

Of marsupials and men: “Backdoor” dihydrotestosterone synthesis in male sexual differentiation

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Following development of the fetal bipotential gonad into a testis, male genital differentiation requires testicular androgens. Fetal Leydig cells produce testosterone that is converted to dihydrotestosterone in genital skin, resulting in labio-scrotal fusion. An alternative ‘backdoor’ pathway of dihydrotestosterone synthesis that bypasses testosterone has been described in marsupials, but its relevance to human biology has been uncertain. The classic and backdoor pathways share many enzymes, but a 3 α -reductase, AKR1C2, is unique to the backdoor pathway. Human AKR1C2 mutations cause disordered sexual differentiation, lending weight to the idea that both pathways are required for normal human male genital development. These observations indicate that fetal dihydrotestosterone acts both as a hormone and as a paracrine factor, substantially revising the classic paradigm for fetal male sexual development.

Abbreviations: AKR1C, AldoKeto Reductase; DHT, Dihydrotestosterone; DHEA, Dehydroepiandrosterone; HSD, Hydroxysteroid dehydrogenase; DSD, disorder of sex development; AMH, Anti Müllerian Hormone; StAR, steroid Acute Response protein; 17OH-Preg, 17-hydroxy pregnenolone; 17OHP, 17-hydroxy progesterone; NAD, Nicotinamide adenine nucleotide; NADPH, Nicotinamide adenine dinucleotide phosphate; mRNA, messenger ribonucleic acid; RT-PCR, reverse transcriptase-polymerase chain reaction; RoDH, Retinol Dehydrogenase.

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1. Role of androgens in fetal male differentiation

Following embryonic sex determination, in which the presence of a Y chromosome drives the differentiation of the primitive, bipotential gonad into a testis, androgens produced by the fetal testis mediate both the stabilization of structures derived from the Wolffian ducts and the closure of the urogenital sinus, resulting in the male phenotype. The indispensable role of androgens is demonstrated by the consequences of human androgen receptor deficiency and androgen-receptor knockout mice, which have female external genitalia despite the presence of testes and abun-

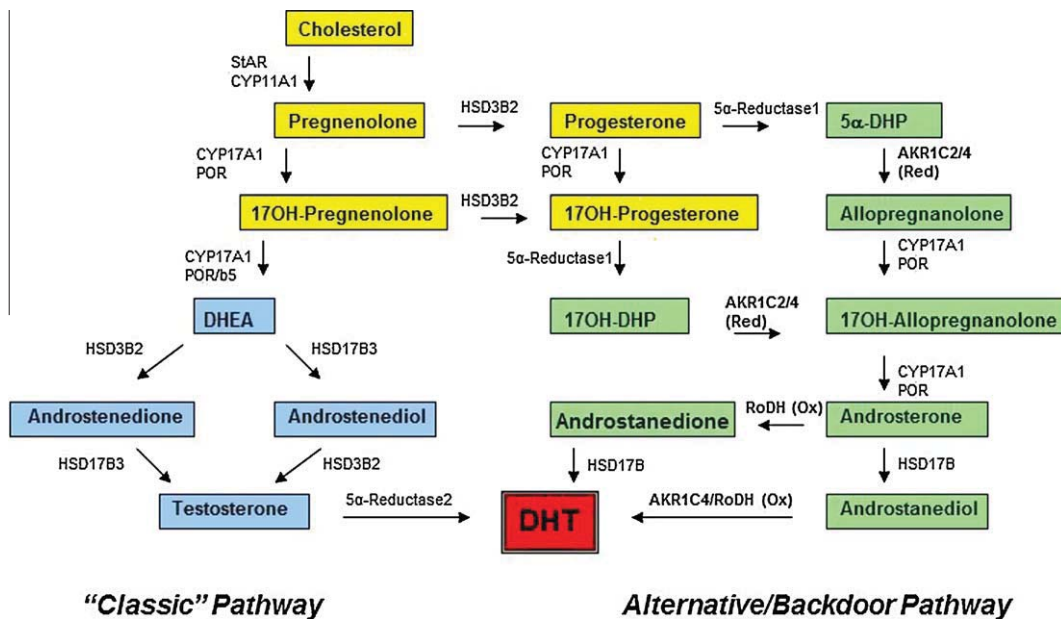


Fig. 1. The classic and alternative ‘backdoor’ pathways of androgen biosynthesis. The classic pathway proceeds from cholesterol via pregnenolone, 17OH-Preg and DHEA to androstenedione or androstenediol and then to testosterone in testicular Leydig cells (shown in blue). Hormonal testosterone from the circulation is then converted to DHT in genital skin. The backdoor pathway proceeds mainly from 17OH-Preg to 17OH-Prog, 17OH-DHP, 17OH-Allo, androsterone, androstenediol (3 α -diol) (all shown in green) and thence to DHT (shown in red), all in the testis. The enzymes and proteins shown in the classic pathway are: CYP11A1 (P450_{sc}, cholesterol side-chain cleavage enzyme), StAR (steroidogenic acute regulatory protein), CYP17A1 (P450_{c17}, 17 α -hydroxylase/17,20-lyase), HSD3B2 (3 β HSD2, 3 β -hydroxysteroid dehydrogenase, type 2), cytochrome b5, P450 oxidoreductase (POR), HSD17B3 (17 β HSD3, 17 β -hydroxysteroid dehydrogenase, type 3), and 5 α Red2 (5 α -reductase, type 2). The alternative pathway is characterized by the presence of three different enzymes: 5 α Red1 (5 α -reductase, type 1); reductive 3 α HSD activity catalyzed by AKR1C2 and AKR1C4; and oxidative 3 α HSD activity, apparently catalyzed by RoDH (also known as 17 β HSD6). Steroid names include: 17OH-Pregnenolone, 17-hydroxypregnenolone; 17OH-Progesterone, 17-hydroxyprogesterone; 17OH-DHP, 17-hydroxydihydroprogesterone (5 α -pregnan-3 α ,17 α -diol-20-one); 5 α -DHP, 5 α -dihydroprogesterone (5 α -pregnane-3,20-dione); 17OH-Allopregnanolone, 17-hydroxyallopregnanolone (5 α -pregnan-3 α ,17 α -diol-20-one); androstenediol, androsta-5-ene-3 β ,17 β -diol.

