Invited Review: Research Update**Regulation of human (adrenal) androgen biosynthesis – new insights from novel throughput technology studies**

Running title: Update on human androgen synthesis and regulation

Sameer S. Udhane and Christa E. Flück

Pediatric Endocrinology and Diabetology of the Department of Pediatrics and Department of Clinical Research, University of Bern, 3010 Bern, Switzerland

Address of corresponding author:

Christa E. Flück

Pediatric Endocrinology and Diabetology

University Children's Hospital Inselspital

Freiburgstrasse 15 / C845

3010 Bern

Switzerland

Phone: +41 31 6320499

Email: christa.flueck@dkf.unibe.ch

Abstract

Androgens are precursors for sex steroids and are predominantly produced in the human gonads and the adrenal cortex. They are important for intrauterine and postnatal sexual development and human reproduction. Although human androgen biosynthesis has been extensively studied in the past, exact mechanisms underlying the regulation of androgen production in health and disease remain vague. Here, the knowledge on human androgen biosynthesis and regulation is reviewed with a special focus on human adrenal androgen production and the hyperandrogenic disorder of polycystic ovary syndrome (PCOS). Since human androgen regulation is highly specific without a good animal model, most studies are performed on patients harboring inborn errors of androgen biosynthesis, on human biomaterials and human (tumor) cell models. In the past, most studies used a candidate gene approach while newer studies use high throughput technologies to identify novel regulators of androgen biosynthesis. Using genome wide association studies on cohorts of patients, novel PCOS candidate genes have been recently described. Variant 2 of the DENND1A gene was found overexpressed in PCOS theca cells and confirmed to enhance androgen production. Transcriptome profiling of dissected adrenal zones established a role for BMP4 in androgen synthesis. Similarly, transcriptome analysis of human adrenal NCI-H295 cells identified novel regulators of androgen production. Kinase p38 α (MAPK14) was found to phosphorylate CYP17 for enhanced 17,20 lyase activity and RARB and ANGPTL1 were detected in novel networks regulating androgens. The discovery of novel players for androgen biosynthesis is of clinical significance as it provides targets for diagnostic and therapeutic use.

1. Current knowledge of human androgen biosynthesis

Androgens are steroids that are able to stimulate the androgen receptor and thereby promote masculine (Greek *andros* - male) effects. In addition, androgens are the precursors of all estrogens. Testosterone is the best known human androgen, whose activity is essential during fetal development to form the complete normal male phenotype in a 46,XY fetus. On the other hand, a 46,XX female fetus will encounter virilization of the external genitalia and the brain under the influence of high androgen levels. Beyond infancy and childhood, normal androgen synthesis is needed for pubertal development, sexual life and fertility in both males and females. Thus, biosynthesis of human androgens must be tightly regulated during fetal and postnatal life. Among all vertebrates, androgen production is also regulated in a species-, sex-, organ- and mostly cell-specific manner. However, its regulation is still not fully understood. Common human disorders that are associated with deregulated androgen production include premature adrenarche, polycystic ovary syndrome (PCOS) and prostate cancer. The treatment of these hyperandrogenic states awaits improvement. However, to optimize the treatment it is crucial to understand androgen regulation in health and disease in great detail. This review aims to summarize the recent gains in knowledge of human androgen production and regulation with a special focus on human adrenal androgens and PCOS. We also allowed ourselves to include our own recent work and put it into the context of current (ongoing) research in the field.

1.1. Biochemistry of androgen biosynthesis

1.1.1. The classic pathway of testosterone synthesis

All steroids (not only androgens and estrogens, but also mineralocorticoids and glucocorticoids) are produced from a cholesterol precursor, which is transported to the inner mitochondrial membrane with the help of the steroidogenic acute regulatory protein (StAR) (Figure 1) [1]. At the inner mitochondrial membrane the first and rate limiting step of the steroid cascade is supported by the so-called side-chain cleavage system consisting of the enzyme CYP11A1 and

its cofactors ferredoxin and ferredoxin-reductase, yielding pregnenolone (Preg) as a conversion product. For testosterone production through the classic pathway, Preg is then converted in a stepwise manner to 17-hydroxypregnenolone (17OH-Preg) and dehydroepiandrosterone (DHEA) by the 17-hydroxylase and 17,20 lyase activities of CYP17 supported by cofactors P450 oxidoreductase (POR) and cytochrome b5 (CYB5). DHEA is a C19 steroid and androgen precursor, which is then either converted to androstenedione (Δ 4A) or androstenediol and finally to testosterone by the enzymes 3 β -hydroxysteroid dehydrogenase (HSD3B) and 17 β -hydroxysteroid dehydrogenase (HSD17B), which both come in different isozymes with tissue-specific expression [1]. 5 α -reductase (SRD5A) can then convert Testosterone to the most potent androgen, the dihydrotestosterone (DHT). Concerning androgen action on the androgen receptor (AR) for biological effects, DHT is the most potent androgen followed by testosterone, while Δ 4A and androstenediol exert only weak activation of the AR [2]. By contrast, DHEA does not bind to the AR, and is therefore only an androgen precursor [3].

1.1.2. The novel, alternative “backdoor” pathway

The existence of an alternative pathway to DHT synthesis was first discovered in the tammar wallaby pouch young, then in mice pups [4-6] (Figure 1). Specific steroid metabolites of this alternative pathway were then found in humans harboring steroid biosynthetic defects due to POR deficiency [7, 8], and later also in patients with congenital adrenal hyperplasia due to 21-hydroxylase (CYP21A2) deficiency [9]. Finally, we identified human genetic mutations in specific genes (aldo-keto reductase family 1, member C2/4 (AKR1C2/4)) of this novel pathway in 46,XY individuals with moderate to severe forms of disordered sex development (DSD) [10]. Although this pathway seems to play a role in human sex differentiation during fetal life, its postnatal role remains largely unknown. This pathway is currently thought to diverge from the classic pathway at the level of the 17-hydroxyprogesterone (17OH-Prog) and proceed through 17OH-dihydroprogesterone (17OH-DHP; 5 α -pregnane-17 α -ol-3,20-dione), 17OH-allopregnanolone

(17OH-Allo; 5 α -pregnane-3 α ,17 α -ol-3,20-dione), androsterone and androstenediol (3 α Diol) to DHT. The stepwise conversion of 17OH-Prog to DHT is supported by the enzymes 5 α -reductase type 1 (SRD5A1), AKR1C2/4, CYP17, HSD17B3/5 and AKR1C2/4 or HSD17B6 (Figure 1) [6, 11].

1.2. Organ specific androgen production

Androgen production is specific for species, sex, during development as well as between organs and tissues. To enable such complex specificity, enzyme activities for androgen synthesis are tightly regulated by various mechanisms including epigenetic, transcriptional, post-transcriptional and posttranslational regulation as well as substrate and cofactor availability. So far, tissue- and cell-specific androgen production seems largely regulated by specific expression of genes for steroid enzymes. However, many aspects of androgen regulation remain obscure, and little to nothing is known about the backdoor pathway of androgen synthesis.

