Research

Androgenic potential of human fetal adrenals at the end of the first trimester

Endocrine **CONNECTIONS**

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Abstract

The onset of steroidogenesis in human fetal adrenal glands (HFA) during the first trimester is poorly investigated. An unresolved question is the capacity of the HFA to produce potent androgen DHT via conventional and/or the backdoor pathway(s) at the end of first trimester, when androgen-responsive organs are developed. Our aim was to explore steroidogenesis and the expression of steroidogenic enzymes and transcription factors in HFA at gestational weeks (GW) 9–12 with focus on their androgenic potential. Steroids in the HFA were analyzed by gas chromatography/mass spectrometry. The expression of steroidogenic enzymes and transcription factors in the HFA at GW9–12 was investigated by qPCR, automated Western blotting and immunohistochemistry. We demonstrated that during GW9–12 HFA produced steroids of the Δ^5, Δ^4 and the backdoor pathways of the biosynthesis of DHT, though the latter was limited to production of 17α-OH-dihydroprogesterone, androsterone and androstanedione without further conversion to DHT. The only androgens identified in the HFA were testosterone and androsterone, a precursor in the biosynthesis of DHT. We also observed higher levels of CYP17A1 but low expression of 3βHSD2 at GW11–12 in the HFA. Elevated levels of CYP17A1 were associated with an increased expression of SF-1 and GATA-6. Altogether, our data demonstrate that of those steroids analyzed, the only potent androgen directly produced by the HFA at GW9–12 was testosterone. The onset of steroidogenesis in the HFA is a complex process that is regulated by the coordinated action of related transcription factors.

Introduction

Steroid hormones produced by the human fetal adrenal glands (HFA) have been proposed to regulate intrauterine homeostasis and the maturation of certain organs required for extrauterine life (1). In this case, appropriate development of and hormonal production by the HFA are critical for normal fetal maturation and survival.

The HFA develop from the intermediate mesoderm and by GW7 have acquired two distinct zones, the inner

fetal (FZ) and outer definitive zone (DZ) (2). In parallel, differentiation of the female and male external genitalia begins at GW7 and is completed by GW10, during which period, an overabundance of androgens can lead to abnormal male-directed development referred to as virilization (3). Deficient activity of cytochrome P450 21-hydroxylase (CYP21A2) is one defect in adrenocortical steroidogenesis well known to result in overproduction of androgens by the HFA in response to ACTH, a disorder referred to as congenital adrenal hyperplasia (4).

It has been reported that the HFA has the potential to produce testosterone from androstenedione by action of 17βHSD5 (5), similar to that observed in the adult adrenals, where this enzyme has been found to be expressed in zona reticularis (6). It is also known that testosterone can be further converted to 5α-dihydrotestosterone (DHT) in the target tissue such as the external genital tissue (7), and this metabolic process from cholesterol to DHT by means of testosterone is called the conventional pathway. In addition to this the frontdoor route, it has been demonstrated that 17-hydroxyprogesterone (17-OHP) can be converted to DHT in the testes of pouch young of the tammar wallaby and immature postnatal testes of rodents via an alternative 'backdoor' pathway that bypasses the conventional intermediates androstenedione and testosterone (8, 9, 10). In this pathway, progesterone and 17OH-progesterone are subsequently converted to dihydroprogesterone (DHP), allopregnanolone, 17-OH allopregnanolone, androsterone, androstanediol and DHT by the action of 5α-reductase 1 (SRD5A1), CYP17A1, the family of 3α-HSD1–4 (AKR1C1–4), 17βHSD3 and 17βHSD6 (HSD17B3, HSD17B6) (9, 11). Recent studies have reported the presence of a backdoor pathway of DHT synthesis in newborn untreated patients with 21-hydroxylase deficiency (21-OHD), suggesting that the HFA could contribute to virilization of the female fetuses with 21-OHD via production of this potent androgen (12). In line with this, the HFA have been reported to express abundantly *AKR1C1*, *AKR1C3* and *AKR1C4* (13), suggesting that synthesis of DHT via the backdoor pathway is probable but not proven in physiological conditions by the HFA. Furthermore, recent study has proposed that DHT can be produced from androstenedione either through 5α-androstanedione (i.e. androstenedione, 5α-androstanedione, DHT) or through 5α-androstanedione, androsterone and androstanediol (i.e. androstenedione, 5α-androstanedione, androsterone, androstanediol, DHT) in the human prostate (14). Given that the HFA produce androstenedione and express many steroidogenic enzymes required for this pathway

(5, 13, 15), such a novel pathway for the biosynthesis of DHT cannot be excluded.

One unresolved question in this context is the capacity of the HFA to produce potent androgen DHT via conventional and/or the backdoor pathway(s) at the end of the first trimester, when androgen-responsive organs are developed. Furthermore, due to limited access to appropriate material for research, little is presently known about the potential relationship between the expression of steroidogenic enzymes and of associated transcription factors by the HFA at early stages of the gland development.

Accordingly, in the present study, we carried out a comprehensive analysis of the profile of steroids produced by the HFA at the end of the first trimester. To extend our knowledge concerning the onset of steroidogenic activity of the HFA, we also explored expression of the corresponding steroidogenic enzymes and associated transcription factors during GW9–12. We found that at the end of the first trimester, the HFA have potential to produce testosterone and 5α-reduced precursors of DHT biosynthesis but not this androgen itself. Moreover, the ontogenic expression profiles of the various steroidogenic enzymes differ and are regulated by appropriate transcription factors.