dant androgens. Two androgens, testosterone and dihydrotestosterone (DHT), are needed to produce the human male phenotype (Wilson et al., 1981). Testosterone produced in the fetal testis reaches the genital skin of the fetal labioscrotal folds, where it is converted to DHT by 5 α -reductase. The resulting DHT induces labio-scrotal fusion, the development of the phallic urethra and some phallic enlargement. Despite acting through a single androgen receptor, distinct roles for testosterone and DHT are shown by 46,XY males with 5 α -reductase deficiency, who typically have an open urogenital sinus, a perineal urethral orifice, a small phallus and chordee (Wilson et al., 1994). In the “classic” pathway of androgen synthesis, DHEA and androstenedione or androstenediol are key intermediates in the synthesis of testosterone, and fetal DHT is produced in situ in its target organ, the genital skin, where it acts as a paracrine factor (Fig. 1). An alternative, or “backdoor” biosynthetic pathway leading to the production of DHT without going through testosterone has been described in marsupials (Renfree et al., 1995; Wilson et al., 2003, 2002) and in rodents (Eckstein et al., 1987; Mahendroo et al., 2004), but the potential relevance of this pathway to human genital masculinization has been unclear. Recent work has indicated this pathway as a player in human male sexual development. Patients with genetic lesions in AKR1C2, a 3 α -hydroxysteroid dehydrogenase (3 α HSD) that participates in the backdoor pathway but not in the classic pathway of testosterone biosynthesis, had a disorder of sexual development (DSD) (Flück et al., 2011). Several mutations were found, and the index family had combined partial disorders of two AKR1C enzymes, AKR1C2 and AKR1C4, whereas another patient had 46,XY DSD only with disordered AKR1C2 (Flück et al., 2011). This unique, newly described form of DSD supports the idea that the backdoor pathway is essential for normal male sexual development, dramatically revising former views of the mechanisms of normal and pathological masculinization.

2. Sexual development and sexual differentiation

Sexual determination and *sexual differentiation* refer to distinct processes. Sexual determination is the process by which the early bipotential gonad develops into a testis or an ovary, whereas sexual differentiation is the subsequent process by which gonadal hormones determine the anatomic phenotype (Biason-Lauber, 2010; Grumbach et al., 2003; MacLaughlin and Donahoe, 2004). The human bipotential gonad develops at about 4–6 weeks post conception, testicular development occurs soon thereafter, primarily between 6 and 8 weeks, while ovarian development occurs a bit later, at 10–14 weeks. Once developed, the fetal ovary is hormonally quiescent (Voutilainen and Miller, 1986), and ovarian function is not needed for the development of female genital organs; this is clearly evidenced by the normal female anatomy of newborns with 45,X gonadal dysgenesis (Turner Syndrome). By contrast, the fetal testis is active by 8 weeks, with the Sertoli cells producing Anti Müllerian Hormone (AMH) and the Leydig cells producing androgens, both of which are needed for normal male development. Fetal testicular androgens are required for male sexual differentiation, the process by which the labio-scrotal folds fuse, and the genital tubercle develops into the penis with the urethra traversing its inferior surface. Thus male sexual differentiation is crucially dependent on androgen biosynthesis and action.

3. The “classic” pathway of androgen biosynthesis

The ‘classic’ pathway of androgen biosynthesis is well-known (Fig. 1) (Miller and Auchus, 2011). After cholesterol reaches the inner mitochondrial membrane through the action of the steroidogenic acute regulatory protein (StAR), it is converted to pregnenolone in a three-step process catalyzed by the cholesterol side-chain

cleavage enzyme, mitochondrial cytochrome P450_{sc}, encoded by the *CYP11A1* gene. While pregnenolone may be converted to progesterone by 3 β -hydroxysteroid dehydrogenase type 2 (3 β HSD2) in the Leydig cells, the principal pathway (the Δ 5 pathway) is that pregnenolone is sequentially converted to 17OH-pregnenolone (17OH-Preg) and thence to DHEA by microsomal cytochrome P450_{c17}, encoded by the *CYP17A1* gene (Flück et al., 2003). The Δ 5 pathway is preferred because the *K_m* (Michaelis constant) for Δ 5 steroids binding to 3 β HSD2 is 5.5 mM whereas the *K_m* for binding to P450_{c17} is 0.8 mM, so that pregnenolone or 17OH-Preg has a 7-fold preference for the Δ 5 pathway (Nikolajeff et al., 1999). Furthermore, human P450_{c17} can convert 17OH-progesterone (17OHP) to androstenedione with only 2–3% of the efficiency of its conversion of 17OH-Preg to DHEA (Flück et al., 2003; Auchus et al., 1998), so that any 17OHP that is produced by 3 β HSD2 will accumulate and act on 3 β HSD2 by end product inhibition to inhibit further conversion of 17OH-Preg to 17OHP. DHEA may be converted to Δ 4-androstenedione by 3 β HSD2 and thence converted to testosterone by 17 β -hydroxysteroid dehydrogenase, type 3 (17 β HSD3), or DHEA may first be reduced to Δ 5-androstenediol by 17 β HSD3 (Geissler et al., 1994) and then converted to testosterone by 3 β HSD2. These two pathways from DHEA to testosterone are quantitatively about equally important. Once produced, testicular testosterone is a classic hormone that is secreted into the circulation and acts at distant targets. One such target is the fetal genital skin, which expresses the enzyme 5 α -reductase, type 2 (5 α Red2), which converts testosterone to its substantially more active derivative, dihydrotestosterone (DHT) (Wilson et al., 1994). The synthesis of DHT in fetal genital skin induces labio-scrotal fusion. It appears that activation of the androgen receptor in these cells induces production of extracellular matrix proteins that promote cellular adhesion, but the exact molecular mechanisms of labio-scrotal fusion remain unclear. The essential role of androgen action in the differentiation of the male external genitalia is demonstrated by the female external genital phenotype of 46,XY individuals with non-functional androgen receptors (complete androgen insensitivity syndrome) (French et al., 1990; McPhaul et al., 1993).