1.2.1. Gonads (testis/ovary)

In the adult human testis, the Leydig cell produces testosterone from cholesterol through the classic pathway of androgen synthesis (Figure 1 and 2). For that, human testosterone synthesis predominantly follows the so-called delta 5 pathway from Preg to 17OHPreg to DHEA and then Δ 4A and testosterone (Figure 1) [12], and the Leydig cell specifically expresses the isozymes HSD3B2 and HSD17B3 [1]. By contrast, testosterone synthesis in the testis of rodents occurs predominantly through the delta 4 pathway from Preg to progesterone (Prog) to 17OH-Prog, then Δ 4A and testosterone [13-15]. Differences in the use of the delta 5 and/or delta 4 pathway for testosterone synthesis are explained by variable catalytic efficiencies for the 17,20 lyase reactions between species. Testosterone production in the testis is confined to the Leydig cell, from which it is secreted. But testosterone is not only a hormonal regulator, it also exerts intratesticular effects on Sertoli cells and spermiogenesis. In principal, luteinizing hormone (LH)

of the pituitary gland, which is under the control of the hypothalamic gonadotropin releasing hormone (GnRH) controls testosterone synthesis in the Leydig cell. The Leydig cell also secretes small amounts of $\Delta 4A$ and estradiol. Tiny amounts of estradiol are also produced from androgens in immature Sertoli cells and developing sperm cells, which both express CYP19A1 (aromatase) [16]. Whether the alternative backdoor pathway for DHT synthesis plays a role in the adult testis is unknown, but its involvement in fetal testicular androgen production has been shown in the context of human mutations [10].

The adult human ovary produces Prog and estradiol from cholesterol in a collaborative two-cell system in a cycle specific pattern under the control of gonadotropins LH and FSH. Prog is produced during the luteal phase and estradiol during the follicular phase. Since the granulosa cell lacks CYP17A1, it converts cholesterol to Preg (by CYP11A1), which is then converted either to Prog (by HSD3B2) or transferred to the theca cell for the production of $\Delta 4A$ by CYP17/POR/CYB5 and HSD3B2. Little $\Delta 4A$ is converted to testosterone likely through HSD17B5/AKR1C3. However, most $\Delta 4A$ is transferred back to the granulosa cell, which expresses aromatase (CYP19A1) and HSD17B1 specifically for the synthesis of estradiol. During normal sex steroid production in the gonads some leakage of metabolites to the periphery occurs. $\Delta 4A$ leakage from theca cells comprises about 50% of circulating androgens in women [17]. In men, circulating estrogens, which represent a fraction of 1/200 of androgens, originate by about 60% from the testes either directly or indirectly [18, 19].

1.2.2. Adrenal androgens

The adult human adrenal cortex is composed of three layers, namely the zona glomerulosa (ZG) for mineralocorticoid production, the zona fasciculata (ZF) for glucocorticoid production and the zona reticularis (ZR) for androgen production. In contrast to the ZG and ZF, which replace the fetal adrenal soon after birth, the ZR is only slowly formed over the course of the first years of life and becomes functionally active after the age of 6-8 years at adrenarche [20].

Development of the adrenal zones is thought to involve centripetal differentiation of progenitor cells into ZR, then ZG and finally ZR cells [21]. The ZR attains full activity in terms of androgen production at around 30 years and declines in activity thereafter [22]. The characteristics of the ZR are high CYP17-17,20 lyase, low HSD3B2 and high sulfonyl-transferase (SULT2A1) activities yielding massive production of DHEA and DHEA-sulfate (DHEA-S) and some $\Delta 4A$ (Figure 1) [23]. A similar steroid biosynthetic profile is found in the fetal zone of the adrenals during intrauterine life, but fetal adrenals involute soon after birth. The physiologic role of fetal DHEA production is unknown. Fetuses without adrenal function develop normally and survive to term [24]. Also, fetal DHEA is immediately metabolized to estrogens by the fetal-placental unit since otherwise elevated DHEA may lead to abnormal virilization of the external genitalia in a female fetus. Similarly, the physiologic role of the ZR and its androgen production remain unknown. Moreover, adrenarche is a phenomenon observed in humans and higher primates only and is not found in rodents [25]. However, premature adrenarche and exaggerated adrenal androgen production may be regarded as pathologic states, since they often lead to PCOS in later life [26, 27]. In healthy women, adrenal androgens contribute about 50% to the pool of circulating androgens [17].

1.2.3. Peripheral androgen synthesis and metabolism

In general, circulating (precursor) androgens may be substrates for the synthesis of more potent androgens in extra-adrenal and extra-gonadal tissues [28]. Thus DHEA/-S and $\Delta 4A$ may be converted to testosterone in peripheral tissues that express HSD3B1 and HSD17B1/5, such as the adipose tissue or the skin (Figure 2). These steroids may be further converted to estrogens in the periphery, e.g. by aromatase activity in the adipose tissue (Figure 2). For instance, the pilosebaceous unit of the skin is equipped with the enzymatic machinery to produce both androgens and estrogens, either in small amounts directly from cutaneous cholesterol or, in larger amounts, from circulating androgen precursors [29]. Most importantly, the skin expresses

SRD5A1 abundantly for the conversion of testosterone to DHT, which cannot be further aromatized to estrogens.

Most steroids are metabolized in the liver to make them water-soluble. Through this process many active and inactive metabolites arise of known and unknown biological function. Finally, over 99% of all steroids are excreted through the kidney in the urine. Therefore, studying urine steroids provides an opportunity to obtain a global idea of endogenous steroid biosynthesis and metabolism and offers a great opportunity for diagnostics [30].

2. Disorders of human androgen biosynthesis

2.1. Hypoandrogenism

Low androgen levels or lack of androgens lead to disorders in both sexes. Causes for hypoandrogenism are manifold such as genetic, environmental (e.g. toxins or drugs) or acquired (e.g. organ loss), and may be at the level of the producing organ (primary) or at the level of organ stimulation (secondary) by hypothalamic-pituitary hormones (e.g. corticotropin-releasing hormone (CRH) - adrenocorticotrophic hormone (ACTH) for adrenal steroids and GnRH - LH/follicle-stimulating hormone (FSH) for gonadal steroids). In general, lack of androgens manifests more severely in males than females since androgens are essential for the development of the male phenotype and physiologically needed hormone levels are much higher in males than females. Therefore, genetic defects of androgen biosynthesis or action lead from mild to severe forms of disorders of sex development in chromosomal males, known as 46,XY DSD. Such defects (e.g. mutations in StAR, CYP11A1, HSD3B2, CYP17A1 or POR) may be associated with life-threatening adrenal insufficiency requiring glucocorticoid and maybe mineralocorticoid replacement therapy for survival [31, 32]. Genes involved in 46,XY DSD and defective androgen biosynthesis only include CYP5, HSD17B3, SRD5A2 and AKR1C2/4. In addition, androgen deficiency in males leads to lack of pubertal development and reproductive functions. Similarly, as all estrogens are produced from androgen precursors, some androgen

biosynthetic defects also affect sex steroid production in females leading to lack of pubertal development, menstrual cycles and thus fertility (e.g. StAR, CYP11A1, HSD3B2, CYP17A1, POR).

