotrophic hormone (ACTH). **Context:** The expression profiles of genes responsible for cortical growth and zonation in the HFA gland are poorly characterized. The neural tube disorder anencephaly is associated with fetal adrenal hypoplasia with a large size reduction of the fetal zone of the HFA. **Objective:** To determine gene expression profile differences in the adrenals of anencephalic compared to normal HFAs to identify genes that may play important roles in adrenal development. **Design and Methods:** Fresh tissues were obtained at the time of autopsy from normal and anencephalic human fetuses delivered at mid-gestation. The following techniques were used: cell culture, messenger RNA (mRNA) extraction, microarray analysis, complementary DNA (cDNA) synthesis, quantitative real-time reverse transcriptase polymerase chain reaction (QT-PCR). **Results:** We identified over 40 genes expressed at levels 4-fold or greater in the normal versus anencephalic HFAs and that 28 genes were expressed at increased levels in the anencephalic HFA. The expression of *FREM2* at approximately 40-fold greater levels in the normal HFA compared to the HFA of anencephalic fetuses was confirmed by QT-PCR. Expression of *FREM2* in the kidney was not significantly different between normal and anencephalic fetuses. In cultured HFA cells, ACTH treatment for 48 hours increased the expression of *FREM2* and a gene responsive to ACTH, CYP17, but not tyrosine hydroxylase. **Conclusions:** Abnormal expression of many genes may be involved in the adrenal hypoplasia seen in anencephaly. *FREM2* appears to be regulated by ACTH and is the most differentially expressed gene, which may be important in the development and function of the HFA, particularly the fetal zone of the HFA.

Keywords

fetal adrenal gland, anencephaly, FREM2, Fras-1-related extracellular matrix protein, ACTH

Introduction

Defects in the neural tube such as anencephaly are severe congenital anomalies that occur in approximately 1 out of every 1000 live births in the United States.[1] In such pregnancies, there is markedly reduced production of estrogen in the placenta, due primarily to severe impairments in production of the major estrogen precursor, dehydroepiandrosterone sulfate, in the adrenals of the anencephalic fetus.[2-4] The adrenals of anencephalic fetuses are typically much smaller than normal, in association with a marked reduction in the mass of the fetal zone of the adrenal cortex but apparently normal development of the adrenal medulla.[5,6] In preliminary studies,[7] we found evidence for reduced volume of the adrenocortical cells in anencephalic adrenals, but no reduction in the proportion

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of adrenocortical cells expressing a marker of cell replication (proliferating cell nuclear antigen [PCNA]) compared to that of normal adrenals, which would be consistent with adrenocortical hypoplasia in anencephaly. Therefore, despite the obvious importance of cell proliferation in the process of adrenal morphogenesis,[8] the effects of anencephaly on adrenal development may, in part, involve a defect in adrenocortical hypertrophy.

The factors responsible for orderly development of the adrenal gland are only partially understood and the expression profiles of genes that are responsible for cortical growth and zonation in the human fetal adrenal (HFA) are not known. We reasoned that by comparing gene expression profile differences between normal and anencephalic adrenal glands, we could identify genes that heretofore have not been considered important in adrenal morphogenesis.

Materials and Methods

Human Tissue Preparation

Portions of HFA gland, liver, and kidney were obtained at the time of autopsy of fetuses at 19 to 21 weeks' gestational age. In addition, fetal adrenal gland, liver, and kidney were obtained from Advanced Biosciences Resources Inc. (Almeda, California) in accordance with the Donors Anatomical Gift Act of the State of Texas. In total, 6 normal adrenal samples and 5 anencephalic adrenal samples were obtained for use in this study. The use of these tissues was approved by the Institutional Review Boards of the University of Alabama at Birmingham and the University of Texas Southwestern Medical Center. Portions of the tissues were immediately processed for cell culture. The remaining tissues were kept frozen at $-80^{\circ}C$ for subsequent RNA extractions.