Multiple lines of evidence demonstrate the importance of the classic pathway of androgen biosynthesis. In 46,XY individuals, complete absence of the activities of StAR (Bose et al., 1996), P450_{sc} (Tajima et al., 2001; Kim et al., 2008), or P450_{c17} (Auchus, 2001) results in a female external genital phenotype; the minimal virilization seen in some patients with complete absence of the activities of 3 β HSD2 (Moisan et al., 1999; Rheaume et al., 1992) and 17 β HSD3 (Geissler et al., 1994; Andersson et al., 1996) has been attributed to the action of the peripheral conversion of DHEA and/or androstenedione to functional androgens. Evidence for the essential role of DHT produced in genital skin comes from the observation that 46,XY individuals with varying defects in 5 α Red2 have a disorder of sexual differentiation called pseudovaginal perineal hypospadias (5 α -reductase deficiency); the partial androgenization seen in these individuals at birth is generally ascribed to the action of testicular testosterone. Thus the known pathways of human androgen biosynthesis and the phenotypic consequences of mutations in the androgenic enzymes in this pathway have been consistent; no additional factors were thought to be needed to explain male sexual differentiation. However, as discussed below, the partial virilization seen in patients with 5 α -reductase deficiency may also be partially attributable to DHT produced in the fetal testis by a pathway that does not require 5 α Red2.

4. The alternative “backdoor” pathway of androgen synthesis

Marsupials give birth to their young at a stage of development approximately comparable to the end of the first trimester of

human pregnancy. The newborn emerges from the birth canal, crawls up the mother's abdomen and into her pouch and attaches itself to one of her teats, whereupon this ‘pouch young’ can be nourished and finish development in the pouch. This mode of delivery and development permits facile investigation of the early physiology of these animals during their early sexual differentiation (Wilson et al., 2002). In male pouch young of the tammar wallaby (*Macropus eugenii*), scrotal development is androgen-independent but testicular androgens mediate virilization of the urogenital sinus at 20–45 days postpartum (Renfree et al., 1995). Studies of early testicular steroidogenesis in the tammar wallaby found that this marsupial synthesizes the androgen androstenediol (5 α -androstane-3 α ,17 β -diol, referred to herein as 3 α Diol) by an alternative pathway that begins with 17OHP and does not utilize DHEA, androstenedione or testosterone as intermediates, but instead proceeds through 17OH-dihydroprogesterone (5 α -pregnane-17 α -ol-3,20-dione), 17OH-allopregnanolone (17OH-Allo) (5 α -pregnane-3 α ,17 α -ol-3,20-dione) and androsterone to 3 α Diol and, apparently, to DHT (Wilson et al., 2003; Auchus, 2004) (Fig. 1).

5. AKR1C2 Mutations in patients: the backdoor pathway is essential for human sex development

The initial characterization of the backdoor pathway focused on the steroids produced, and the enzymes responsible for the steroidal conversions were inferred. A key mode of identifying human steroidogenic pathways has been from studying diseases resulting from mutations in the genes encoding steroidogenic enzymes and other factors (Miller and Auchus, 2011). The steroidal conversions in the backdoor pathway involve a 5 α -reductase, 17,20 lyase, a 17 β -hydroxysteroid dehydrogenase and both reductive and oxidative 3 α -hydroxysteroid dehydrogenases (3 α HSD) (Fig. 1). Studies with mice lacking 5 α -reductase type 1 (5 α Red1) or 5 α Red2 indicated that the type 1 enzyme participates in the backdoor pathway (Mahendroo et al., 2004), and studies in vitro showed that human P450_{c17} readily catalyses 17,20 lyase conversion of 17OH-Allo to androsterone (Gupta et al., 2003). The 17 β HSD in the backdoor pathway is thought to be 17 β HSD3 (Auchus, 2004), although 17 β HSD5 (AKR1C3) and other forms of 17 β HSD have not been formally excluded. However, the nearly wholly female phenotype in 46,XY patients with complete absence of 17 β HSD3 suggests that absence of this one enzyme suffices to eliminate essentially all androgen production, suggesting it is the principal 17 β HSD in both the backdoor pathway as well as in the classic pathway. The enzymes catalyzing the reductive and oxidative 3 α HSD activities have been less clear; however, substantial data indicate that 3 α HSD activities are catalyzed by members of the AKR1C family of aldo-keto reductases (Couture et al., 2005; Dufort et al., 2001; Jin and Penning, 2006; Penning et al., 2000, 2004, 1996; Rizner et al., 2003) (Fig. 1).

Because many of these enzymatic steps are common to both the classic and backdoor pathways, it has been difficult to determine the relative importance of the backdoor pathway by studying patients with disorders of androgen biosynthesis. The description of 46,XY patients with disordered sexual differentiation due to mutations in AKR1C2 has sustained the hypothesis that both the classic and backdoor pathways are needed for normal human male sexual differentiation (Flück et al., 2011).

5.1. Family A

This family, firstly described in 1972 as having “17,20 desmolase deficiency” (Zachmann et al., 1972) was recently re-investigated. The patients had presented at birth with cryptorchidism and undervirilized external genitalia, and a 46,XY maternal aunt had ambiguous genitalia and no uterus. Studies of urinary steroids