In principal, genetic defects affecting androgen biosynthesis are known for all genes of the classic and some genes of the alternative, backdoor pathway (Figure 1). We have recently reviewed these genetic disorders and their phenotype in great detail [31].

2.2. Hyperandrogenism

In adulthood, androgen excess is less of a concern in males than in females. Hormonally active tumors of the testis or the prostate (or the adrenal cortex) and drug abuse are clinically most relevant in adult males. By contrast, androgen excess during infancy and childhood may lead to precocious pseudopuberty and growth acceleration in boys and girls, and is mostly due to genetic defects (e.g. mutations in the CYP21A2 gene) causing non-classic congenital adrenal hyperplasia. However, although rarer, androgen excess in childhood may also be found with tumors of the adrenal cortex or the testis. In females, androgen excess especially during early pregnancy, may result in moderate to severe virilization of the external genitalia (also known as 46,XX DSD). Milder signs of virilization (cliteromegaly, acne and hirsutism) may also occur postnatally under the influence of androgens. More important however in females is that androgen overproduction will lead to amenorrhea and thus infertility. The most common genetic steroid biosynthesis disorder leading to androgen excess is congenital adrenal hyperplasia due to 21-hydroxylase deficiency, but other genes may also cause 46,XX DSD (e.g. HSD3B2, CYP11B1, POR, CYP19A1) [24]. In childhood, the most common disorder of androgen excess is premature adrenarche, which is defined as the awakening of the adrenal ZR function before the age of 8 years. Premature adrenarche is visible as the premature appearance of pubic and/or axillary hair, oily skin and stinky feet. Epidemiologic studies suggest that premature adrenarche may lead to PCOS in later life [27] and therefore be regarded as risk factor or even

early manifestation of PCOS. PCOS is the most common androgen excess syndrome in adult life affecting 5-10% of women. PCOS is defined by androgen excess, menstrual disorders and polycystic ovaries [33]. In PCOS both, the ovaries and the adrenal cortex contribute to excessive androgen production [34]. Although PCOS comprises a large health burden, treatment options are very limited because its pathophysiology is poorly understood. Currently, two hypotheses are discussed in the scientific community: In one hypothesis key to the underlying defect seems to be insulin resistance, while in the other hypothesis androgen excess plays an important role [35]. Given that PCOS manifests with different phenotypes, different underlying pathomechanisms in a connected biological network may be expected. Treatment of PCOS is currently symptomatic with common drugs targeting the reproductive and/or metabolic consequences of the disorder, e.g. oral contraceptives for menstrual irregularities and metformin for pathologic glucose tolerance [36].

3. Models to study androgen production

3.1. Experiments of nature in humans

Clinical, biochemical and genetic studies of patients presenting with inborn errors of androgen biosynthesis have contributed essentially to our current knowledge of androgen production and regulation. To date, human genetic mutations are described for almost all genes involved in androgen synthesis (Figure 1 and [24]). The latest mutations identified in subjects with 46,XY DSD due to a defect in androgen synthesis were found in the backdoor pathway [10]. In addition, genome-wide association studies (GWAS) in large cohorts of PCOS patients have recently provided novel candidate genes, which were hitherto out of focus [37, 38]. Follow-up studies of one of those genes, DENN/MADD domain containing 1A (DENND1A), revealed that variant 2 of this gene is an important regulator of androgen biosynthesis [39]. Furthermore, clinical studies in offspring of mothers with PCOS or their parents have revealed insight into the heritability and the evolution of the syndrome [40-42]. Thus, clinical studies of affected subjects

and analysis of their biomaterials have been and will always be of outmost importance to understand disease mechanisms and treatment effectiveness.

3.2. Tissue and cell models for studying androgen biosynthesis

Models to study the regulation of androgen biosynthesis of the human adrenal cortex and the ovary, when interested in premature adrenarche and PCOS, are scarce. In general, no good small animal models are available as steroid biosynthesis is highly species-specific, and studies using higher primates, which resemble the human system, are rightfully very restricted.

Studies in sheep or rhesus monkeys have revealed that fetuses, which were exposed to high concentrations of androgens in utero, developed a PCOS-like phenotype postnatally, indicating that the syndrome is due to abnormal androgen programming during development and may not only be caused by endogenous (genetic), but also exogenous (environmental) factors [43].

Obtaining “fresh” human ovarian tissue, which is steroidogenically active and therefore originating from women during their fertile years, is understandably rare to obtain. In addition, establishing primary cultures from ovarian tissue will only generate a very short lived culture model as steroid production has been shown to cease after 4-6 days in culture [44]. Only the McAllister laboratory is successfully performing studies on human androgen producing theca cells originating from ovaries of healthy and PCOS women who donated leftover cell material for scientific use during invasive fertility procedures [45]. However, current fertility procedures no longer produce such byproducts. In addition, no immortalized tumor cell line exists for studying theca cell steroidogenesis and available granulosa tumor cell lines may not suffice to address the biology of ovarian androgen production, because these cells do not express CYP17 for androgen biosynthesis [46]. Human granulosa cells used for in vitro studies mainly originate from patients donating biomaterial whilst undergoing in vitro fertilization (IVF). These women and thus their granulosa cells have been stimulated with FSH and human chorionic gonadotropin (hCG). The isolated granulosa cells are therefore fully luteinized and have a

limited life span in vitro [47]. Although it has been also challenging to establish primary cultures of granulosa cells, unlike for theca cells, there are several granulosa cell lines established from various species including rodent, bovine, porcine and primate [46]. These cell lines have been developed with different methods such as spontaneous immortalization, oncogenic transformation, chemical mutagenesis, explants of human tumors, and tumorigenesis in transgenic animal models [46]. In the past, we have tried to develop a primary cell culture model consisting of mixed granulosa and theca cells from pig ovaries (unpublished data). But cell cultures showed steroid biosynthesis only during the first 2-3 passages and the steroid profile did not reflect physiological values/ circumstances since CYP17 activity was minimal.

Furthermore, there is no proper human testicular Leydig cell model to study human androgen biosynthesis. Though, some rodent Leydig cell models like mouse Leydig MA-10 cells exist, these cells cannot be used as a model to study human androgen production since the androgen biosynthesis pathway varies between humans and rodents [12, 48].

Similarly, models for studying human adrenal androgen biosynthesis are also limited: firstly, adrenal androgen production and zonation differs profoundly between various species such that a rodent model may not be used to address questions concerning human adrenal steroidogenesis [49, 50]; second, adrenarche is a phenomenon limited to humans and few higher primates (chimpanzees, gorillas and orangutans) [51, 52]. Therefore, current studies investigating adrenal androgen synthesis use tissue material originating from the human adrenal cortex or derived cell cultures. Tissue material has been employed for immunohistochemical studies [53] or gene expression studies [54-56], but is difficult to use for functional studies. Thus far, all attempts to establish a human primary cell line from the human adrenal cortex have remained unsuccessful [57], but decades ago an immortalized cell line NCI-H295 was established from an adrenocortical carcinoma tumor harboring characteristics of all three zones

of the adrenal cortex [58]. Since then two adherent strains of this cell line (H295R (ATCC; CRL-2128) and H295A [59] are almost exclusively used for functional studies of human adrenal steroidogenesis. Although the two cell lines originated from the same tumor, they differ. H295A cells produce more mineralocorticoids, whereas H295R cells produce more androgens likely due to lower HSD3B2 but higher CYP17-17,20 lyase activity [60]. This “androgenic” character makes H295R cells a good model to study androgen biosynthesis, even more so since their androgen production may be enhanced by starvation growth conditions [61].