Materials and methods

Ethical approval

These experimental procedures were approved by the Regional Ethics Committee of Stockholm (EPN dnr 2014/1022-32).

Human fetal adrenal collection

HFA were obtained from aborted fetuses in connection with elective termination of pregnancy during the first trimester (9–12 weeks of gestation) at Karolinska University Hospital, Stockholm, Sweden. Fetuses were transported to the laboratory within 30 min of delivery. Gestational age was validated by ultrasound (crown-rump length) and measuring fetal limb length to obtain more precise information about the age of the fetuses as described previously (16). Average value of each gestational week (GW) of the fetuses was as follows: $GW9.3 \pm 0.04$ ($n=9$), $GW10.3 \pm 0.06$ ($n=10$), $GW11.2 \pm 0.05$ ($n=14$) and $GW12.0 \pm 0$ ($n=5$). The fetuses were dissected under a binocular microscope in ice-cold PBS and the HFA were removed aseptically. The HFA were isolated from

individual fetuses within few minutes and immediately snap-frozen in dry ice and stored at –85°C during one–two weeks before performing analysis of steroidogenic genes expression, steroid levels assay and Western blotting or fixed overnight in neutral-buffered formaldehyde, transferred to 70% ethanol and processed for histology. Adrenals from 38 fetuses were divided into two groups (GW9–10 and GW11–12) and the sexes were pooled.

Isolation of RNA and generation of cDNA

Total RNA was extracted with the RNeasy Mini Kit (74104, Qiagen), in accordance with the manufacturer's instructions. This RNA was then pretreated with DNAse (RNase-free DNase Set, Qiagen), again as specified by the manufacturer and then quantified by spectrophotometry (BioPhotometer, Hamburg, Germany). The RNA was maintained at –80°C until further processing with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) employing the manufacturer's protocol.

Analysis of gene expression by qPCR

The samples were prepared for qPCR utilizing iQ SYBR Green Supermix (170-8882, Bio-Rad Laboratories) and after determining the optimal conditions by running a temperature gradient, cycles run at 95°C for 10s, 60–62°C for 45s, 95°C for 60s and 55°C for 60s, followed by performance of a melting curve from 55°C to 95°C in steps of 0.5°C and then maintenance at 4°C (iCycler iQ, Bio-Rad Laboratories). To compensate for possible variations in RNA concentration, all values were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, the product of a housekeeping gene. To monitor efficiency, negative control (RT-) was added to each qPCR assay. The $2^{-\Delta Ct}$ method was applied to calculate fold changes in gene expression. An overview of the primers and running conditions employed are presented in Table 1.

Automated Western blotting

All reagents for Wes-automated Western blotting were prepared and used in accordance with the manufacturer's recommendations (ProteinSimple, San Jose, CA, USA; www.proteinsimple.com/simon.html). The HFA were lysed in CelLytic cell reagent (Sigma) and the lysates diluted with sample buffer to a protein concentration of 0.3µg in 4µL were mixed together with the 5x Master Mix (DTT, fluorescence labeled marker, SDS) in a ratio 5:1 and then incubated at 95°C for 5min. The samples, the biotin-labeled protein ladder, blocking reagent, primary antibodies, HRP-conjugated secondary antibodies, chemiluminescent substrate and stacking matrices were loaded into individual wells of the sample plate. Antibodies were diluted with antibody diluent buffer. After plate loading, the separation electrophoresis and immunodetection steps took place in the capillary system and were fully automated in Wes instrument. Briefly, the capillaries first fill with separation matrix for 200s, and then stacking matrix for 15s and finally sample for 9s with vacuum injection. Separation was then performed at 375V for 25min in each capillary. After separation, the capillaries were exposed to UV light, activating the proprietary linking chemistry and locking the separated protein to the capillary wall. Subsequently, the matrix was removed and washed with washing buffer for three times. The capillaries were then blocked with antibody diluent to prevent non-specific binding, and target proteins were immunoprobed with primary antibodies, followed by HRP-conjugated secondary antibodies.

The 30-min incubations with primary antibodies against StAR, CYP17A1, GAPDH and CYP11A1 and 60-min incubations with SF-1, 3βHSD2, DAX1 and GATA6 antisera were followed by incubation with HRP-conjugated goat anti-rabbit secondary antibody for 30min. The detail information about the antibodies used is presented in Table 2.

Luminol and peroxide (ProteinSimple) were then added to generate chemiluminescence. The digital images obtained were analyzed with the Compass software (ProteinSimple). Band densities were normalized against GAPDH and the ontogenetic expression of steroidogenic enzymes and related transcription factors were expressed as percentages of the corresponding values of GW9 values. The lane views of the expression of relevant steroidogenic enzymes and system software–generated electropherograms are shown in Supplementary Fig. 1 (see section on supplementary data given at the end of this article).

GC–MS/MS analysis of steroids

Steroids from the HFA at GW9–10 ($n=5$) and GW11–12 ($n=7$) were extracted twice with diethyl ether and prepared for analysis as described earlier (17). Briefly, tissue samples (from 0.5 to 15mg) were ground in the presence of methanol/water 80:20 (v:v). The resulting

Table 1 qPCR primer sequences and running conditions, bp-base pair.