Cell Culture

Fragments of the HFA (N = 3) were minced and dissociated into single-cell suspensions by repeated exposure of the tissue fragments to 0.4 mg/mL collagenase (Sigma Aldrich, St. Louis, Missouri) in phosphate-buffered saline (PBS) enriched with 10% bovine serum albumin (Sigma Aldrich) at 37°C for 15 to 30 minutes. After separation of the cells from the collagenase mixture by centrifugation, the cell pellets were suspended in culture medium containing McCoy's 5A medium (Gibco, Grand Island, New York) that contained 5% fetal bovine serum (Hyclone, Logan, Utah) and antibiotics/antimycotics consisting of penicillin/streptomycin, gentamycin, and amphotericin (Gibco). Cells were cultured initially for 2 to 3 days in T-75 culture flasks in a humidified atmosphere of 95% air/5%CO_2 at 37°C before use or stored by freezing at $-80^{\circ}C$ for subsequent utilization. For experiments, HFA cells were then subcultured and plated onto 24-well culture dishes at a density of 200 000 cells per well and allowed to grow for 5 days in compete growth medium consisting of Dulbecco modified Eagle's medium (DMEM)/F12 medium (Invitrogen, Carlsbad, California) containing 10% cosmic calf serum (Hyclone), 1% insulin transferrin selenium (ITS) (BD Biosciences, Bedford, Massachusetts) and antibiotics and antimycotics consisting of penicillin/streptomycin, gentamycin, and amphotericin (Gibco). Cells were cultured in the presence and absence of 10 nmol/L adrenocorticotrophic hormone ([ACTH] Organon, Bedford, Ohio) using the same medium as described above, with the exception that the serum concentration was reduced to 1% the day before treatment. The cells were then lysed for RNA isolation as described below. All experiments were repeated in triplicate.

RNA Extraction

RNA was isolated from frozen, thawed tissues using TRIzol reagent (Invitrogen) following the manufacturer's instructions. RNA was isolated from cells using the RNeasy kit (Qiagen, Valencia, California). RNA quantification was performed by measuring the optical density (OD) 260 and 260/280 ratio using a Nanodrop ND 1000 UV spectrometer (Thermo Scientific, Marietta, Ohio). RNA quality was analyzed using the Agilent 2100 Bioanalyzer (Applied Biosystems, Foster City, California).

Microarray Analysis

A mixture of equal amounts of RNA from anencephalic adrenal tissues (N = 2, 1 male and 1 female) and from normal fetal adrenal glands (N = 2, 1 male and 1 female) was prepared. Extracted RNA was submitted to the Medical College of Georgia Microarray Core Facility and was scanned at high resolution using an Affymetrix GeneChip Scanner 3000 (Santa Clara, California). Microarray analyses were performed using the Affymetrix Human Genome U133 Plus 2.0 Chip. Results between arrays were studied using the GeneSpring 6.1 software (Silicon Genetics, Redwood City, California). Briefly, the raw GeneChip files were uploaded, background-subtracted, variance stabilized, and normalized with gene chip robust multi-array averaging (GC-RMA) method. The control group was used as a baseline to calculate the intensity ratio/fold changes of the treated versus the control group. The ratio was log2-transformed before further statistical analysis. The *P* values were obtained by an unpaired *t* test assuming unequal variance.

Complementary DNA Synthesis, and Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction Analyses

Reverse transcription was performed using the high-capacity complementary DNA (cDNA) reverse transcription kit (Applied Biosystems) following the manufacturer's instructions. Quantitative polymerase chain reactions were performed in the ABI 7900HT Fast Real-Time PCR system (Applied Biosystems). Taqman gene expression assays (Applied Biosystems) were used to quantify the levels of *FREM2* and several other genes that were strikingly differentially expressed based on the microarray analyses. Quantitative normalization of cDNA in each sample was performed using expression of 18S ribosomal RNA (rRNA) as an internal control. The 18S quantification was also performed using a TaqMan Gene

Expression Assay (Applied Biosystems). The relative messenger RNA (mRNA) expression levels of the anencephalic samples were normalized to the normal fetal samples. Final results were expressed as the fold differences in gene expression to the normalized calibrator, calculated by the $\Delta\Delta C_T$ method as follows: n-fold $-2^{-(\Delta CT\ sample\ -\ \Delta CT\ calibrator)}$ where ΔC_T values of the sample and calibrator were determined by subtracting the average of the transcript of investigation from the average C_T value of the 18S rRNA gene for each sample. All samples were repeated in triplicate.

Data Analysis and Statistical Methods

The quantitative real-time reverse transcriptase polymerase chain reaction (QT-PCR) validation of microarray data was compared using the student *t* test to determine the overall significance between groups. Statistical significance of the differences between pooled results from triplicate cultured fetal adrenal cell experiments also was analyzed using the *t* test. Logarithmic transformation was applied to data sets that had abnormal distributions. *P* values less than .05 were considered significantly different. Data are presented as means ± standard error of the mean (SEM).