4. Knowledge gain using the old candidate gene or hypothesis driven approach

In the past, researchers have mostly used a candidate gene or hypothesis driven approach to study synthesis and regulation of androgens. In the following section, we will show recent contributions that were obtained in the field using this approach.

4.1. Regulation of specific genes involved in androgen production

CYP17 and HSD3B2 are essential for androgen biosynthesis (Figure 1) and therefore studies of their regulation are key to understanding androgen over- or underproduction.

4.1.1. CYP17

The CYP17 enzyme (localized in the endoplasmic reticulum) possesses 17 α -hydroxylase and 17,20 lyase activities (Figure 1). In the human adrenal cortex CYP17 is expressed in the ZF and the ZR enabling glucocorticoid and androgen synthesis, but only androgen production requires both hydroxylase and lyase activities. Lyase activity of CYP17 is supported in the ZR by at least three factors. First, the amount of POR for electron transfer [62], second, presence of allosteric activator microsomal CYB5 [63], and third, phosphorylation of the CYP17 protein at serine residues [61, 64, 65]. Protein phosphatase 2A (PP2A) was shown to dephosphorylate CYP17 and diminish lyase activity and androgen synthesis [66]. Although phosphorylation of CYP17

has been shown to be enhanced by cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) [64, 65], and rho-associated, coiled-coil containing protein kinase 1 (ROCK1), this phosphorylation does not increase lyase activity [67]. Only recent studies identified the mitogen-activated protein kinase 14 (MAPK14, p38 α) as the responsible kinase enhancing 17,20 lyase activity by phosphorylation [68]. In line with this finding, involvement of the mitogen-activated protein kinase (MEK) and the extracellular signal-regulated kinase (ERK) signaling was also reported for the hyperandrogenic state of PCOS theca cells [69], and for the mechanism of action of the drug pioglitazone in down regulating androgen production in H295R cells [70].

Given the key role of CYP17 in androgen production, finding specific CYP17 inhibitors for clinical use is of great interest. Abiraterone is a CYP17 inhibitor already in use for prostate cancer treatment but unfortunately it also alters activities of other CYP enzymes [71, 72]. Therefore, with new drug development strategies more selective CYP17-17,20 lyase inhibitors are currently designed and being tested (e.g. orteronel, galeterone and VT-464).

4.1.2. HSD3B2

In humans, there are two HSD3B isozymes, which share 93.5% identity at the protein level, have the same function in steroidogenesis, but show tissue specific expression [73, 74]. HSD3Bs are membrane bound enzymes, which reside in the mitochondrion or the ER depending on the cell type they are expressed in [74, 75]. HSD3B2 is exclusively expressed in the adrenal cortex and the gonads. During adrenarche the gene expression profile of the ZR changes; the expression of CYP17, CYB5 and POR increases while the expression of HSD3B2 decreases [20, 76, 77]. By contrast, with PCOS expression of both CYP17 and HSD3B2 increases in the theca cells compared to controls [78], indicating that androgen regulation differs in various situations.

As shown in cell based studies, transcription factors like nerve growth factor IB (NGFIB/Nur77), GATA and steroidogenic factor-1 (SF-1)/liver receptor homologue 1 (LRH1) seem essential for

regulating HSD3B2 [79, 80]. Nur77 plays an important role for HSD3B2 expression during fetal development [81] and adrenal zonation [82]. We found differential regulation of HSD3B2 under serum starvation and cAMP stimulation conditions [79]. Short cyclic AMP stimulation inhibits HSD3B2 activity while long cAMP stimulation enhances HSD3B2 expression and activity [79]. Further studies revealed that PKA and MEK/ERK signaling are involved in modulating HSD3B2 [79], but the exact network regulating HSD3B2 for androgen synthesis remains unknown. However, it is reported that membrane translocase Tim50 protein regulates HSD3B2 expression and activity, implying a role for translocases in steroidogenesis [83]. Additionally, other factors like pH have also been reported to modulate HSD3B2 conformation and thus enzyme activity [84].

4.2. Role of specific signaling pathways for androgen regulation

A complex network with cross talks between multiple signaling pathways appears to modulate androgen production (Figure 3) [85, 86]. Accordingly, androgen excess and PCOS may be caused by various defects within this network, but only a few pathways are known to be involved. With regard to androgen production, the adrenocorticotrophic hormone (ACTH)/cAMP/PKA and the Mitogen-activated protein kinase (MAPK) signaling are most widely studied.

4.2.1. Androgen regulation and the ACTH/cAMP/PKA pathway

ACTH stimulates adrenal glucocorticoid biosynthesis and co-regulates adrenal androgen production [20, 87]. ACTH is also a developmental and growth factor for the adrenal cortex [88]. It binds to the melanocortin 2 receptor (MC2R) at the cell surface, which is a G-protein coupled receptor (GPCR) that triggers the production of the second messenger cAMP [85, 89]. Likewise, FSH and LH stimulate sex steroid biosynthesis in the gonads by binding to their specific

GPCRs, production of cAMP and activation of the PKA signaling leading to phosphorylation of various proteins and transcription factors involved in androgen synthesis [85]. Although the cAMP/PKA signaling seems to play an important role, second messenger cAMP can also cross talk to other signaling pathways such as MEK/ERK to target CYP17 and HSD3B2 [69, 79].

4.2.2. Androgen production and the MAPK pathway

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases. In mammalian cells, the MAPK family comprises four major groups: 1) mitogen-activated protein kinase 1/2 (MEK1/2)/ extracellular signal-regulated kinase 1/2 (ERK1/2), 2) p38MAPKs, 3) c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs), and 4) extracellular signal-regulated kinase 5 (ERK5) [90]. MAPKs transmit signals from a large array of extracellular stimuli such as growth factors, hormones, cytokines and neurotransmitters. So far MEK/ERK1/2 and p38MAPKs signaling has been reported to being involved in the regulation of androgen production [48, 68-70, 91]. Inhibition of MEK/ERK signaling affects CYP17 and HSD3B2 expression and thus androgen synthesis [61, 79]. Recently, we showed that under hyperandrogenic starvation conditions, kinases MEK and ERK were less phosphorylated in H295R cells [48, 86]. This finding fits with the report that phosphorylation and activity of MEK/ERK kinases are decreased in hyperandrogenic PCOS theca cells [69]. On the other hand, kinase p38 α has recently been shown to phosphorylate CYP17 for enhanced 17,20 lyase activity and thus production of androgens [68].