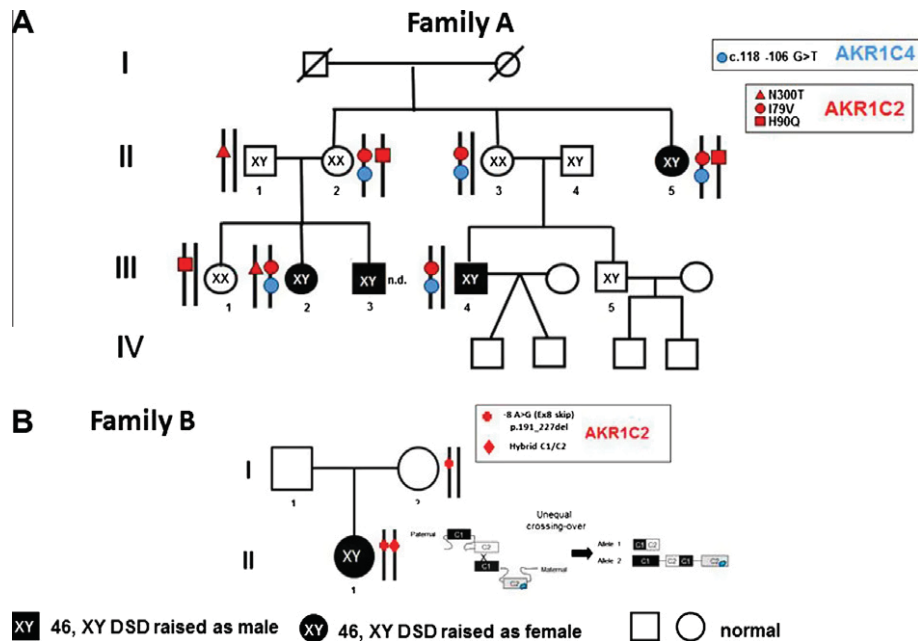


Fig. 2. Genetics of identified AKR1C mutations. A. AKR1C mutations correlate with phenotypic severity in the index family A. Females are unaffected, irrespective of AKR1C mutation status, as androgens are not needed for female sexual development or reproduction. The Ile79Val mutation in AKR1C2 and the AKR1C4 splice mutation co-segregate, suggesting they lie on one allele. A male having one mutation (individual II.1) is normal; a male carrying two mutations on one allele (individual III.4) had impaired fertility, as his reproduction required in vitro fertilization. Two genetic males carrying mutations on both alleles (individuals II.5 and III.2), had severe DSD and were raised as females. B. An unequal crossover event ablates AKR1C2 activity in another individual (Family B) with 46,XY DSD. The AKR1C1 and AKR1C2 genes, which share about 90% nucleotide sequence identity, apparently mis-paired during meiosis (left). The result is that the affected individual's Allele 1 lacked an intact AKR1C2 gene and had only the nonfunctional C1/C2 gene from the crossover. Allele 2 in the affected individual had an intact AKR1C1 gene from the father, the hybrid C1/C2 gene and an intact AKR1C2 gene from the mother. However, the maternal AKR1C2 gene carried the missense mutation H222Q (indicated by the light blue spot); this mutation destroyed all measurable 3 α HSD activity (Flück et al., 2011). N.d. = not done.

indicated normal production of cortisol and impaired production of androgens (Zachmann et al., 1972). Recent studies showed that the affected individuals lacked mutations in the CYP17A1 gene encoding P450c17, cytochrome P450 oxidoreductase (POR), cytochrome b5, steroidogenic factor-1, and the androgen receptor (Flück et al., 2011). Hormonal data from 1972 appeared to be inconsistent with deficiencies in 17 β HSD or 5 α -reductase, hence mutations in an alternative pathway were sought. Genes were investigated encoding the AKR1C enzymes that catalyze the 3 α HSD reactions that are unique to the backdoor pathway, beginning with AKR1C2, an enzyme with reductive 3 α HSD activity (Jin and Penning, 2006; Rizner et al., 2003).

AKR1C2 mutations were found in all affected individuals (Flück et al., 2011) (Fig. 2A). The 46,XY proband with severe undervirilization and a female sex assignment was a compound heterozygote for the AKR1C2 missense mutations I79V and N300T. A 46,XY heterozygote carrying only I79V was moderately undervirilized, and a heterozygote carrying N300T had normal male external genitalia; both fathered children, although the former required in vitro fertilization. The proband's 46,XX mother was a compound heterozygote for I79V and H90Q and was phenotypically normal and bore three children, but her identically affected 46,XY sibling was severely undervirilized, lacked a uterus and was raised as a female. Thus the disorder only affected genetic males (sex-limited recessive); this was an expected result because androgens are not required for female sexual differentiation, puberty or fertility.

The discovery of AKR1C2 mutations did not exclude the possibility of mutations in other genes. A genome-wide scan of microsatellite markers in nine members of the index family identified loci of potential interest on chromosomes 22 and 10. Finding potential loci on these chromosomes, but not elsewhere, excluded several factors potentially involved in androgen synthesis, including cytochrome b₅ on chromosome 18, 5 α Red1 on chromosome 5

and 17 β HSD6 (also known as RoDH) on chromosome 12, an enzyme that may catalyze the oxidative 3 α HSD activity needed to convert 3 α -Diol to DHT (Biswas and Russell, 1997). The microsatellite markers on chromosome 10 with the highest linkage significance (D10S1713 and D10S2382 gave log of the odds scores of 6.3 and 6.2, respectively) lie within the AKR1C locus, providing very strong additional genetic evidence for the involvement of an AKR1C gene. However, the AKR1C locus includes five closely related AKR1C genes (AKR1C1, C2, C3, C4 and CL1), so that it was possible that the identified AKR1C2 mutations were merely polymorphisms in linkage disequilibrium with hypothetical, unidentified mutation(s) in another AKR1C gene(s). This possibility was tested by sequencing the exons of AKR1C1, C2, C4 and CL1 in DNA from the proband, but identified no further mutations (Flück et al., 2011).

AKR1C enzymes catalyze both oxidative and reductive reactions, depending on cofactor availability (Table 1); oxidative reactions require nicotinamide adenine dinucleotide (NAD⁺), whereas reductive reactions require reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Penning et al., 2000; Penning et al., 1996). The AKR1C enzymes have a high affinity for NADP(H) (K_d = 100–200 nM) and a low affinity for NAD(H) (K_d = 200 μ M) (Penning et al., 2000; Penning et al., 1996), making NADP(H) the preferred cofactor in vivo, so that AKR1C2 functions primarily as a reductase. When transfected into COS-1 cells, the available concentrations of cofactors result in AKR1C2 functioning as a reductase, but not as an oxidase (Rizner et al., 2003), and when 1 mM NAD⁺ is supplied to recombinant AKR1C2 in vitro to permit oxidation of 3 α Diol to DHT, the reaction can be blocked by only 10 μ M NADPH (Rizner et al., 2003; Steckelbroeck et al., 2004). Consistent with this co-factor requirement, other members of the AKR1C family do not oxidize 3 α Diol effectively (Penning et al., 2000; Dufort et al., 1996), although under non-physiologic conditions

Table 1
Characteristics of AKR1Cs and HSD17B6.