Oligo	Sequence	Prod. length (bp)	Temp (°C)
GAPDH	F: 5′-gaaggtgaaggtcggagtcaac-3′ R: 5′-cagagttaaaagcagccctggt-3′	71	55–65
CYP11A1	F: 5′-ctcagtcctggtcaaaggct-3′ R: 5′-cttctccctgtaaatcgggc-3′	250	60
CYP17A1	F: 5′-ggttcgtatgggcaccaaga-3′ R: 5′-agagttgccatttgaggccg-3′	111	60
GATA4	F: 5′-gcggaaagagggggatccaaa-3′ R: 5′-gaaggagctgctggtgtctt-3′	75	60
SF-1	F: 5′-tggtgtgaggggggtttctgc-3′ R: 5′-aagaagcccttgcagctctca-3′	249	60
GATA6	F: 5′-agaagcgcgtgccttcatc-3′ R: 5′-catagcaagtggtctgggca-3′	158	60
CEBPB	F: 5′-gcacagcgacgagtacaaga-3′ R: 5′-tgcttgaacaagttccgcag-3′	192	60
MRAP	F: 5′-agatgaggaacagccccaag-3′ R: 5′-gggtcagttcccagaggaga-3′	243	60
SULT2A1	F: 5′-tcagttccaaggccaaggtg-3′ R: 5′-tcctgtgtcctgtttcagctc-3′	248	60
AKR1C4	F: 5′-gaggaacagagctgtgtagaggtcacc-3′ R: 5′-tgaaagaaagtgcaccaaagct-3′	185	60
AKR1D1	F: 5′-cttggggaggttgagtgccat-3′ R: 5′-cgctggatgttgaaacgcaa-3′	226	60
SP1	F: 5′-cagcgtccgcgtttttcc-3′ R: 5′-tgatcttggtcgctcatggt-3′	93	60
MC2R	F: 5′-gcacagttcatgtgggatga-3′ R: 5′-cacacgaggacagtcggaat-3′	232	60
HSD17B3	F: 5′-caccggatgaaatccagagcc-3′ R: 5′-gacagcatatggggtcagcac-3′	253	60
STAR	F: 5′-tgggagctcctacagacaca-3′ R: 5′-ttccagccgagaaccgagta-3′	163	60
AKR1C3	F: 5′-aagaagtaaagctttggaggtcaca-3′ R: 5′-cgatgaaaagtggaccaaagct-3′	183	60
DAX-1	F: 5′-tgctctttaacccggacggagtg-3′ R: 5′-gcgtcatcctggtgtgttca-3′	102	60
COUP-TFII	F: 5′-tcctgttcacctcagatgcc-3′ R: 5′-ctttccgaatctcgtcggct-3′	131	60
LRH-1	F: 5′-tcctggttactgggcaacaa-3′ R: 5′-gaggttgttgagggtggctc-3′	71	60
HSD3B2	F: 5′-tctaagttacgccctcttctgg-3′ R: 5′-ttgtccaaggccctgatctc-3′	207	60
POR	F: 5′-tgccagcgtttcatgatcaac-3′ R: 5′-gagacccacgatgagcgaaa-3′	132	60
CYB5A	F: 5′-ggagctccatccagaaactct-3′ R: 5′-ttcctgcgctgacttctgag-3′	157	60
AKR1C2	F: 5′-taaaagtaaagctctagaggccgt-3′ R: 5′-cgatgggaattgctccaaagctt-3′	183	60
SRD5A1	F: 5′-tctgatgcgaggaggaaagc-3′ R: 5′-catgcccgttaaccacaagc-3′	175	60

mash was transferred in glass tubes, and the methanol layer was evaporated under N_2 stream. Then, an enzymatic deconjugation was performed for hydrolysis of glucuronide and sulfate forms by β-glucuronidase and arylsulfatase, respectively. The deconjugation was operated overnight at 37°C. All total (free+deconjugated) steroids were then extracted twice with diethyl ether. A liquid/liquid partitioning with pentane was used to separate androgens/progestogens from estrogens, and the two resulting fractions were finally submitted to a further purification through silica (SiOH) solid-phase extraction cartridges.

Detection and quantification of androgens and estrogens were performed on a Scion 436 gas chromatograph coupled to a Scion TQ triple quadrupole mass spectrometer (Bruker, Fremont, CA, USA) as described

Table 2 List of antibodies used in the study.

Peptide/protein target	Name of antibody	Manufacturer, catalog no. or name of source	Species raised in monoclonal or polyclonal	Dilution used
SF-1	NR5A1	NovusBiologicals, NBP1-52823	Rabbit polyclonal	1:50
DAX1	DAX1/NR0B1	NovusBiologicals, NBP1-32832	Rabbit polyclonal	1:50
CYP17A1	Anti-cytochrome P450 17A1 antibody (EPR6293)	Abcam, ab125022	Rabbit monoclonal	1:50
CYP11A1	Anti-CYP11A1 antibody	Abcam, ab175408	Rabbit polyclonal	1:300
GATA-6	GATA-6 (H-92)	Santa Cruz, sc-9055	Rabbit polyclonal	1:25
3β-HSD	3β-HSD (H-143)	Santa Cruz, sc-28206	Rabbit polyclonal	1:25
StAR	StAR (FL-285)	Santa Cruz, sc-25806	Rabbit polyclonal	1:100
GAPDH	GAPDH (FL-335)	Santa Cruz, sc-25778	Rabbit polyclonal	1:150
5αR1	5αRED1(H-105)	Santa Cruz, sc-20658	Rabbit polyclonal	1:100
AKR1C3	Human Aldo-keto Reductase 1C3/AKR1C3	R&D Systems, MAB7678	Mouse monoclonal	1:100
5βR1	AKR1D1	Thermo Fisher Scientific, PA5-28963	Rabbit polyclonal	1:100
AKR1C1/AKR1C2	Anti-AKR1C1/AKR1C2 antibody –C-terminal	Abcam, ab170613	Rabbit polyclonal	1:100