4.2.3. Other signaling pathways involved in androgen production

In a recent review we published some preliminary results establishing a possible role for protein kinase C (PKC) and protein kinase D (PKD) signaling in androgen production [86]. Studies for phosphoinositide-3-kinase (PI3K)/protein kinase B (PKB/Akt) signaling revealed mixed results [67, 86], so that its role remains unsolved. Additionally, non-GPCR and non-cAMP-dependent

signaling pathways were found to influence androgen biosynthesis [85]. Stimulators of such pathways include growth factors like insulin-like growth factor-1(IGF-1), epidermal growth factor (EGF), steroidogenic-inducing protein (SIP), macrophage derived factors and chloride or calcium channel messengers [85]. In Leydig cells and adrenal cells, growth factors EGF and IGF-1/insulin have been shown to regulate steroidogenesis [88, 92].

5. New(er) approaches: high throughput techniques

The major problem with using a candidate gene or hypothesis driven approach for studying disease mechanisms is that the unknown or unthought of possibilities are left out. Hence, the candidate based approach is useful when strong candidates are available. By contrast, to find novel candidates regulating human androgen biosynthesis, which are out of focus of current thinking, high throughput approaches like GWAS and transcriptome profiling may be used (or: are a good alternate option).

5.1. GWAS to solve PCOS

PCOS seems to be a complex disorder caused by environmental (e.g. obesity) as well as genetic factors [93]. It has a high incidence in different populations and puts family members at risk of also developing PCOS [94, 95]. Recent GWAS on large PCOS cohorts revealed several loci suggesting that inheritance of PCOS is likely multifactorial [37, 38, 96-98]. Two GWAS studies on large cohorts of Han Chinese people identified a total of 11 loci and revealed the following genes associated with PCOS: thyroid adenoma associated (THADA), DENND1A, insulin receptor (INSR), follicle-stimulating hormone receptor (FSHR), luteinizing hormone/choriogonadotropin receptor (LHCGR), high mobility group AT-hook 2 (HMGA2), Yes-associated protein 1 (YAP1), chromosome 9 open reading frame 3 (C9orf3), TOX high mobility group box family member 3 (TOX3), Ras-related protein Rab-5B (RAB5B) and SUMO1 pseudogene 1 (SUMO1P1)/zinc finger protein 217 (ZNF217) [37, 38]. Importantly, these

candidate genes were also identified in GWAS of PCOS women in the European population [96, 97, 99, 100].

One of these candidates, DENND1A was recently further explored and confirmed to cause PCOS [39]. Through alternative splicing the DENND1A gene yields two principal transcripts, namely DENND1A variant 1 (DENND1A.V1) and variant 2 (DENND1A.V2). Both variants harbor clathrin-binding domains but possess distinct C-terminal domains and therefore interact differentially with membrane adaptor proteins [51]. DENND1A is highly expressed in androgen producing tissues [98]. McAllister et al. reported just recently that expression of DENND1A variant 2 is increased in PCOS theca cells, while variant 1 is decreased compared to normal theca cells [39]. They showed that overexpression and knockdown of DENND1A.V2 in normal theca cells altered CYP17 and CYP11A1 gene transcription and androgen production accordingly, but the exact mechanism of action for connecting DENND1A to androgen synthesis remains unclear. DENND1A.V2 perhaps alters the signaling to steroidogenesis via gonadotropin or insulin receptors [101]. Nuclear localization of DENND1A.V2 suggests that it modulates gene transcription or that it functions as scaffold for transcription factors that regulate gene expression of steroidogenic enzymes. Current interest in DENND1A.V2 includes whether it might be used as a diagnostic marker for PCOS, and whether it could be a target for future treatment.

Besides DENND1A, loci harboring receptors LHCGR, FSHR and INSR have also been identified and replicated in GWAS studies in several PCOS cohorts [96, 97]. These receptors are activated by LH, FSH and insulin and their involvement in controlling follicular growth, differentiation and ovarian steroidogenesis is established [102-105].

5.2. Tissue transcriptome profiling

Tissue profiling has been shown to be a powerful tool for basic and translational research as well as for diagnostic applications and drug discovery [106]. Through the use of transcriptome analysis, new information may be generated on (physiologic) mechanisms, pathways and genes involved in specific biologic processes.

Several studies have addressed the specific development and function of the fascinating zonation of the adrenal cortex both in humans and other mammals, but so far many aspects remain unrevealed. This is particularly true for the ZR, which develops continuously after birth and produces adrenal androgens in a life-time specific fashion that is found similarly only in higher primates [25]. However, it is key to understand these regulations for the development of new treatment strategies against hyperandrogenic disorders such as premature adrenarche and PCOS.

In the past, remarkable knowledge on adrenal zonation and function was gained through (immune) histological and biochemical studies. Already in 1996 Endoh et al [107] isolated ZF and ZR cells from the human adrenal cortex and demonstrated that the ZR in comparison to ZF secretes large amounts of DHEA due to little expression of HSD3B2. Wang et al [54] described in 2001 the differential gene expression profiles of the human ZR and ZF using RNA isolation and cDNA hybridization on a microarray. He found that 17 of the 750 genes included in the array differed in expression by more than 2-fold; among them were transcripts of HSD3B2, aldoketo reductase family 1, member B1 (AKR1B1) and SULT2A1. Meanwhile several gene expression studies, were performed on rodent tissues and cells [108-110]. However, with these studies questions on human ZR may not be addressed since rodents do not have a functional ZR [49, 50, 111]. Only recently transcriptome profiling of the human adrenal ZF and ZR was repeated with an array containing 47,231 transcripts of the human genome [55]. In this study, expression of 347 genes varied by 2-fold between ZF and ZR. Interestingly, in the group of steroidogenic enzymes, only HSD3B2, SULT2A1, AKR1C3 and CYB5 were significantly

different, with HSD3B2 being less and the other three genes being more highly expressed in the ZR [55]. Using this unbiased method, novel players were identified including a network containing bone morphogenetic proteins (BMPs) (e.g. BMP4), their receptors (BMPR1A and BMPR2) as well as other signaling proteins like SMAD 1,4 and 5 that are involved in BMP signaling [55]. BMP4 was up-regulated in ZG and ZF compared to ZR. Further studies established a role for BMP4 in the regulation of androgen biosynthesis. BMP4 altered phosphorylation of SMADs and decreased the synthesis of C19 steroids (DHEA, DHEA-S and $\Delta 4A$) in human adrenal H295R cells by suppressing CYP17 expression (Figure 4) [56]. Hence, transcriptome analysis has clearly enhanced our understanding of adrenal androgen production.

5.3. Transcriptional profiling of human adrenal NCI-H295 cells

As mentioned earlier in this review, the NCI-H295 cell model is almost the only available cell model to study human steroidogenesis [57]. It represents steroidogenesis of all three layers of the human adrenal cortex and has been shown to follow regulations similar to those seen in human biology [57, 58]. For instance, ACTH/cAMP/PKA signaling plays an essential role in the regulation of steroid production of H295R cells [89, 112, 113], and regulation of CYP17, which is essential for human androgen production, seems similar in this cell system [31, 114].