Enzyme	3 α HSD1	3 α HSD2	3 α HSD3	3 α HSD4	RODH
Directional preference	Reductive	Reductive	Reductive	Reductive	Oxidative
Gene	<i>AKR1C4</i>	<i>AKR1C3</i>	<i>AKR1C2</i>	<i>AKR1C1</i>	<i>HSD17B6</i>
Cofactors	NADP(H)	NADP(H)	NADP(H)	NADP(H)	NAD(H)
Major reactions:					
Reduction					
3 α HSD ^a	High	Moderate	Moderate-high	Moderate	Moderate
3 β HSD ^b	Nil	Nil	Nil	Nil	Moderate
17 β HSD ^c	Nil	moderate-high	nil	low	low
20 α HSD ^d	Low	Low	Low	Moderate-high	Nil
other substrates		DOC, PGF2 α			
Oxidation					
3 α HSD ^a	Low	Nil	Low	Low	High
3 β HSD ^b	Nil	Nil	Nil	Nil	High
17 β HSD ^c	Nil	Low	Nil	Nil	Moderate
20 α HSD ^d	Nil	Nil	Nil	Low	Nil
Tissue distribution	Liver \gg adrenal/gonad	Prostate, breast, liver, adrenal, testis, lung	Liver, prostate, lung, uterus, brain	Liver, testis, lung, breast, uterus, brain	Prostate

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^a Reductive reactions include: DHT \rightarrow 5 α -androstane-3 α ,17 β -diol, 5 α -androstane-3,20-dione \rightarrow androsterone, and 5 α -pregnane-3,20-dione \rightarrow allopregnanolone; oxidative reactions include the reverse reactions.
^b Reductive reactions include: DHT \rightarrow 5 α -androstane-3 β ,17 β -diol, 5 α -androstane-3,20-dione \rightarrow 3 β -androsterone, and 5 α -pregnane-3,20-dione \rightarrow 3 β -allopregnanolone; oxidative reactions include the reverse reactions.
^c Reductive reactions include: androsterone \rightarrow 5 α -androstane-3 α ,17 β -diol, androstenedione \rightarrow testosterone, estrone \rightarrow estradiol; oxidative reactions include the reverse reactions.
^d Reductive reactions include: progesterone \rightarrow 4-pregnen-20 α -ol-3-one (20 α -dihydroprogesterone) and DOC \rightarrow 4-pregnene-20 α ,21-diol-3-one (20 α -dihydroDOC).

in vitro, AKR1C3 can oxidize 3 α Diol to androsterone and AKR1C4 can oxidize 3 α Diol to DHT (Penning et al., 2000; Lin et al., 1997). Thus AKR1C2 functions almost exclusively as a reductive 3 α HSD.

To ascertain the enzymatic consequences of the identified AKR1C2 mutations, each was recreated in an AKR1C2 cDNA expression vector, expressed in bacteria and purified to apparent homogeneity. The activities of these biosynthetic AKR1C2 proteins were assessed by measuring their ability to reduce 5 α DHP to Allo and their ability to oxidize 3 α Diol to DHT, and the data were expressed as the enzymatic maximal velocity divided by the Michaelis constant (Vmax/Km). The activities of AKR1C2 mutants I79V, H90Q, and N300T to convert 5 α DHP to Allo were only modestly reduced, to 82%, 22%, and 41% of wild-type, respectively (Table 2). Similarly, the capacity of these AKR1C2 mutants to oxidize 3 α Diol to DHT was only modestly reduced (to 28–48%) (Flück et al., 2011). Thus, the identified AKR1C2 mutations partially impaired 3 α HSD activity, but not to the degree typically seen in recessive disorders of steroidogenesis. These data thus suggested that another factor in addition to AKR1C2 was disordered in the index family. Because the microsatellite data only pointed to the AKR1C locus, the expression of the other genes in this locus was examined more closely by analysis of leukocyte RNA from the affected family members. As shown by reverse transcription followed by polymerase chain reaction (RT-PCR), the mRNAs for four of the five AKR1C enzymes were spliced correctly, but for AKR1C4 there was both correct splicing and incorrect splicing: both the expected product and a shorter product were seen. Sequencing of the AKR1C4 transcripts showed that the incompletely spliced product lacked all of exon 2; this abnormally spliced AKR1C4 mRNA was found in all affected family members, but not in unaffected controls (Flück et al., 2011). The sequence of the AKR1C4 gene in the genomic DNA of affected individuals was normal, except for a G to T mutation in an intron, 106 bases upstream from exon 2. Most intronic mutations that cause aberrant RNA processing are close to intron/exon borders (for example, in the neurofibromatosis 2 gene, 178 of 183 splicing mutations were within 10 bases of an intron/exon border

(Baser, 2006), hence finding a mutation 106 bases away would not normally be predicted to alter mRNA splicing. However, assessment of this intronic mutation in vitro by an exon trapping assay showed that it resulted in the loss of exon 2 from the AKR1C4 mRNA. When the encoded truncated protein was assayed in vitro, it had only about 10% of WT activity. Thus the disordered androgen biosynthesis in the index family appeared to arise by ‘digenic inheritance’, wherein partial defects in two genes, AKR1C2 and AKR1C4, combine to yield the phenotype (Fig. 2A and Table 2).