Results

Microarray Analysis of Normal and Anencephalic Fetal Adrenal Glands

To examine the potential differences in gene expression between adrenal glands from normal and anencephalic fetuses, we compared the RNA expression profiles by microarray analysis between RNA specimens (N = 2 in each group). Based on the gene list generated by statistics, further filtering was conducted by raw intensity and fold changes. To avoid differences due simply to gender, we also eliminated genes that are located on the Y-chromosome. Then, we identified the genes that were either increased or decreased by at least 4-fold in both of the normal fetal adrenal specimens compared to the anencephalicadrenals. The resulting gene list was clustered using a hierarchical clustering algorithm. Genes that were identified as being highly differentially expressed between normal and anencephalic fetal adrenal glands are summarized in Tables 1 and 2.

Gene Expression Differences Between Normal and Anencephalic Fetal Adrenal Glands

The results of the microarray analyses were validated using QT-PCR in the fetal adrenal samples utilized for the microarray as well as in additional normal and anencephalic adrenal glands as an independent sample series; RNA from 5 anencephalic adrenals and 6 normal adrenals were used for these analyses. There are clear-cut differences between the expression levels of genes commonly associated with the adrenal medulla and those associated with the cortex as a function of experimental group: Genes associated with cholesterol, lipid, and steroid metabolism were clearly increased in the normal adrenals versus the anencephalic adrenals as shown in Figure 1A. In contrast, genes involved in catecholamine and neuropeptide regulation were strikingly higher in the anencephalic adrenals than in the normal adrenals as shown in Figure 1B.

FREM2 *Gene Expression in the Fetal Adrenal and Kidney*

One of the most striking differences in expression between normal and anencephalic fetal adrenal glands was that of the *FREM2* gene, which was expressed at 50-fold greater levels in the normal adrenal as shown by our QT-PCR results in Figure 2A. To further characterize the expression of *FREM2* between normal and anencephalic fetal tissues, we also examined the expression of *FREM2* in the fetal kidney by QT-PCR as shown in Figure 2B. As expected, the expression of *FREM2* was high in the fetal kidney. However, no significant difference was found between normal and anencephalic kidney expression of *FREM2*.

Effects of ACTH Treatment on Fetal Adrenal Cell Expression of CYP17, FREM, *and* TH.

Treatment of cultured fetal adrenal cells with 10 nmol/L ACTH for 48 hours caused a significant increase (32-fold) in *CYP17* expression as measured by QT-PCR of *CYP17* mRNA (Figure 3A). This increased expression of *CYP17* confirms previous reports that ACTH is an activator of steroidogenesis and steroidogenic enzymes in cultured fetal adrenal cells.[6] Treatment of cultured adrenal cells with ACTH resulted in a 360-fold increase in *FREM2* gene expression compared to cells treated for 48 hours in experimental medium only (Figure 3B). On the other hand, ACTH treatment of fetal adrenal cells for 48 hours caused a slight, but nonsignificant increase in the expression of the neuroendocrine gene, *TH* (Figure 3C).

Discussion

Genes involved in the development of the HFA gland have not been well characterized to date. By comparing the gene expression differences between normal HFA development with HFA that undergo abnormal development (anencephalics), we have established several genes that are likely to play a critical role in growth and zonation in the HFA. Several genes were noted to be upregulated in anencephalic fetuses as compared to normal fetuses including genes involved in catecholamine and neuropeptide production. Previously, it has been shown that the adrenal medulla in anencephalic fetuses appears to be histologically advanced as compared to age-matched controls.[9] Our study supports this observation in that various neuropeptide and catecholamine modulating genes in the adrenal medulla appear to be upregulated in anencephalic fetal adrenal glands. These genes include tyrosine hydroxylase, proenkephalin, secretogranin II, and adrenomedullin.[10-16] The differential expression of H3 histone, family 3B (H3F3B) found in our

Table 1. Microarray Results for Genes Expressed at Higher Levels in Normal Human Fetal Adrenals Than in Anencephalic Adrenals