Therefore, transcriptome profiling of H295A cells has been employed to identify specific kinases involved in the phosphorylation of CYP17, which is known to enhance 17,20 lyase activity for androgen production [67]. As it has been shown that cAMP promotes CYP17 phosphorylation [64], stimulation with cAMP on H295A cells was used to study the kinome with the Affymetrix U133 plus 2.0 chip, which provides full kinase coverage [67]. In H295A cells, 145 of the 278 known serine/threonine kinases were expressed, but only six showed significant differential expression (>2-fold) upon 8Br-cAMP treatment [67]. Although several kinases modulating pathways targeting CYP17 (e.g. PKA/PI3K/Akt, calcium/calmodulin/MAPK) were studied, none

was found to enhance 17,20 lyase activity. ROCK1 kinase was shown to phosphorylate CYP17 but did not lead to increased 17,20 lyase activity [67]. Only recently the kinase promoting CYP17 phosphorylation and activity was identified as p38 α (MAPK14) [68]. In theory, the network connecting this kinase to other regulators of androgen biosynthesis is currently drawn as follows (Figure 4): DENND1A, ROCK1, hormones or other factors activate mitogen-activated protein kinase kinase kinases (M3Ks), which trigger mitogen-activated protein kinases (such as MAP2K3, 4 or 6) that target p38 α and finally CYP17 [114]. However, confirmation and further details of this network are needed. Understanding of this network may uncover targets for diagnostic or therapeutic opportunities.

Similarly, we used transcriptome analysis of H295R cells grown under starvation conditions to study the regulation of human androgen biosynthesis [48]. Previously, we described that H295R cells produce more androgens than H295A cells [60], and that serum starvation of H295R cells leads to a marked shift towards androgen production (Figure 4A) [48, 61]. In starved H295R cells increased 17,20 lyase activity and decreased HSD3B2 expression and activity were found. These findings resemble some changes seen in the human adrenal during adrenarche [77, 115]. Therefore, we used our starvation model and performed gene expression profiling and bioinformatics analysis to identify novel regulatory genes and networks underlying androgen biosynthesis. Interestingly, only 14 genes were found significantly altered (>2.0-fold) after starvation [48]. As expected, genes involved in steroid biosynthetic processes (HSD3B1, HSD3B2 and CYP21A2), energy metabolism and signal transduction (especially MAPKK kinases) were identified. However, we also found two genes downregulated after starvation, namely retinoic acid receptor beta (RAR β) and angiopoietin-like protein 1 (ANGPTL1), which were novel to the scene of androgen regulation [48].

According to GEO profiling, RAR β is expressed in normal human adrenal and gonadal tissues (GEO GDS3113/109692/RAR β). Our functional studies revealed that RAR β regulates the promoters of StAR, CYP17 and HSD3B2 (Figure 4B) [48], and that overexpression of RAR β

and its ligand all-trans retinoic acid (ATRA) enhance $\Delta 4A$ production in starved H295R cells (Figure 4C). We also found that RARB co-operates with transcription factor Nur77 for modulating HSD3B2. As Nur77 has been shown to play an essential role in the adrenal zonation [82] and in fetal developmental specific steroid production [81], the role of RARB relating to these events remains to be explored.

Depending on the cell context ANGPTL1 is a known regulator of angiogenesis or anti-angiogenesis [116, 117]. In endothelial cells it exerts its anti-apoptotic activity by stimulating phosphorylation of ERK1/2 and Akt [117]. We found that ANGPTL1 induces ERK1/2 phosphorylation and represses CYP17 expression in starved H295R cells (Figure 4D and 4E). ERK regulation of CYP17 phosphorylation has been reported in several studies [69, 70].

When analyzing the transcriptome data of our starved H295R cell model with a cut-off level of >1.5-fold ($p < 0.05$), 77 genes are differentially expressed; 60 are up- while 17 are down-regulated. The corresponding heat map and analysis for gene ontology (GO) processes are shown in Figure 5. Details on enrichment analysis for GO pathways and disease biomarkers are given in Table 1. The table describes the statistical ranking of different pathways/disease biomarkers based on the p-values. GO process analysis suggests an effect of serum starvation on steroidogenesis, MAPK signaling and on the regulation of cell differentiation. This is conceivable since serum contains several growth factors, cytokines and hormones, which are known to play an important role for cell differentiation.

Interestingly, our study also identified a network containing 7 genes, among them CYP21A2, for androgen regulation (Figure 6). CYP21A2 is important for cortisol production and its deficiency causes androgen excess in humans, also known as congenital adrenal hyperplasia (CAH). The other genes were MEK kinase 5 (MAP3K5) also known as apoptosis signal-regulated kinase 1 (ASK1), thyroid stimulating hormone receptor (TSHR), glucosaminyl (N-Acetyl) transferase 1, core 2 (GCNT1, also known as G6NT), complement factor I (CFI, also known as factor 1), Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin) member 5 (SERPINA5,

also known as Protein C inhibitor) and GPCRs (Figure 7). The center of this network consists of the transcription factor SP1, which regulates CYP21A2, and this regulation seems to be controlled by the kinase ASK1 (MAP3K5). In general, ASK1 activates MAP2K4 that activates c-Jun N-terminal kinase 1 (JNK1) [118], which then phosphorylates transcription factor SP1 [119]. Thus, androgen regulation may not only include the CYP21A2 gene in case of CAH caused by 21-hydroxylase deficiency, but this gene may also be a factor of the normal regulatory network.

6. Future directions

In the near future, use of additional high throughput techniques, including next generation sequencing (NGS), micro-RNA profiling, proteomics and metabolomics, will enhance our knowledge on human androgen biosynthesis in health and disease. Specifically, NGS will provide genetic diagnosis in patients who are not currently diagnosed. Most likely involvement of multiple genes/networks will be found explaining the different phenotypes of PCOS. Micro-RNA studies may reveal novel regulatory modules for androgen production at the post-transcriptional level so far undiscovered. Micro-RNAs are predicted to influence 30 to 80% of human genes [120-124], hence it might be wise to consider a role of micro-RNAs in disorders of androgen production. With a phospho-proteomics approach proteins or pathways, which are modulated by phosphorylation, can be further studied in order to put the current knowledge on involvement of single phospho-regulated proteins in androgen production in a bigger perspective. Finally, metabolomics profiling shows consequences of regulatory mechanisms, and untargeted analysis of biomaterials using massspectrometric methods will allow detection of steroid metabolites unthought of, which will eventually lead to novel pathways as recently illustrated by the studies on the backdoor pathway. Ultimately, all this knowledge will help in finding and developing new diagnostic tools and drugs. These are especially needed for the treatment of PCOS, which affects almost 10% of women.