5.2. Family B

The central role of AKR1C2 was clarified by finding that an unrelated, 46,XY patient who had female external genitalia and intra-abdominal testes had a complex chromosomal rearrangement in the AKR1C locus that resulted in no functional AKR1C2 enzyme (Flück et al., 2011) (Fig. 2B and Table 2). An unequal crossover between the AKR1C2 gene and the adjacent, 90% identical AKR1C1 gene resulted in the patient having a single C1/C2 hybrid gene on one allele and paternal C1, the C1/C2 hybrid and a maternal C2 on the other allele. However, the intact maternal C2 gene carried the missense mutation H222Q, so that neither allele encoded authentic AKR1C2 (Fig. 2B). When this H222Q mutant was assessed in vitro it lacked any detectable activity to convert 3 α Diol to DHT (Flück et al., 2011). Thus this single patient in an unrelated family had a similar but distinct monogenic disorder in which only AKR1C2 was disordered, but in which the AKR1C2 defect was apparently complete. Thus ablation of all AKR1C2 activity appears to be sufficient to interrupt backdoor testicular synthesis of DHT and cause 46,XY DSD.

6. Sites of DHT synthesis

These studies have expanded and revised the understanding of the sites of fetal androgen synthesis. The testis appears to be the unique site of expression of 17 β HSD3 (Geissler et al., 1994), so that

Table 2
Phenotype/genotype correlation in AKR1C2 mutant subjects.

Subject	Phenotype	Karyotype	Mutation		Function [intrinsic clearance (% of WT)]		
			AKR1C2	AKR1C4	Red		Ox*
					AKR1C2	AKR1C4	AKR1C2
Family A							
II.1	Normal male Fertile	46,XY	N300T/WT	–	41/100	–	28/100
II.2	Normal female Fertile	46,XX	I79V/H90Q	V29_K84del/WT	82/22	10/100	48/41
II.3	Normal female Fertile	46,XX	I79V/WT	p.V29_K84del/WT	82/100	10/100	48/100
II.4	Normal male Fertile	46,XY	–	–	100/100	–	100/100
II.5	Ambiguous external genitalia (Prader III) No Müllerian structures Tall stature Undescended non dysgenetic testes	46,XY	I79V/H90Q	p.V29_K84del/WT	82/22	10/100	48/41
III.1	Normal female	46,XX	H90Q/WT	–	22/100	–	41/100
III.2	Female external genitalia No Müllerian structures Undescended non dysgenetic testes Obesity	46,XY	I79V/N300T	p.V29_K84del/WT	82/41	10/100	48/28
III.3	Hypospadia Cryptorchidism	46,XY	n.d.	n.d.	n.d.	n.d.	n.d.
III.4	Hypospadia Cryptorchidism	46,XY	I79V/WT	p.V29_K84del/WT	82/100	10/100	48/100
III.5	Normal male fertile	46,XY	–	–	100/100	–	100/100
Family B							
I.1	Normal male Fertile	46,XY	–	–	–	–	–
I.2	Normal female Fertile	46,XX	H222Q/WT	–	n.d.	–	undetectable/100
II.1	Female external genitalia No Müllerian structures Undescended non dysgenetic testes	46,XY	H222Q/hybrid	–	n.d.	–	undetectable/n.d.

Intrinsic clearance = Vmax/Km. n.d. = not done.
* There is no detectable oxidative activity of AKR1C4 in our experimental system.

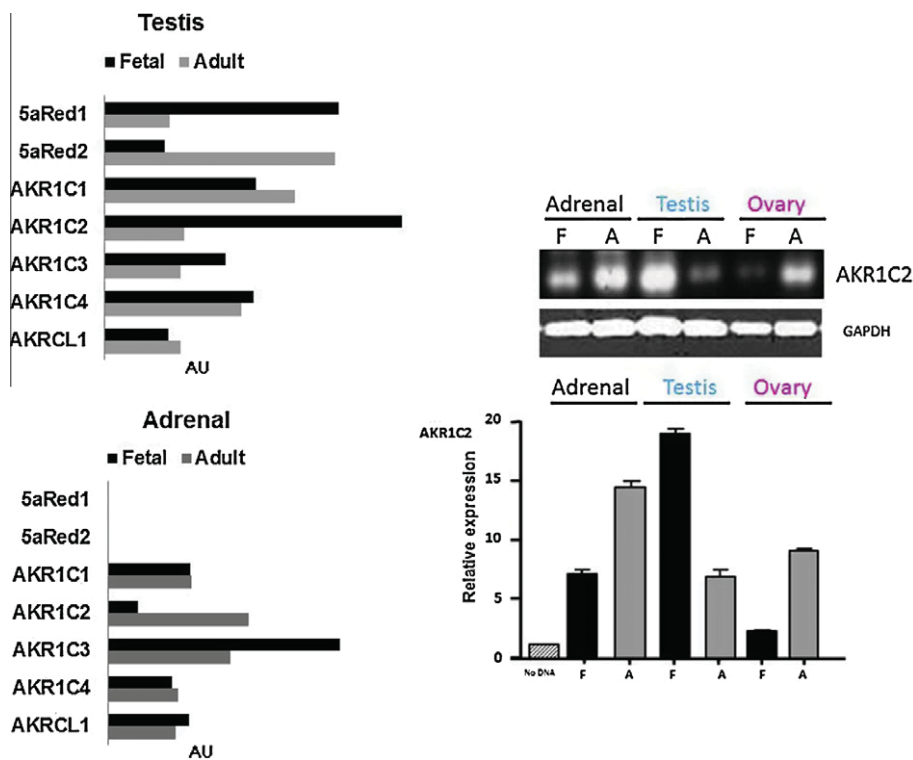


Fig. 3. Relative expression levels of AKR1Cs and 5 α -reductase 1/2 in fetal and adult testis (A) and adrenal (B). C: comparison of qualitative and quantitative AKR1C2 expression levels between fetal (F) and adults (A) human adrenal, testis and ovaries. Data are originally given in 2^{- $\Delta\Delta$ ct} and derive from quantitative RT-PCR performed with specific primers on total RNA extracted from fetal testis, ovaries or adrenal tissues around mid- gestation or tissues from mid-adulthood (Flück et al., 2011).