previously ([17](#), [18](#)). The steroids assayed, all involved in the biosynthesis of androgens and estrogens via the Δ^4, Δ^5 and backdoor pathways for DHT synthesis were as follows: pregnenolone, progesterone, 17-hydroxypregnenolone, 17-hydroxyprogesterone, androstenedione, DHEA, 17β-testosterone, 17α-testosterone, DHT, androsterone, etiocholanolone, 17α-hydroxydihydroprogesterone, 5α-androstanedione, 17αOH-allopregnanolone, allopregnanolone, androstanedione, androstanediol, 5-androstene-3α,17β-diol, 5-androstene-3β,17α-diol, 5α-androstane-3β-17α-diol, epiandrosterone, 5-androstene-3β,17β-diol, 5α-androstane-3β-17β-diol and 17β-estradiol. The coefficients of determination (R^2) of the calibration curves performed for each batch of analysis were higher than 0.99. The inter-day repeatability was lower than 10% for a majority of substances, and globally lower than 20% (Supplementary Table 1).

Immunohistochemical analysis

Paraffin-embedded fetal adrenal tissue was cut to a thickness of 5 μm and mounted on microscope slides (P/N10143352, Superfrost Plus, Thermo Scientific) and placed at 60°C for 40 min in the oven. Tissue sections were dewaxed with xylene (P/N 02080, HistoLab, Gothenburg, Sweden) for 20 min and then rehydrated in graded ethanol (99.6, 96 and 70%). Antigen retrieval in 0.01 M citrate buffer (pH 6.0) for 20 min in a water bath was used for all slides.

Samples were incubated with 3% H_2O_2 in 96% methanol for 10 min at RT for non-specific endogenous peroxidase blocking. After washing with PBS with or without 0.01% Tween 20 (P/N P1379, Sigma Aldrich), the sections were treated with a mixture of 3% goat serum and 1% bovine serum albumin (BSA) in PBS for 60 min at RT to avoid non-specific binding. Slides were subsequently incubated with primary mouse monoclonal antibody to 17βHSD5 and rabbit polyclonal antibodies against AKR1C1/AKR1C2, 5βR1 (AKR1D1) and 5αR1 (Table 2) or unspecific IgGs (for negative control) dissolved in 3% goat serum in PBS overnight at 4°C. After washing with PBS and 0.01% Tween 20, the slides were incubated with biotinylated secondary antibody (ab64256, Abcam), and then with avidin–biotin–peroxidase complex prepared using Vectastain ABC kit (PK-6100, Vector Laboratories, Burlingame, CA, USA) for 60 min at RT. Finally, the slides were stained with DAB (SK-4105, Vector Laboratories) for 20–40 s at RT, washed twice with H_2O, counterstained with Hämalaun solution (Merck), rinsed for five min with running tap water, dehydrated with gradually increasing concentrations of ethanol, cleared with xylene and mounted with cover glass. IgG-negative sections exposed to non-immune rabbit and mouse serum in the absence of primary antibody were included in all immunohistochemistry runs and showed no positive immunostaining.

Statistical analyses

Differences between values were analyzed for statistical significance with Student's *t*-test for pairwise comparison and by the one-way analysis of variance (ANOVA) for multi comparison followed by Student–Newman–Keuls analysis or Dunn's analysis if the normality test failed

using the SigmaStat (v 11.00) package (SPSS). $P < 0.05$ was considered to be statistically significant.

Results

The level of steroids produced by HFA at the end of first trimester

We observed that the HFA at the end of the first trimester produced substantial amount of steroids of Δ^5 and Δ^4 pathways and several 5α-and 5β-reduced metabolites of DHEA and androstenedione (e.g. androstanedione, epiandrosterone, etiocholanolone) as well as some precursors of DHT biosynthesis via the backdoor pathway (e.g. 17OH-DHP and androsterone), but DHT itself was not detected in the HFA at GW9–12 (Fig. 1). The only androgens identified in the HFA were testosterone, which was found in the form of 17α- and 17β-testosterone and androsterone, a precursor in the biosynthesis of DHT. We also found significant attenuation of the production of reduced steroids, androstanedione and etiocholanolone (by 78 and 92%, $P < 0.001$, $P < 0.01$, respectively) by the HFA at GW11–12 compared to GW9–10 (Fig. 1).

Ontogeny of steroidogenic enzyme expression in the HFA at the end of the first trimester

Upon exploring the ontogeny of the expression of steroidogenic enzymes and of the ACTH receptor (MC2R) in the HFA at the end of the first trimester as an indication of steroidogenic potential, we found higher level of MC2R

mRNA at GW11–12 then at GW9–10 (by 2.9-fold, $P < 0.05$), but the expression of its accessory protein MRAP was similar at both ages (Fig. 2A), indicating ongoing differentiation. Similarly, the levels of mRNA encoding steroidogenic genes StAR, CYP11A1 and SULT2A1 were significantly higher in the HFA from GW11–12 then at GW9–10 (Fig. 2A). We also observed that the HFA highly expressed CYP17A1 and genes coding P450 oxidoreductase (POR) and microsomal cytochrome b_5 (CYB5A) (Fig. 2B), the accessory proteins that support the catalytic activity of CYP17A1.