Gene	Chromosomal Location	Average N/AN	Gene Symbol
Fras-1-related extracellular matrix protein 2	chr13q13.3	43.0292	*FREM2*
Phosphoserine phosphatase	chr7p15.2-p15.1	24.46784	*PSPH*
Tetraspanin 12	chr7q31.31	22.37974	*TSPAN12*
Ankyrin repeat and BTB (POZ) domain containing 2	chr11p13	16.77311	*ABTB2*
Hypothetical protein LOC283551	chr14q22.1	12.52372	*LOC283551*
potassium channel, subfamily K, member 5	chr6p21	11.91095	*KCNK5*
Elastase 2A	chr1p36.21	9.734687	*ELA2A*
Phospholipase D family, member 5	chr1q43	8.912774	*PLD5*
Hypothetical LOC440731	chr1q42.2	8.878863	*LOC440731*
Amidohydrolase domain containing 1	chr12q23.1	8.307319	*AMDHD1*
Ectonucleotide pyrophosphatase/phosphodiesterase 3	chr6q22	7.571086	*ENPP3*
UV radiation resistance associated gene	chr11q13.5	7.566602	*UVRAG*
Cytochrome P450, family 21, subfamily A, polypeptide 2	chr6p21.3	7.533817	*CYP21A2*
Tumor necrosis factor (ligand) superfamily, member 13b	chr13q32-34	7.482117	*TNFSF13B*
Solute carrier family 6 (neurotransmitter transporter, taurine), member 6	chr3p25-p24	7.456271	*SLC6A6*
Urotensin 2	chr1p36	7.355153	*UTS2*
Chymotrypsinogen B1, chymotrypsinogen B2, similar to - Chymotrypsinogen B precursor	chr16q23-q24.1, chr16q23.1	7.138787	*CTRB1, CTRB2, LOC647971*
Arylacetamide deacetylase (esterase)	chr3q21.3-q25.2	7.073137	*AADAC*
Chromosome 18 open reading frame 17	chr18q11.2	7.054895	*C18orf17*
Acyl-CoA synthetase short-chain family member 2	chr20q11.22	6.645874	*ACSS2*
ATP-binding cassette, subfamily B (MDR/TAP), member 1	chr7q21.1	6.29569	*ABCB1*
Calcium/calmodulin-dependent protein kinase II inhibitor 2	chr3q27.1	6.224097	*CAMK2N2*
Solute carrier family 26 (sulfate transporter), member 2	chr5q31-q34	6.189978	*SLC26A2*
Lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	chr21q22.3	5.999915	*LSS*
Solute carrier family 1 (glial high-affinity glutamate transporter), member 2	chr11p13-p12	5.831966	*SLC1A2*
Thromboxane A2 receptor	chr19p13.3	5.563456	*TBXA2*
Inositol 1,4,5-triphosphate receptor, type 1	chr3p26-p25	5.511998	*ITPR1*
HRAS-like suppressor	chr3q29	5.454378	*HRASLS*
Zinc finger-like	chr19q13.41	5.386079	*LOC400713*
Arginine vasopressin-induced 1	chr10q24.2	5.300153	*AVPI1*
UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 12 (GalNAc-T12)	chr9q22.33	5.240025	*GALNT12*
ATP-binding cassette, subfamily A (ABC1), member 5	chr17q24.3	5.222057	*ABCA5*
Sterol-C4-methyl oxidase-like	chr4q32-q34	4.902113	*SC4MOL*
Insulin induced gene 1	chr7q36	4.795448	*INSIG1*
Glutathione S-transferase A3	chr6p12.1	4.748845	*GSTA3*
Squalene epoxidase	chr8q24.1	4.726801	*SQLE*
Hypothetical protein FLJ10847	chr17p11.2	4.607179	*FLJ10847*
Kalirin, RhoGEF kinase	chr3q21.1-q21.2	4.490525	*KALRN*
Nexilin (F actin binding protein)	chr1p31.1	3.600014	*NEXN*
Transmembrane protein 144	chr4q32.1	4.316513	*TMEM144*
Melanocortin 2 receptor accessory protein	chr21q22.1	4.305429	*MRAP*
Farnesyl diphosphate farnesyltransferase 1	chr8p23.1-p22	4.234349	*FDFT1*
RAS protein activator like 2	chr1q24	4.2139	*RASAL2*
Mevalonate (diphospho) decarboxylase	chr16q24.3	4.107099	*MVD*
Formiminotransferase cyclodeaminase	chr21q22.3	4.101851	*FTCD*
PNC1 protein	chr1p36.22	4.096771	*BMSC-MCP*
Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	chr5q22.3	4.052776	*KCNN2*
Niemann-Pick disease, type C1	chr18q11-q12	3.994877	*NPC1*

Abbreviations: N, normal; AN, anencephalic.

microarray comparison was not confirmed by our independent sample series due to the fact that the differences noted by QT-PCR were not statistically significant.