the testis is the primary site of testosterone biosynthesis, although the fetal adrenal can produce small amounts of testosterone via 17 β HSD5 (Goto et al., 2006). Immunoblotting of human adult

and fetal tissues has shown that 5 α Red2 is expressed in genital skin but earlier studies did not identify either 5 α Red2 or 5 α Red1 in fetal testes (Thigpen et al., 1993); however, more sensitive stud-

ies using RT-PCR found abundant mRNA for 5 α Red1 but not for 5 α Red2 in fetal testes, and abundant 5 α Red2 but not 5 α Red1 in adult testes (Flück et al., 2011). Similarly, RT-PCR showed that AKR1C2 is abundantly expressed in the fetal testis but minimally expressed in the adult testis (Flück et al., 2011) (Fig. 3A). Thus AKR1C2 appears to participate in the backdoor pathway to DHT in the human fetal testis, but not in the adult testis, and its mutation appears to cause incomplete male genital development. RoDH (17 β HSD6) was also detected in the fetal testes, suggesting it may catalyze the oxidative 3 α HSD reaction needed to complete the backdoor pathway to DHT, but this identification remains uncertain. Adult adrenals expressed abundant AKR1C2 (Fig. 3B), so that the backdoor pathway to DHT production may be important in situations where intra-adrenal 17OHP concentrations are high, such as in 21-hydroxylase deficiency. AKR1C2 is not expressed in the fetal ovary, consistent with the fact that androgens are not crucial in female sex development, whereas its mRNA is readily detectable in the adult gonad (Fig. 3C).

7. Modeling structural studies

The AKR1C enzyme family has high amino acid sequence conservation, and the same catalytically important residues are found in the same positions (Asp50, Tyr55, Lys84 and His117) in all family members (Fig. 4A). For catalytic activity, Tyr55 acts as an acid, Lys84 decreases the pKa of the tyrosine, and Asp50 forms a salt-bridge to the lysine. The substrate-binding pocket consists of 5 loops; loop A (117–143), B (217–238), C (299–323), β 1- α 1 (23–32) and β 2- α 2 (51–57); allowing binding of structurally diverse substrates through an “induced-fit” mechanism (Couture et al., 2005). Two of the amino acids that were mutated in our patients, Ile79 and Asn300, are at the beginning of loops that make up the steroid binding pocket (Fig. 4B); amino acid changes at these locations may disturb the Lys84, Trp86 and Leu308 residues further down the loops that interact with the substrates at the catalytic site. The His90 residue that was found to be mutated is in close proximity (5.2Å) to His117 and in contact with Ile116 and Phe118, which are part of catalytic center. In our analysis, the His117-NADPH contact was broken in the His90Gln variant, potentially from the additional interactions of Gln90 with side chains of neighboring residues. His117 is essential for the activity in all AKR family members, as it interacts with a central acidic ion to act as a bridge between steroid substrate and NADPH. A change in interaction between NADPH and His117 will likely affect co-factor binding and electron transfer. Comparison of the structures of rat AKR1C9 and human AKR1C2 shows that changing His222 to Ser222 pushes the side-chain of neighboring Trp227, resulting in testosterone binding deeper than in human AKR1C2, where testosterone binds closer to surface. The substitution of His for Gln (His222Gln), as found in one of our patients, does not shift the side-chain of Trp227, so that the steroid binding pocket remains unchanged. However, flipping of the side-chain of Gln222 pushed it into the NADPH binding tunnel (Fig. 4B), potentially blocking NADPH entry. In human AKR1C3 and AKR1C4, His222 is naturally replaced with Gln222, but this change is accommodated by other amino acid replacements (as compared to AKR1C2) that may alter the co-factor binding patterns. A major factor in AKR1C2 reactions is the release of cofactors following catalysis; minor changes in charge caused by Asn300Thr may influence how a particular steroid or co-factor could exit the catalytic site. An enzyme with slower release of product or co-factor will impair binding of the next molecule of substrate or the co-factors. The three-dimensional model of AKR1C2 and C4 with mutations is depicted in Fig. 4.