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Abbreviations

AKR1C2/4, aldo-keto reductase family 1, member C2/4; ASK1, apoptosis signal-regulated kinase 1; ANGPTL1, angiopoietin-like protein 1; ATRA, all-trans-retinoic acid; BMPs, bone morphogenetic proteins; C9orf3, chromosome 9 open reading frame 3; CRH, corticotropin-releasing hormone; CFI, complement factor I; CYB5, cytochrome b5; CYP19A1, aromatase; CYP21A2, 21-hydroxylase; DENND1A, DENN/MADD domain containing 1A; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; GCNT1, glucosaminyl (N-Acetyl) transferase 1, core 2; GWAS, genome-wide association studies; HMGA2, high mobility group AT-hook 2; HSD3B, 3 β -hydroxysteroid dehydrogenase; HSD17B, 17 β -hydroxysteroid dehydrogenase; INSR, insulin receptor; JNK/SAPK, c-Jun N-terminal kinases/stress-activated protein kinases; LH, luteinizing hormone; LHCGR, luteinizing hormone/choriogonadotropin receptor; LRH1, liver receptor homologue; MAPK14, mitogen-activated protein kinase 14; MC2R, melanocortin 2 receptor; NGFIB/Nur77, nerve growth factor IB; PCOS, polycystic ovary syndrome; PKA, protein kinase A; PKB/AKT, protein kinase B; PKC, protein kinase C; POR, P450 oxidoreductase; PP2A, protein phosphatase 2A; RAB5B, Ras-related protein Rab-5B; RARB, retinoic acid receptor beta; ROCK1, rho-associated, coiled-coil containing protein kinase 1; SERPINA5, Serpin peptidase inhibitor, clade A member 5 ; SF1, steroidogenic factor-1; SIP, steroidogenic-inducing protein; SRD5A, 5 α -reductase; StAR, steroidogenic acute regulatory protein; SULT2A1, dehydroepiandrosterone sulfotransferase; SUMO1P1, SUMO1 pseudogene 1; THADA, thyroid adenoma associated; TOX3, TOX high mobility group box family member 3;

TSHR, thyroid stimulating hormone receptor; YAP1, Yes-associated protein 1; ZNF217, zinc finger protein 217; ZF, zona fasciculata; ZR, zona reticularis;

Figure Legends:

Figure 1 – Scheme showing the classic and the alternative, backdoor pathway for the synthesis of dihydrotestosterone (DHT) from cholesterol. Intermediate products, enzymes and cofactors needed for the catalytic processes are depicted. Note that in the alternative pathway 17-hydroxyprogesterone (17OH-Prog) is 5 α , 3 α reduced and then stepwise further converted to DHT without going through testosterone.

Figure 2 – Organ specificity of androgen biosynthesis. The classic androgen synthesis pathway, as established in the adrenal cortex, the human testis and ovary, and in peripheral tissues, such as the adipose tissue, is shown. The scheme shows the pathways starting from cholesterol as substrate and gives intermediate products as well as responsible enzymes and/or cofactors enabling the stepwise conversion for the production of testosterone. Organ specific characteristics are shown in colors: green, adrenal cortex; violet, ovarian theca cell; blue, testis Leydig cell; red, adipose tissue. Prog, progesterone; Preg, pregnenolone; DHEA, dehydroepiandrosterone; (DHEA-S), DHEA-sulfate; 17OH Preg, 17 α -hydroxypregnenolone; 17OH P, 17-hydroxyprogesterone; 11DOC, 11-deoxycortisol; Δ 4A, androstenedione; E1, estrone; E2, estradiol; T, Testosterone.

Figure 3 – Signaling network of androgen production. Scheme summarizing published signaling pathways, regulating and regulated genes, proteins and co-factors. Newer genes and networks involved in the regulation of androgen biosynthesis are highlighted in yellow. GWAS of PCOS cohorts helped in discovering the recently described DENND1A gene [37, 38]. Overexpression

of variant 2 of DENNDA1 in PCOS is responsible for augmented androgen production by altering the expression of CYP17A1 and CYP11A1. A possible mechanism how DENND1A (as transcription factor or via signaling) may alter the expression of steroid enzymes is shown in dotted lines [39]. Transcriptome profiling of adrenal cortex layers recently discovered differential expression of BMP4 between the zona glomerulosa and reticularis [56]. BMP4 phosphorylates SMADs and decreases androgen synthesis by suppressing CYP17A1 expression. The exact signaling between phospho-SMADs and CYP17A1 gene (dotted line) remains unknown. Transcriptome profiling of H295A cells revealed that ROCK1 kinase phosphorylates CYP17A1; however, this phosphorylation does not enhance 17,20 lyase activity [67]. Recent p38 α (MAPK14) was described to phosphorylate CYP17A1 and confer increased 17,20 lyase activity, which is essential for the production of androgens [68]. It has been suggested that ROCK1 regulates MAPK signaling (dotted line) and targets p38 α for CYP17 phosphorylation [68]. Several studies show crosstalk pathways between MAPK signaling (especially MAPK kinase p38) and SMADs signaling (dotted line) [125, 126]. Gene expression profiling of starved, hyperandrogenic H295R cells revealed a role for two novel gene networks around RARB and ANGPTL1 for androgen production [48]. Transcription factor RARB stimulates the StAR, CYP17 and HSD3B2 promoters. Secretory protein ANGPTL1 modulates CYP17 expression by inducing ERK1/2 phosphorylation.

Figure 4 - Impact of serum starvation on human adrenal H295R cells and the role of RARB and ANGPTL1 regulators for adrenal androgen biosynthesis. A) Serum starvation induces a hyperandrogenic state in H295R cells. A steroid profile (thin layer chromatogram) of H295R cells grown under starvation growth condition (SM) for 48 h is shown in comparison to the profile obtained under normal growth conditions (GM). Quantitative analysis is shown on the right. For Materials and Methods see [48]. * $p < 0.05$, ** $p < 0.01$. Prog, progesterone; $\Delta 4A$, androstenedione; Preg, pregnenolone; DHEA, dehydroepiandrosterone; 17OH Preg, 17 α -

hydroxypregnenolone; 17OH Prog, 17-hydroxyprogesterone; 11DOC, 11-deoxycortisol. B) Role of the nuclear transcription factor RARB in altering HSD3B2 gene expression. H295R cells were transfected with the human -1.05kb HSD3B2 promoter construct along with an expression vector for the RARB transcription factor in the presence or absence of ATRA. Promoter activation was assessed by the dual luciferase assay (Promega) [48]. * $p < 0.05$, ** $p < 0.01$. C) Effect of RARB overexpression and ATRA treatment on H295R steroidogenesis. H295R cells were transiently transfected with RARB and optionally treated with 1 μ M ATRA. Steroid production was assessed as described in [48]. Quantitative data for androstenedione ($\Delta 4$ A) are depicted. * $p < 0.05$, ** $p < 0.01$. D) Role of ANGPTL1 in androgen production. Effect of rANGPTL1 on ERK1/2 kinase phosphorylation and on CYP17 expression is shown (Western blots from [48]). E) Scheme showing the suggested interplay between ANGPTL1, ERK phosphorylation and inhibition of CYP17 expression.

Figure 5 - Heat Map of Affymetrix microarray data showing differentially expressed genes in H295R cells grown under normal versus hyperandrogenic starvation conditions. Microarray data were analyzed by Cluster 3.0 and JTreeview software for creating a representative heat map of the 77 genes, which were identified at a significance level of >1.5 fold change. In the heat map graphic, rows show individual genes. Gene expression levels are displayed for each independent sample. In the picture we also show the analysis for GO processes for the identified genes, like the regulation of cell differentiation, response to steroid hormones, steroid biosynthetic process and MAPKKK cascade. Over-expression is shown in red, under-expression in green.

