The molecular basis of male sexual differentiation

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Abstract

Male sexual differentiation is the result of complex mechanisms involving developmental genetics and endocrinology. Formation of the bipotential gonads and subsequently the testes is dependent on a series of sex chromosome-linked and autosomal genes. The testes secrete both peptide and steroid hormones essential for the formation of internal and external genitalia. Hormone action is mediated via specific receptors, functioning as transcription regulators. Disruption of these genetic events leads to sexual dimorphism involving external and internal genitalia, and may also interfere with the development of other organs.

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Introduction

Male sexual differentiation can be divided into several steps. The genetic sex is mediated through the chromosomal set, which is usually 46,XY. This chromosomal pattern is the beginning of a cascade of genetic events leading to the development of the male gonads, the testes. This is referred to as the gonadal sex. The gonads, in turn, secrete both steroidal as well as peptide hormones which are essential for the development of the internal and external genitalia. Hormone action mediates the phenotypic sex. The term sexual determination is also used for the developmental processes leading to global testicular function. In contrast, sexual differentiation describes the specific hormone actions leading to the sexual phenotype of an individual. This accounts both for the development of the internal and external genitalia as well as the progression of sexual maturation during puberty. The gender of an individual is the sex of assignment and usually depends both on normal sexual determination and differentiation.

Sexual determination

About 40 years ago it was recognised that the presence of the Y-chromosome is usually associated with male development. However, rare sex reversal may occur producing a male phenotype with the 46,XX karyotype or a female phenotype with 46,XY karyotype. Even more rarely, differentiation of both gonadal structures, testicular and ovarian tissue, occurs in true hermaphroditism. In the latter cases, the chromosomal set may be 46,XX or 46,XY or a chromosomal mosaic or chimera (1).

The development of the gonadal, adrenal, urogenital, and renal systems are closely linked. Several genes are known to be involved in this process leading to the creation of the undifferentiated gonad (Fig. 1). Abnormalities in the Wilms' tumour 1 (WT1)-gene are associated with failure of gonadal differentiation, nephropathy, development of Wilms' tumours (in Denys-Drash syndrome) and gonadoblastoma (in Frasier syndrome), and in the Wilms' tumours, aniridia, genital abnormalities, mental retardation (WAGR) syndrome they are associated with anomalies of the eye (aniridia) and mental retardation (1–4). The role of the WT1-gene in gonadal differentiation is not sex specific, as gonadal dysgenesis also occurs in 46,XX individuals. This suggests its importance in the formation of the bipotential gonad rather than in testicular development. However, the genital abnormalities in Denys-Drash syndrome are limited to 46,XY patients.

Another gene involved in the development of the bipotential gonad and the kidneys is the recently cloned LIM1-gene (2). Homozygous deletions in this gene in mice lead to developmental failure of both gonads and kidneys. To date, no human mutations have been described in this gene, although a phenotype of renal and gonadal developmental defects in association with brain abnormalities might be perceived.

Exciting new implications for the role of steroidogenic factor 1 (SF1) in gonadal formation have recently been reported (5). SF1 is the product of the FTZ1-F1-gene and is believed to be a nuclear orphan hormone receptor due
to the presence of two zinc fingers and a ligand binding domain in its molecular structure (6). FTZ1-F1 mRNA is present in the urogenital ridge which forms both gonads and adrenals, and is also found in developing brain regions. Mice lacking SF1 fail to develop gonads, adrenals, and the hypothalamus. Recently, a phenotypically female patient with a 46,XY karyotype was described, who had presented with primary adrenal failure during the first weeks of life. Normal Müllerian structures were found on ultrasound, and no androgenic response was elucidated after human chorionic gonadotrophin (hCG) stimulation. Histology of the gonads revealed poorly differentiated tubules and connective tissue. Within the FTZ1-F1-gene, a heterozygous deletion was characterised which eliminated binding of SF1 to a canonical binding site (5). This case demonstrates the important role of SF1 for normal adrenal and gonadal formation. SF1 is probably also involved in other aspects of sexual development, as it regulates enzymes mediating steps in steroid formation as well as the transcription of the anti-Müllerian hormone (AMH) (7, 8).

Further progression from the bipotential gonad towards testicular differentiation is mediated through gonosomal and autosomal genes (Fig. 1). It was long believed and has now been proven that a specific testis-determining factor (TDF) was essential for testicular development, and that the encoding gene was located on the Y-chromosome. This gene, termed sex-determining region of the Y-chromosome (SRY) is a single-exon gene which encodes a protein with a DNA-binding motif that acts as a transcription factor and which, in turn, regulates the expression of other genes (9). Evidence has been provided that SRY binds to the promoter of the AMH-gene and also controls the expression of steroidogenic enzymes (10). Thus, SRY probably induces the expression of AMH to prevent the formation of Müllerian duct derivatives. Indication that SRY is the TDF was demonstrated when Koopman et al. (11) introduced the mouse homologue sry into female mouse embryos and produced a normal male phenotype in these genetically engineered animals. Furthermore, naturally occurring mutations of SRY have been described in humans and are usually associated with a complete sex reversal in 46,XY individuals. However, in two cases, the development of both ovarian and testicular tissue in true hermaphroditism has been described in association with mutations of SRY (12, 13).