At the protein level, expression of CYP17A1 was upregulated at GW11–12 compared to GW9–10, while the level of 3βHSD2 protein peaked at GW10 and was later reduced at GW11 (Fig. 3A). Immunohistochemical analysis of cross sections of the HFA revealed expression of several androgen-metabolizing enzymes such as 5αRI, 5βRI, AKR1C1/AKR1C2 and AKR1C3 (Fig. 3B). This observation agrees well with our finding of several steroidogenic products (e.g. 17OHDHP, androsterone and etiocholanolone) as a result of the activities of these enzymes.

The observed differences in the temporal profiles of the expression of steroidogenic enzymes by the HFA suggest a link between the onset of steroidogenesis and the expression of certain transcription factors.

Expression of transcription factors by the HFA during GW9–12

To test this suggestion, we characterized the expression of transcription factors involved in both positive and negative regulation of steroidogenic gene expression.
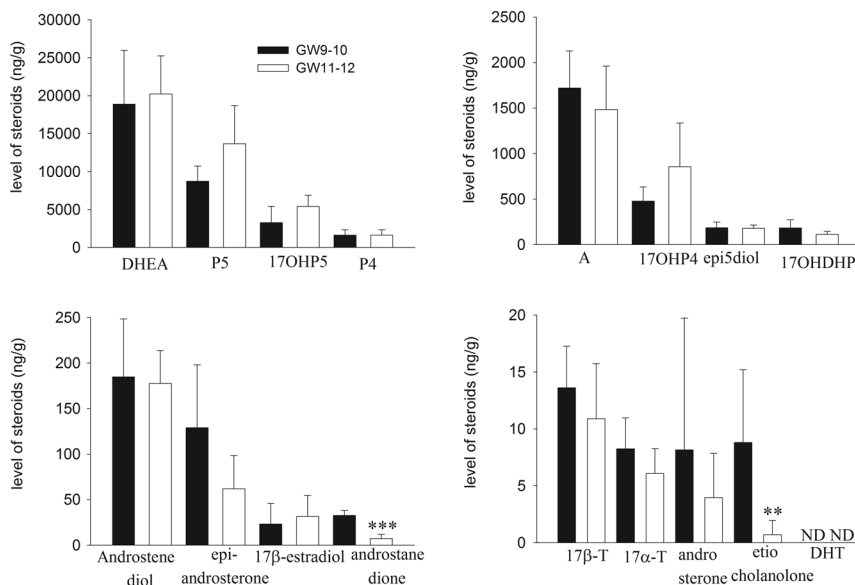