Figure 6 - Network analysis of differentially expressed genes (>1.5 fold change) in starvation conditions. GeneGo Metacore software was used for analyzing the microarray data to obtain common gene networks between significantly changed genes. Networks found for analyzing at

>2.0 fold change are published in [48]. Seven genes can be found in an additional common network when analyzing at >1.5 fold change, namely CYP21A2, ASK1 (MAP3K5), TSHR, G6NT (GCNT1), complement factor 1 (CF1), protein C inhibitor (SERPINA5) and GPCRs like GPR56. The center of this network consists of the transcription factor SP1, which seems to regulate CYP21A2, and this regulation seems to be controlled by ASK1 (MAP3K5) kinase. The core of the identified network is highlighted in grey color. Only down-regulated genes were found in this network and they are marked with small red circles. Green lines show activating relationships; red lines show inhibition; gray lines show relationships of unknown quality; turquoise bold lines connect members of the RAS superfamily.

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Table 1: Identification of 77 differentially expressed genes in hyperandrogenic human adrenal H295R cells.

Gene expression microarray analysis was performed on human adrenal H295R cells grown under normal growth (GM) vs hyperandrogenic, starvation (SM) conditions. Analysis with a fold change cut-off at 1.5 (with an adjusted p-value of < 0.05) revealed 77 gene transcripts that were differentially expressed. Enrichment analysis was then performed with GeneGo MetaCore software to assess the biological function of the identified genes; this included gene involvement in different signaling pathways and diseases states. Results provided in the table are grouped according to biological processes. Analysis significance levels (p-values) and false discovery rate (FDR) are given. N means the number of genes found in the specific group.

Enrichment analysis		GM vs SM *xls (>1.5 fold analysis)			
	Pathway Maps	p-value	FDR	N	Genes
1	Cortisol biosynthesis from cholesterol	1.317E-04	1.040E-02	3	HSD3B2, CYP21A2, HSD3B1
2	Neurophysiological processes, circadian rhythm	1.012E-02	1.928E-01	2	DEC2, DEC1 (Stra13)
3	Androstenedione and testosterone biosynthesis and metabolism	1.274E-02	1.928E-01	2	HSD3B2, HSD3B1
4	Development, role of proteases in hematopoietic stem cell mobilization	5.707E-02	1.960E-01	1	Carboxypeptidase M
5	Apoptosis and survival, NO signaling in apoptosis; oxidative stress, role of ASK1 under oxidative stress	7.236E-02	1.960E-01	1	ASK1 (MAP3K5)
6	Immune response, IFN alpha/beta signaling pathway	7.538E-02	1.960E-01	1	IRF9
7	Transcription, transcription regulation of amino acid metabolism	7.840E-02	1.960E-01	1	ASSY
8	Signal transduction, Erk interactions: inhibition of Erk	1.051E-01	1.960E-01	1	MKP-3
9	Transcription, role of the non-genomic action of retinoic acid and phosphorylation of retinoic acid receptors in the initiation of transcription	1.051E-01	1.960E-01	1	RARbeta
10	Cell adhesion, PLAU signaling	1.197E-01	1.960E-01	1	SERPINA5
	Diseases (by biomarkers)	p-value	FDR		Genes
1	Endocrine system diseases	3.839E-07	2.221E-04	38	FHL2, ANGPTL1, HSD3B2, KLRG1, NELL2, ERM, DOCK4, CYP21A2, Platin, hASH1, MKP-3, PLK2, Apo-

					2L(TNFSF10), OLR1, ASK1 (MAP3K5), ASSY, Kv4.2 channel, NOV, IGFBP7, UNC5B, RNS2, CD22, RARbeta, GPR56, HSD3B1, SERPINA5, Neurotrimin, DEC1 (Stra13), CCDC80, FAP, TR-beta1, LAPT4B, NPTX2, Nidogen-2, KCNK2
2	Gonadal disorders	2.407E-05	3.567E-03	24	FHL2, HSD3B2, ERM, DOCK4, CYP21A2, Plastin, MKP-3, PLK2, Apo-2L(TNFSF10), IGFBP7/8, UNC5B, RNS2, CD22, RARbeta, GPR56, HSD3B1, SERPINA5, FAP, TR-beta1, Thyroid hormone receptor, LAPT4B, Nidogen-2, KCNK2
3	Ovarian diseases	5.123E-05	4.316E-03	23	FHL2, ERM, DOCK4, CYP21A2, Plastin, MKP-3, PLK2, Apo-2L(TNFSF10), IGFBP7, UNC5B, RNS2, CD22, RAR, RARbeta, GPR56, HSD3B1, SERPINA5, FAP, TR-beta1, Thyroid hormone receptor, LAPT4B, Nidogen-2, KCNK2
4	Ovarian neoplasms	2.695E-04	1.015E-02	21	FHL2, ERM, DOCK4, Plastin, MKP-3, PLK2, Apo-2L(TNFSF10), IGFBP7, UNC5B, RNS2, CD22, RARbeta, GPR56, SERPINA5, TR-beta1, Thyroid hormone receptor, LAPT4B, Nidogen-2, KCNK2
5	Female genital diseases	1.263E-02	8.197E-02	50	FHL2, HSD3B2, SLC9A2, ERM, DOCK4, OLFML2B,

					G6NT, CYP21A2, ABCA5, Zimp10, Plastin, SLC1A3, MKP-3, MKP-5, PLK2, Apo-2L(TNFSF10), ASK1 (MAP3K5), Factor I, ASSY, Kv4.2 channel, NOV, IGFBP7, IGFBP7, UNC5B, RNS2, Trp53inp2, CD22, RARbeta, BY55, GRP-R, GPR56, TRIB2, HSD3B1, SERPINA5, Neurotrimin, C1orf192, DEC1 (Stra13), CCDC80, ARHGAP20, FAP, NR3A, TR-beta1, LAPTM4B, PGM2L1, SV2B, Nidogen-2, KCNK2, SVEP1
6	Hyperandrogenism	5.559E-03	5.454E-02	2	HSD3B2, CYP21A2
7	Male genital diseases	3.223E-04	1.015E-02	46	FHL2, HSD3B2, NELL2, Lphn3, SLC9A2, ERM, OLFML2B, G6NT, CYP21A2, Zimp10, Plastin, hASH1, CCDC3, MKP-5, PLK2, Apo-2L(TNFSF10), ASK1 (MAP3K5), CFI, ASSY, Kv4.2 channel, IGFBP7, UNC5B, Carboxypeptidase M, ENC1, CD22, RARbeta, GRP-R, GPR56, TRIB2, HSD3B1, SERPINA5, CRMP4, Neurotrimin, C1orf192, CCDC80, ARHGAP20, FAP, TR-beta1, PGM2L1, NPTX2, SV2B, Nidogen-2, SVEP1
8	Disorders of sex development	3.420E-04	1.015E-02	4	HSD3B2, CYP21A2, GPR56, HSD3B1

9	Precocious puberty	1.596E-04	7.867E-03	3	HSD3B2, CYP21A2, GPR56
10	Small cell carcinoma	8.748E-05	5.982E-03	5	hASH1, RAR, RARbeta, GRP-R, GPR56

Classic Pathway

Alternative Backdoor Pathway

Adrenal Cortex

Ovary Theca Cell

Testis Leydig Cell

Peripheral Tissues e.g. adipose tissue

DHEA-S, Androstenediol, Δ4A, T

Circulation