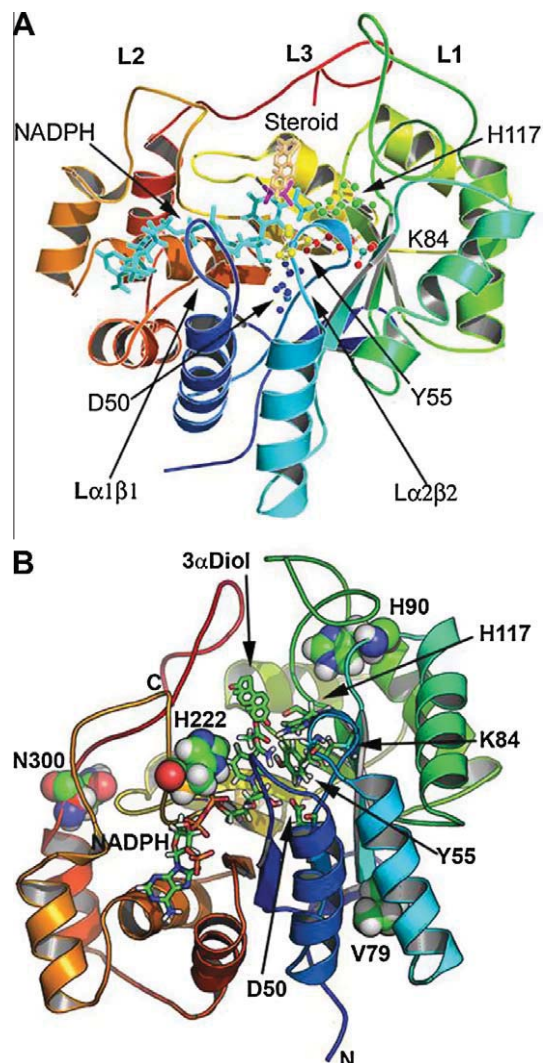


Fig. 4. AKR1C2/4 Structure and Mutations. A The AKR enzyme family has high amino acid sequence conservation, and the same catalytically important residues are found in the same positions (Asp50, Tyr55, Lys84 and His117) in all family members. For catalytic activity, Tyr55 acts as an acid, Lys84 decreases the pKa of the tyrosine, and Asp50 forms a salt-bridge to the lysine. The substrate-binding pocket consists of 5 loops; loop A (117–143), B (217–238), C (299–323), β 1- α 1 (23–32) and β 2- α 2 (51–57); allowing binding of structurally diverse substrates through an “induced-fit” mechanism (Couture et al., 2005). Molecular dynamic simulation analysis of the AKR1C4 structure revealed movements of short flexible fragments comprising AA 9–14 and 50–59, opening and closing access to the catalytic center. A model of AKR1C4 lacking the 56 amino acids (Val29–Lys84) encoded by exon 2 results in a stable structure that may still bind NADPH, but which lacks several catalytically essential residues, rendering the truncated mutant inactive. The active site tyrosine (Tyr55) and Asp50, Leu54 and Lys84 are missing from the truncated AKR1C4 mutant. Leu54 is potentially involved in determining substrate specificity in AKR1C4, and Asp50 and Lys84 together balance the pKa of the active site similarly to their role in AKR1C2, and are necessary for transferring electrons from NADPH to the steroid substrate. B. Ile79 and Asn300 (mutated in some patients) are near the steroid-binding pocket; mutations may disturb the interaction of the steroid with the catalytic site. His90 is in close proximity (5.2Å) to His117, which acts as a bridge between the steroid substrate and NADPH. The His117-NADPH contact was broken in the His90Gln mutant. His222 is situated in loop B, which is involved in binding the steroid and the co-factors. The His222Gln mutation results in the side-chain of Gln222 to be pushed into the NADPH binding tunnel, potentially altering interaction with NADPH. The Asn300Thr mutation is adjacent to Arg301 (whose mutation is important for steroid binding by providing base-acid potential with Tyr55), and may influence how a particular steroid or co-factor could enter or exit the catalytic site due to a change in the charge/hydrophobicity of the steroid-binding tunnel.

Molecular dynamic simulation analysis of the AKR1C4 structure revealed movements of short flexible fragments comprising AA 9–14 and 50–59, opening and closing access to the catalytic center. A

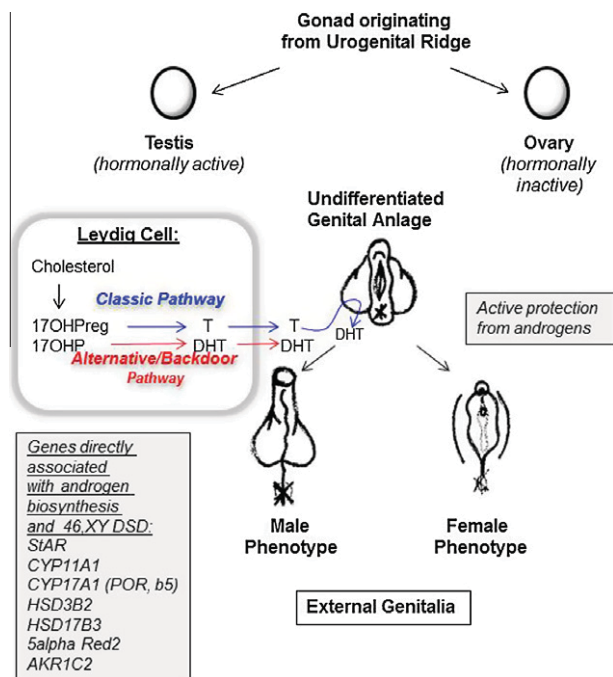


Fig. 5. Role of androgen biosynthesis in the sexual differentiation of the external genitalia of the human fetus. After 6–8 weeks gestation the bipotential gonad develops either into a testis or an ovary. While the ovary remains hormonally quiescent, the testis becomes hormonally active and produces androgens in the Leydig cells. Previously it was thought that intratesticular androgen synthesis stops at the level of testosterone (T) production which will then reach the skin of the external genitalia by circulation where the more potent dihydrotestosterone (DHT) will be formed through the activity of 5 α -reductase type 2. We now have data indicating that the fetal testis produces DHT intratesticularly through the alternative/backdoor pathway (Fig. 1) and that this DHT production seems to be required for the normal differentiation of the male external genitalia. Thus we add a novel genetic cause, *AKR1C2* deficiency, to the list of 46,XY disorders of sexual development (DSD) associated with androgen biosynthesis.

model of *AKR1C4* lacking the 56 amino acids (Val29-Lys84) encoded by exon 2 results in a stable structure that may still bind NADPH, but which lacks several catalytically essential residues, rendering the truncated mutant inactive. The active site tyrosine (Tyr55) and Asp50, Leu54 and Lys84 are missing from the truncated *AKR1C4* mutant. Leu54 is potentially involved in determining substrate specificity in *AKR1C4*, and Asp50 and Lys84 together balance the pKa of the active site similarly to their role in *AKR1C2* (Fig. 4A), and are necessary for transferring electrons from NADPH to the steroid substrate.

8. Conclusions

The conventional model of male external genital development has been that fetal testicular testosterone is a circulating hormone that is converted to DHT by 5 α Red2 in genital skin, which then acts as a paracrine factor to stimulate fusion of the labio-scrotal folds (Figs. 1 and 5). The essential role of testicular testosterone is confirmed by the complete lack of virilization in individuals with mutations in *StAR* (Bose et al., 1996), *P450scc* (Tajima et al., 2001; Kim et al., 2008) and *P450c17* (Auchus, 2001) and the nearly complete lack of external genital development in persons with severe deficiencies of 3 β HSD2 (Moisan et al., 1999; Rheume et al., 1992) and 17 β HSD3 (Geissler et al., 1994). Similarly, the necessary but not sufficient role of DHT synthesized in genital skin is evidenced by the incomplete virilization in persons with defective 5 α Red2 (Russell and Wilson, 1994). However, it has long been known that this model is incomplete (Wilson et al., 2002). The

finding that human *AKR1C2* mutations cause disordered sexual differentiation hints to the fact that DHT produced in the fetal testis via the backdoor pathway seems to act as a hormone that collaborates with DHT produced in genital skin by 5 α Red2 to induce labio-scrotal fusion. Thus both the classic and backdoor pathways participate in human fetal androgen synthesis, and DHT acts in male genital development as both a paracrine factor and as a hormone; both pathways appear to be needed for male differentiation of the urogenital sinus and external genitalia.

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