Figure 1

The tissue levels (ng/g) of steroids in the HFA at the end of the first trimester. Concentrations of pregnenolone (5P), 17-OH pregnenolone (17-OHP5), progesterone (P4), 17-OH progesterone (17-OHP4), DHEA, androstenedione (A), 17β-testosterone (17β-T), 17α-testosterone (17α-T), androstenediol, androsterone, 17α-OH-dihydroprogesterone (17OHDHP), 5-androstene-3β,17α-diol (epi5-diol), epiandrosterone, androstenedione, etiocholanolone, DHT and 17β-estradiol in the HFA were measured by GC–MS/MS as described in the 'Materials and methods' section. The values shown are means ± S.D. for five and seven HFA isolated from individual fetuses at GW9–10 and GW11–12, respectively. **$P < 0.01$, ***$P < 0.001$ compared to GW9–10. ND, not detectable.
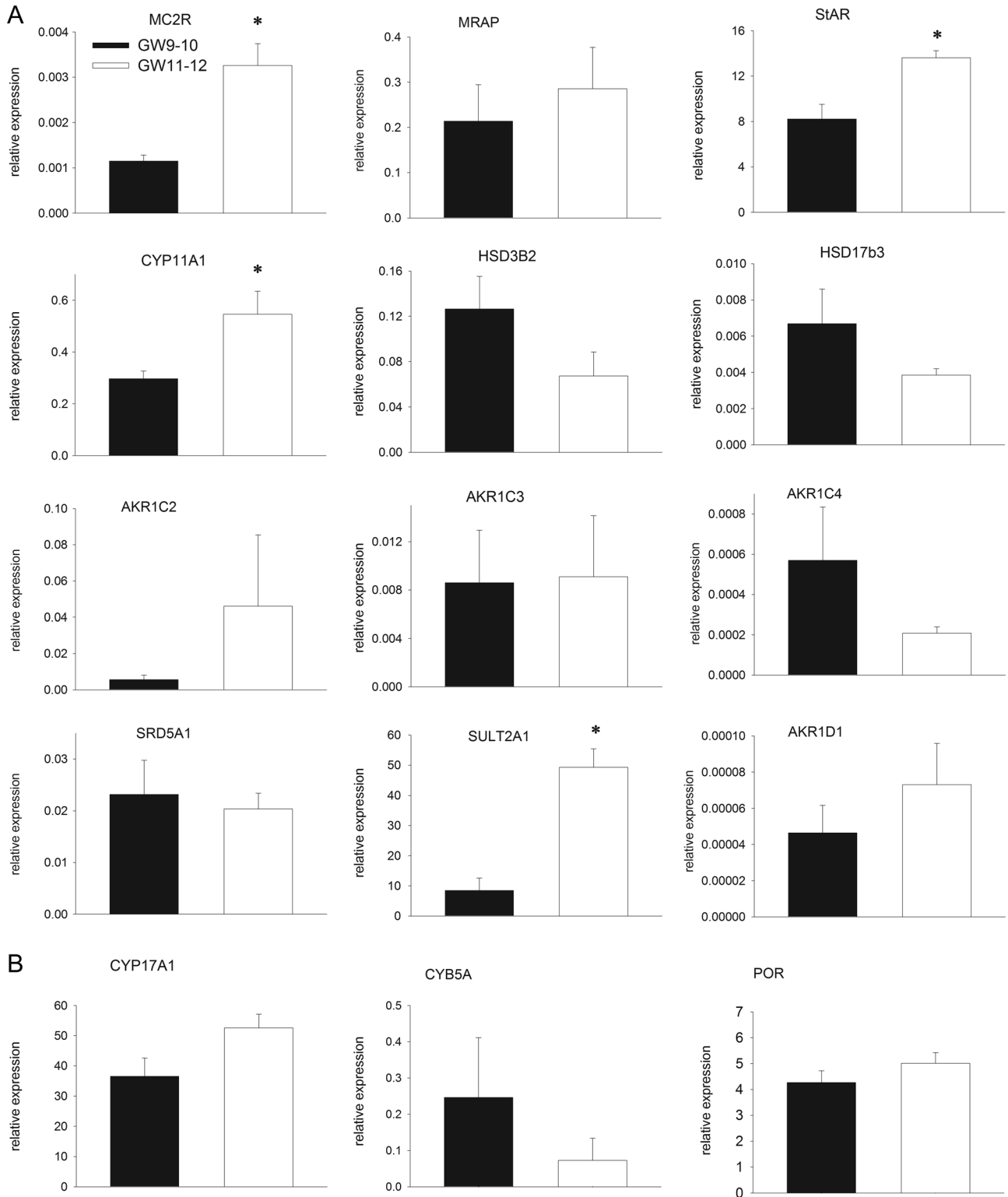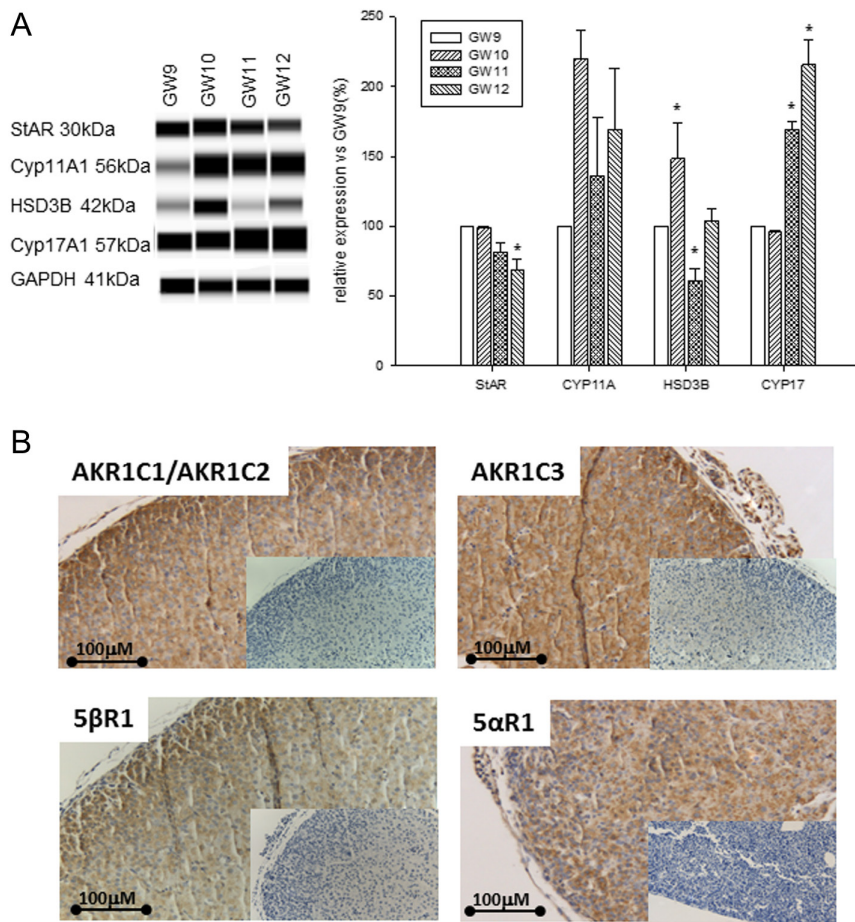
Figure 2

Levels of mRNAs encoding steroidogenic genes in the HFA during GW9–12. The mRNA levels were normalized to GAPDH as a housekeeping gene. The values presented are means±s.e. for eight and six HFA at GW9–10 and GW11–12, respectively. *$P < 0.05$ compared to GW9–10.

Figure 3

Expression of steroidogenic enzyme proteins by the HFA during GW9–12. (A). Protein levels were analyzed by Wes-automated Western blotting and representative blots are shown to the left. To the right, band densities are expressed as a percentage of the corresponding density of GW9. The values shown are means ± s.e. for three (GW9–11) and two (GW12) HFA isolated from individual fetuses. *$P < 0.05$ compared to GW9. (B) Expression of androgen-metabolizing enzymes in the HFA at GW10 identified by immunohistochemistry. Inserts show the absence of immunostaining in the presence of non-immune serum instead of primary antibody. Scale bar: 100 μm.