Many other genes appear to be upregulated in normal HFA glands as compared to those of the anencephalic fetus. Some expression differences in genes involved in steroid

Table 2. Microarray Results for Genes Expressed at Higher Levels in Anencephalic Adrenals Than in Normal Fetal Adrenals

Gene	Chromosomal Location	Average N/AN	Gene Symbol
Tyrosine hydroxylase	chr11p15.5	0.011308	*TH*
Interleukin 13 receptor, α2	chrXq13.1-q28	0.016917	*IL3RA2*
Proenkephalin	chr8q23-q24	0.019325	*PENK*
Stanniocalcin 1	chr8p21-p11.2	0.038231	*STC1*
Insulinoma-associated 1	chr20p11.2	0.046262	*INSM1*
Secretogranin II (chromogranin C)	chr2q35-q36	0.047662	*SCG2*
ADP-ribosylation factor-like 4C	chr2q37.1	0.051258	*ARL4C*
Adrenomedullin	chr11p15.4	0.056739	*ADM*
H3 histone, family 3B	chr17q25	0.063834	*H3F3B*
Dopa decarboxylase	chr7p11	0.073581	*DDC*
Solute carrier family 18, member 1	chr8p21.3	0.092123	*SLC18A1*
Solute carrier family 35, member D3	chr6q23.3	0.092245	*SLC35D3*
Coiled-coil domain containing 4	chr4p13	0.093009	*CCDC4*
Cysteine-rich, angiogenic inducer, 61	chr1p31-p22	0.094103	*CYR61*
Neuroexophilin 1	chr7p22	0.096216	*NXPH1*
Regulator of G-protein signaling 4	chr1q23.3	0.097789	*RGS4*
Dopamine β-hydroxylase	chr9q34	0.103711	*DBH*
Sparc/osteonectin, cwcv, and kazal-like domains proteoglycan (testican) 3	chr4q32.3	0.104892	*SPOCK 3*
RAB27B, member RAS oncogene family	chr18q21.2	0.111925	*RAB27B*
Chromogranin A	chr14q32	0.114851	*CHGA*
Dermatopontin	chr1q12-q23	0.128873	*DPT*
Secreted phosphoprotein 1	chr4q21-q25	0.132109	*SPP1*
Neurexin 3	chr14q31	0.132264	*NRXN3*
Collectin subfamily member 12	chr18pter-p11.3	0.134074	*COLEC12*
Activity-regulated cytoskeleton-associated protein	chr8q24.3	0.140424	*ARC*
Monooxygenase, DBH-like 1	chr6q23.1-23.3	0.1447	*MOXD1*
Insulin-like growth factor 1	chr12q22-q23	0.150605	*IGF1*
Early growth response 1	chr5q31.1	0.156053	*EGR1*

Abbreviations: N, normal;AN, anencephalic.

biosynthesis and cholesterol metabolism and trafficking were expected, given the marked reduction in steroid production by the hypoplastic adrenal glands found in anencephalics. HMG CoA synthase 1 (HMGCS1) is known to condense acetyl-CoA with acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl (HMG)-CoA in the cholesterol biosynthesis pathway and is a key rate-limiting enzyme in cholesterol biosynthesis. Increased expression of HMGCS1 in normal adrenal glands is likely necessary to provide the cholesterol substrate for steroid production as compared to anencephalic adrenal glands, which produce reduced amounts of steroids. In fact, excessive levels of circulating low-density lipoprotein cholesterol (LDL-C) have been noted in the blood of anencephalic fetuses,[5] presumably as a consequence of significantly reduced rates of uptake and utilization as steroid substrate in the hypoplastic adrenals of the anencephalic fetus. Insulin-induced gene 1 (INSIG-1) encodes an endoplasmic reticulum protein that participates in the feedback control and cholesterol homeostasis in the lipid synthesis pathway.[17,18] The increased utilization of steroid precursors such as cholesterol in the normal adrenal as compared to the anencephalic fetal adrenal also appears to be reflected in increased expression of regulatory mechanisms such as INSIG-1.

Other genes upregulated in normal as compared to anencephalic fetal adrenal glands may play a role in the structural development of the gland. The enzyme phosphoserine phosphatase catalyzes the last step in the synthesis of the amino acid L-serine, which is necessary for protein and nucleotide synthesis.[19] Phosphoserine phosphatase has been shown to play a role in neural stem cell proliferation and signaling[20] but has not been examined to date in the HFA gland. Tetraspanins are transmembrane proteins that organize into specialized signaling microdomains in the plasma membrane and may have diverse functions including participating in epithelial tissue development.[21] The possible role of tetraspanin 12 in the development of the HFA has not been explored. Solute carrier family 26 member A2 is a sulfate/chloride transporter that may play a role in fibronectin matrix assembly in the extracellular matrix.[22]

Of all the genes that demonstrated increased expression in normal as compared to anencephalic fetal adrenal glands, the differential expression of *FREM2* was most marked. Interestingly, this expression difference of *FREM2* was not found between the kidneys of normal and anencephalic fetuses, which are not known to show developmental differences. Fras-1-related extracellular matrix protein (FREM2) is an extracellular matrix protein that localizes in the lamina densa of epithelial basement membranes in a macromolecular complex with other structurally similar proteins including Fras-1 and Fras-1-related extracellular matrix protein (Frem1 or Qbrick1).[23,24]

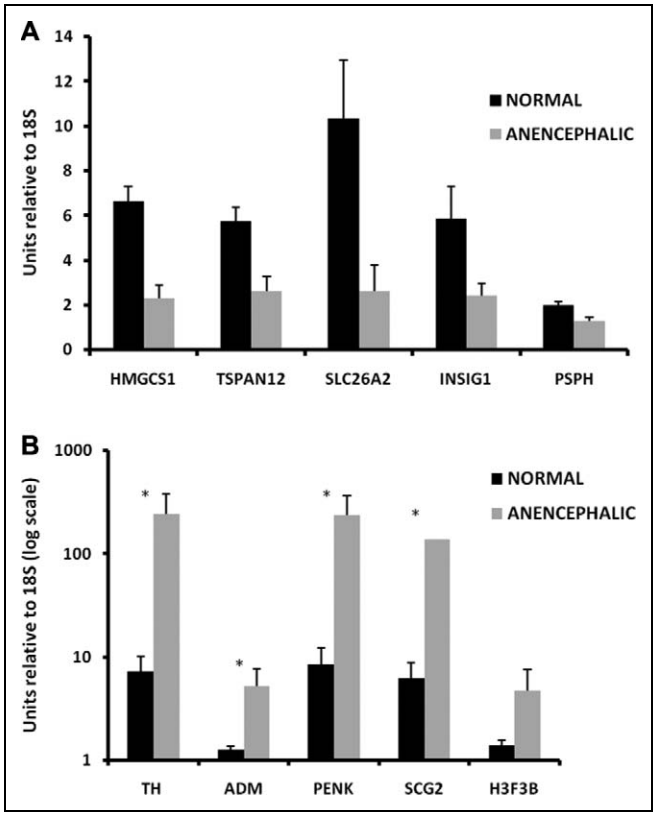

Figure 1. Relative mean expression of genes in normal (N) and anencephalic (AN) adrenals. Validation of microarray data using QT-PCR. A, Genes higher in normal adrenals. Results for the mean units relative to 18S for both normal and anencephalic specimens. $P < .05$ for all genes. B, Genes higher in anencephalic adrenals. Results for the mean units relative to 18S for both normal and anencephalic specimens. *$P < .05$. QT-PCR indicates quantitative real-time reverse transcriptase polymerase chain reaction.