We observed that the level of mRNA encoding the transcription factor COUP-TF2 declined significantly in the HFA at GW11–12 and no significant differences in the levels of mRNA encoding the other transcription factors examined were detected in the HFA at GW9–12 (Fig. 4A). However, at the protein level, expression of SF-1 and GATA-6 was significantly upregulated during GW10–12 and GW10–11, while expression of DAX-1 was slightly, but significantly attenuated by GW10 (Fig. 4B).

Discussion

In the present study, we analyzed the steroidogenic machinery of the HFA during a critical phase of the first trimester, when androgen-dependent sexual dimorphism of the external genitalia is established. To our knowledge, this is the first detailed analysis of steroid production by the HFA at the end of the first trimester employing highly sensitive GC–MS/MS technique and linking this production to the levels of steroidogenic enzymes and related transcription factors. We demonstrated that

during GW9–12, HFA produced steroids of the Δ^5, Δ^4 and the backdoor pathways of the biosynthesis of DHT, though the latter was limited to production of 17α-OH-dihydroprogesterone, androsterone and androstanedione without further conversion to DHT. The only androgens identified in the HFA were testosterone and androsterone, a precursor in the biosynthesis of DHT.

Our study has demonstrated that the capacity of the HFA to synthesize testosterone was limited and only less than 1% of androstenedione was converted to the androgen. This finding agrees well with the study reported that organ culture of the HFA at GW8 can produce low levels of testosterone in unstimulated and activator-stimulated conditions (5). It should also be noted that the abundance of DHEA vs androstenedione and androstenediol compared to testosterone in the HFA is likely associated with low expression of 3βHSD2 reported previously (5, 19) and confirmed in the present study.

We also observed for the first time that HFA express 5α-reductase 1 (SRD5A1), whose activity is critical for the operation of the backdoor pathway (8, 20, 21). Similarly, detection of two forms of testosterone,
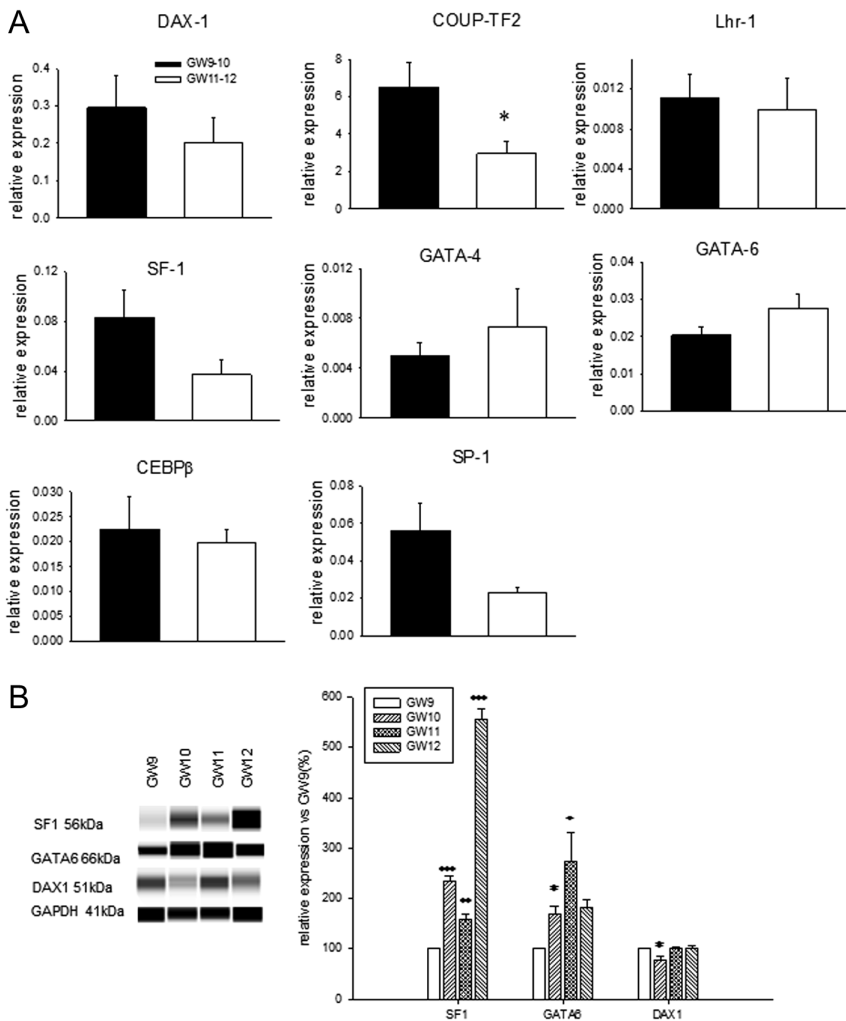
Figure 4

Levels of transcription factors involved in the regulation of steroidogenic enzyme expression in the HFA during GW9–12. (A) The mRNA and (B) protein levels were measured by qPCR and Wes-automated Western blotting, respectively. The values presented are means±s.e. for the same number of samples as indicated in Figs 2 and 3. (A) *P < 0.05 compared to GW9–10, (B) *P < 0.05, **P < 0.01, ***P < 0.001 compared to GW9.

17α- and 17β-testosterone may indicate that the activities of 17αHSD, 17βHSD3 and 17βHSD5 were present in the HFA to convert androstenedione to both forms of testosterone as reported previously (5, 13, 22). Recently, several studies have reported that 11-oxygenated metabolite of testosterone, 11-ketotestosterone (11KT) can be produced by the human adrenals (23), and it has capacity similar to testosterone to bind to the human androgenic receptor (hAR) and regulates AR-dependent gene expression (23, 24, 25, 26), suggesting that androgenic potential of HFA at the end of first trimester cannot be limited to testosterone action only.