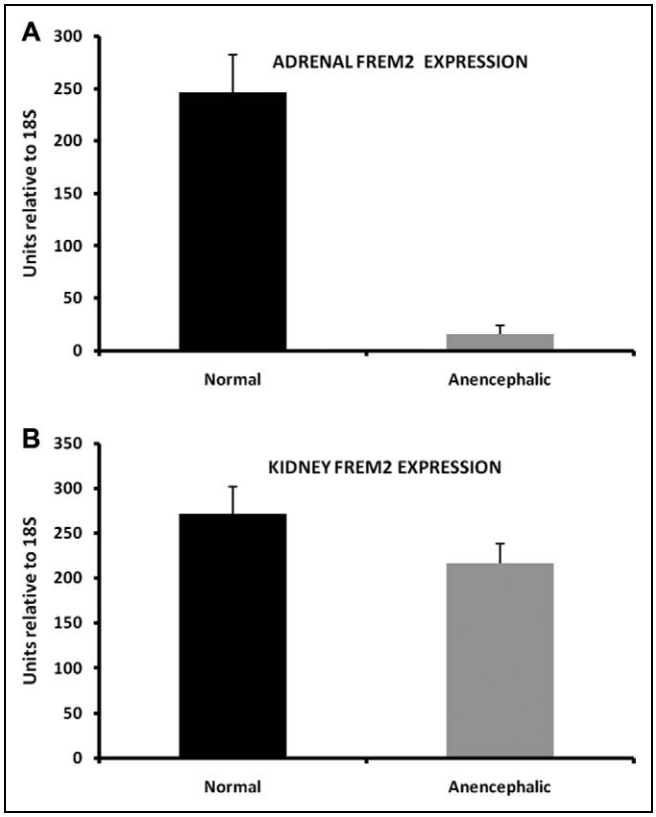

Figure 2. Relative mean expression of *FREM2* in normal and anencephalic tissues. Validation of microarray data using QT-PCR. A, Gene expression of *FREM2* in the fetal adrenal. Results for the mean units relative to 18S for 6 normal and 5 anencephalic specimens. $P < .05$. B, Gene expression of *FREM2* in the fetal kidney. Results for the mean units relative to 18S for 6 normal and 5 anencephalic specimens. $P =$ not significant. QT-PCR indicates quantitative real-time reverse transcriptase polymerase chain reaction; FREM2, Fras-1-related extracellular matrix protein.

Expression of FREM2 appears to provide a substrate for cell migration and morphogenetic rearrangements during embryogenesis.[25] In the murine model, mutation of FREM2 is associated with the myelencephalic blebs (*my*) strain that demonstrate defects including renal agenesis, exencephaly, craniofacial abnormalities, developmental hemorrhages, and degeneration of the eye.[26] FREM2 in the murine model is expressed during organogenesis in epithelial basement membranes of many structures, including the eye, epidermis, kidney, ear, gastrointestinal (GI) tract, lungs, limbs, and central nervous system (CNS)[27] but has not been examined in the development of the adrenal gland. *FREM2* is expressed in epithelial cells as well as in cells from all germ layers.[25] To date, the role of *FREM2* in development of the HFA gland has not been characterized. Global defects in human gene expression of *FREM2* are associated with the Fraser Syndrome phenotype, which is characterized by dysplastic development of various organs in which *FREM2* is highly expressed including the eye, limbs, and kidney.[28] This underlies the critical role *FREM2* may play during organogenesis, and our results are suggestive that it may apply to the development of the fetal adrenal gland as well.

In our study, we did not demonstrate an expression difference of *FREM2* between normal and anencephalic fetal kidneys. The regulatory mechanisms responsible for the expression difference may be related to the dysfunctional development of the hypothalamic–pituitary axis found in the anencephalic fetus. Fetuses affected by anencephaly demonstrate markedly reduced circulating levels of ACTH[29]; this deficiency in pituitary production of ACTH and related by-products of proopiomelanocortin may have played a role in the reduced *FREM2* expression in the anencephalic fetal adrenal gland. Using cultured fetal adrenal cells as a model, in this study we have demonstrated that ACTH treatment appears to significantly upregulate expression of *FREM2*. Adrenocorticotrophic hormone is known to upregulate genes involved in steroidogenesis[30] and has been recently demonstrated to affect expression of a variety of genes in HFA cells.[31] Studies examining primary cultures of fetal adrenal cells have shown that other components of the extracellular matrix such as collagen and fibronectin may interact with hormonal regulation pathways including ACTH. Fetal adrenal cells cultured on collagen have demonstrated cortisol production and increased

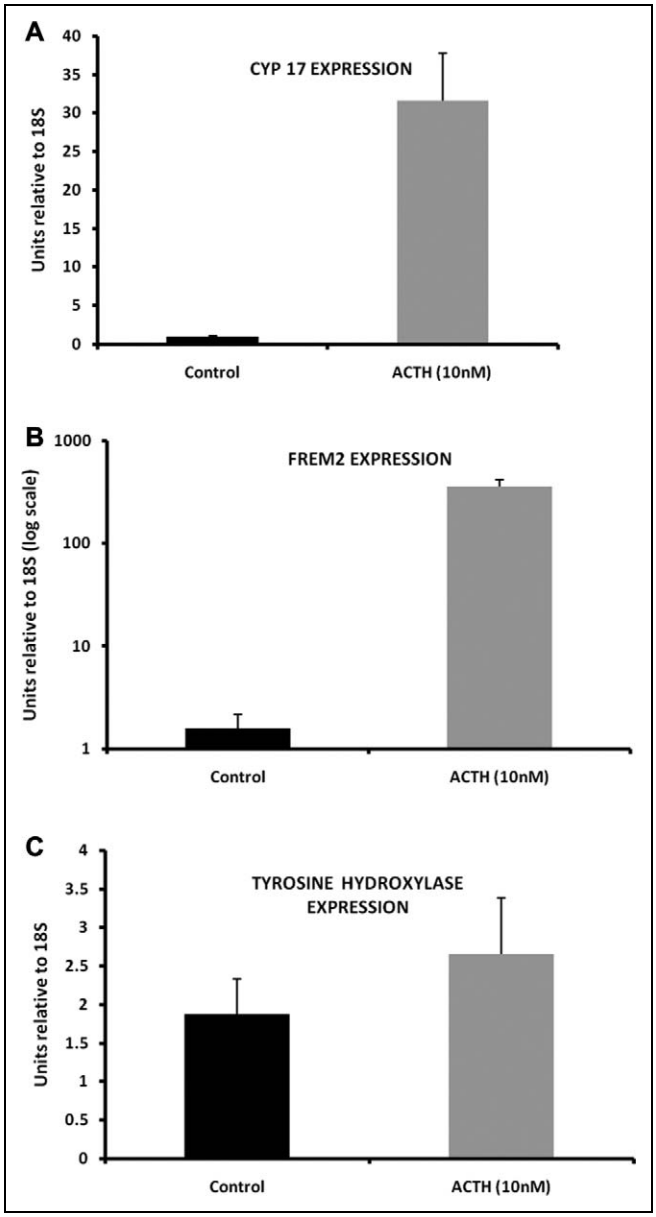

Figure 3. Relative mean expression of genes in cultured fetal adrenals cells at 48 hours with and without ACTH treatment as determined with QT-PCR. Results for the mean units relative to 18S. These data represent the mean $\pm$ the SEM of 3 independent experiments. A, Effects of ACTH treatment on *CYP17* expression. $P < .05$ versus no treatment. B, Effects of ACTH treatment on *FREM2* expression. $P < .05$ versus no treatment. C, Effects of ACTH treatment on TH expression. $P =$ not significant. QT-PCR indicates quantitative real-time reverse transcriptase polymerase chain reaction; FREM2, Fras-1-related extracellular matrix protein; ACTH, adrenocorticotrophic hormone.

expression of the rate-limiting enzyme 3 beta hydroxysteroid dehydrogenase (3β-HSD) in response to ACTH. Cultured fetal adrenal cells on fibronectin matrices have been shown to potentiate dehydroepiandrosterone sulfate (DHEAS) production in the presence of ACTH.[32] Based on expression studies in mice, the FREM2 complex appears to act as an anchoring assembly for the epithelial lamina densa and the collagen-rich extracellular matrix to adhere during embryonic development. The FREM family of proteins shares a common chondroitin sulphate proteoglycan (CSPG)-binding domain that may interact with other extracellular matrix components, including collagen, although a direct interaction has not been demonstrated to date.[23] Given that the extracellular matrix environment appears to be important in adrenal development and hormone production, the possible relationship between ACTH and *FREM2* expression warrants further investigation.

In summary, by comparing gene expression differences between normal and an abnormal (anencephalic) model of fetal adrenal gland development, we have identified a number of genes that are differentially expressed. Of those, *FREM2*, which we found to be highly expressed in the normal HFA, was expressed at extremely reduced levels in the hypoplastic adrenal of anencephalic fetuses. In addition, expression of *FREM2* in the fetal adrenal may be regulated by ACTH, the production of which is known to be limited in the developmental anomaly anencephaly.. Further studies are warranted to investigate the role in adrenal development of genes that we found to be differentially expressed in the adrenals of normal and anencephalic fetuses and particularly that of *FREM2*.

Declaration of Conflicting Interests

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