In the present study, we observed that the HFA at GW9–12 had capacity to produce androstanedione and androsterone, the precursors in the biosynthesis of DHT (8, 9). However, further conversion of these steroids into DHT was blocked in the HFA apparently due to inability of 17βHSD3 to convert androstanedione to DHT and androsterone to androstanediol, which were not

detectable in all samples (data not shown). The observed restriction of the HFA to produce potent androgen DHT at the end of the first trimester may play an important role in protection of the developing female external genitalia from deleterious effects of androgens and to avoid their masculinization. However, evidence for the presence of the backdoor pathway in newborn untreated patients with 21-hydroxylase deficiency (21-OHD) suggests that the HFA could contribute to DHT biosynthesis in patients with 21-OHD (12).

It has been reported that androsterone can be synthesized from two steroid precursors, androstanedione and 17α-OH-allopregnanolone by reductive activity of 3αHSD and the 17–20 lyase activity of CYP17A1, respectively (8, 20). We did not detect 17α-OH-allopregnanolone in the HFA but taking into account that this steroid is the most efficient substrate for the 17–20 lyase activity (27), one can suggest that this intermediate steroid was rapidly and effectively

converted to androsterone by the action of CYP17A1 in the HFA.

Our observation that the expression of MC2R (the receptor for ACTH) in the HFA was elevated during final two weeks of the first trimester may indicate of active differentiation and maturation of the human fetal adrenocortical cells. These findings agree well with an earlier report that the human fetal adrenal cortex is responsive to ACTH and produces corticosteroids and DHEA as early as GW8 (5, 28), strongly indicating functional activity of MC2R at early stages of the HFA development.

In the present study, we found that the major steroid produced by the HFA was DHEA, which was associated with high levels of expression of CYP17A1, its accessory proteins, P450 oxidoreductase (POR) and CYB5A. In addition, low level of expression of 3βHSD2 observed in the HFA (5, 19) may also contribute to excessive production of DHEA. To our knowledge, this is the first time expression of POR and CYB5A by the HFA at the end of the first trimester has been reported. The catalytic activity of CYP17A1 requires a supply of electrons from NADPH via POR (29, 30) and its 17,20-lyase activity is enhanced significantly by microsomal CYB5A (29, 31), which supports interactions with POR allosterically (32). Accordingly, our present findings indicate that the CYP17A1-POR-CYB5 complex is fully functional already at early stages of the HFA development. CYP17A1 is expressed exclusively in the fetal zone (FZ), which produces large amounts of DHEA starting from GW7–8 (33), but the biological role of this steroid in the development and maturation of fetal organs during the first trimester is not yet known. In mice, DHEA has been reported to modulate the growth of embryonic neocortical neurons and may thereby play a crucial role in organizing the neocortex (34). Whether this steroid performs a similar function in the human fetus remains to be determined. DHEA has also been shown to be a ligand for many hepatic nuclear receptors and G-protein-coupled receptors (35).

Further, we also demonstrated the elevated expression of SULT2A1 in the HFA at GW11–12 compared to GW9–10, strongly suggesting that this enzyme plays an important role in the regulation of the availability of pregnenolone, 17OH-pregnenolone and DHEA for androgen production by the HFA (36, 37). In the human adrenals, SULT2A1 converts pregnenolone, 17α-hydroxypregnenolone, DHEA, androsterone and androstanediol to their respective sulfated products (36, 37), which are no longer available as substrates for corresponding enzymes (38).

Our current characterization of the expression of steroidogenic enzymes and related transcription factors by the HFA at the end of the first trimester revealed a partial ontogenetical relationship between the levels of transcription factors that promotes steroidogenic gene expression (e.g. SF-1 and GATA6) and of CYP11A1 and CYP17A1. It has been reported that GATA-6 can work in concert with SF-1 to maximize expression of the enzymes involved in the synthesis of adrenal androgens (39) and can control transcription of CYP17A1 both in a human adrenocortical cell line (40) and virilizing carcinomas (41) as well as is involved in regulating CYP5A transcription (42). Thus, GATA-6 appears to play several roles in connection with development, differentiation and regulation of steroidogenesis in the adrenal cortex (41). Similarly, we observed elevated expression of 3βHSD2 in association with high levels of SF-1 and GATA-6 proteins in the HFA at GW10, while the significant reduction in the level of 3βHSD2 at GW11 was not correlated with the decline in the expression of these steroidogenic factors. It was previously demonstrated that nerve growth factor IB (NGFI-B), an orphan nuclear receptor, can regulate the expression of 3βHSD2 in the HFA during the end of the first trimester (5), indicating that SF-1 and GATA-6 may not be primary transcriptional regulators of the expression of this steroidogenic enzyme.

Altogether, our current study demonstrates that the early onset of steroidogenesis in the HFA is characterized by production of steroids of the Δ^5, Δ^4 and the backdoor pathways of the biosynthesis of DHT, though the latter was limited to production of 17α-OH-dihydroprogesterone, androsterone and androstanedione without further conversion to DHT. Of those steroids analyzed in the present study, the only potent androgen directly produced by the HFA was testosterone. The similar profiles of the expression of steroidogenic cytochromes and their regulatory transcription factors (e.g. SF-1 and GATA-6) may indicate that these regulatory proteins play a key role in the onset of steroidogenesis in the HFA at a very early stage in their development.

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