Autosomal genes related to SRY in their genetic structure and which, to some extent, are involved in testicular development have been described. SOX (SRY-box-related) 9 is connected with chondrogenesis and gonadal differentiation. This gene is transcribed especially following SRY expression in male gonadal structures. Additionally, SOX 9 is an activator of the type II collagen gene which, in turn, is essential for formation of the extracellular matrix of cartilage. Therefore, defects in SOX 9 lead to sex reversal in 46,XY individuals, as well as to skeletal malformations known as campomelic dysplasia (2).

A gene which is involved in adrenal as well as ovarian and testicular development is DAX 1 (8, 14). This gene is located on the X-chromosome and was termed the dosage-sensitive sex reversal locus-adrenal hypoplasia congenita-critical region on the X, gene 1. DAX 1 is expressed during ovarian development but is suspended during testicular formation, implying a critical role for this gene in ovarian formation. Interestingly, DAX 1 is repressed by SRY during testicular development. However, if a duplication of the DAX 1 region on Xp21 is present in a 46,XY patient and the activity of its gene product is thus enhanced, testicular formation is hindered. In contrast, mutations in DAX 1, diminishing its activity, lead to a lack of adrenal formation and to hypogonadal hypogonadism in congenital adrenal hypoplasia (2).

Several other factors may play an important role in male sexual determination. Deletions of chromosomes 9p and 10q have been associated with sex reversal in 46,XY individuals. In the case of chromosome 9p, associated malformations included facial abnormalities such as premature closure of the frontal suture, in addition to hydrenephrosis and developmental delay (15). On chromosome 9p24.3, two genes named DMRT1 and DMRT2 were identified (16). Both genes were deleted in some cases of sex-reversing 9p deletions, suggesting that gonadal dysgenesis might be due to combined hemizygosity of DMRT1 and DMRT2. Also, terminal deletions of chromosome 10q have been
associated with genital malformations in association with other phenotypic abnormalities and mental retardation (17).

Defects in developmental genes responsible for gonadal differentiation lead to a complete or partial gonadal dysgenesis and, in turn, to a global failure of testicular function. Thus, in the phenotypic sex, both internal and external genitalia are abnormal (1). Associated malformations of the adrenal, urogenital, skeletal, or central nervous systems will aid with the identification of the responsible gene. However, in the majority of partial gonadal dysgenesis in 46,XY patients, no genetic defect can be distinguished today. Several other, as yet, unknown genetic factors have been implicated in testicular differentiation. The characterisation of these genes in the, hopefully, near future will be fundamental to the diagnosis, treatment, and counselling of patients with a sexual determination disorder.

**Sexual differentiation**

**Anti-Müllerian hormone**

In early gestation, both the anlagen for the Wolffian and Müllerian ducts are present in the fetus regardless of the karyotype. If testicular formation is unhindered, the Sertoli cells will produce AMH. To exert the action of AMH, high concentrations of this hormone and active binding to a membrane receptor in the mesenchymal cells surrounding the Müllerian ducts are necessary (4). Therefore, reduced excretion of AMH due to a lowered number of Sertoli cells is responsible for partial uterus formation in sex determination disorders (1). The AMH-gene is under the transcriptional control of several other proteins involved in sexual differentiation (18). SF1 binds directly to the AMH-gene promoter and activates its transcription in the Sertoli cells (19). Also, a regulatory effect of SRY on AMH receptor expression has been reported (20). Both lack of AMH, as well as insensitivity to this hormone, have been described in human disease. In ‘persistent Müllerian duct syndrome’ (PMDS), 46,XY males are characterised by the presence of fallopian tubes and uterus. The external genitalia are unequivocally male, as steroid hormone formation is normal. In AMH deficiency, mutations within the AMH-gene have been demonstrated (4). While patients with AMH deficiency have low AMH levels in serum, in approximately 50% of the cases AMH is within the normal range or even elevated (4). Thus, a receptor defect is assumed. The type II AMH receptor, which is necessary for binding of ligand and exertion of AMH action, has been cloned, and functionally relevant mutations have been demonstrated in patients with PMDS (4). The AMH-type II receptor gene has been localised on chromosome 12. PMDS due to both AMH and AMH-type II receptor gene mutations is inherited in an autosomal recessive fashion.

**Luteinizing hormone receptor**

Undiminished androgenic steroid hormone formation and action is necessary for the development of the external genitalia. Testosterone synthesis in the developing testes is controlled during early fetal life by hCG and only later by the fetal luteinizing hormone (LH) itself (21) (Fig. 2). Both hCG and LH stimulate testosterone synthesis via the LH receptor (LHR). The LHR belongs to a family of G-protein-coupled receptors with seven transmembrane helices (22). It has a long extracellular domain involved in ligand binding, in contrast to other receptors of this family, e.g. the thyrotrophin receptor. The genetic organisation was elucidated in 1995. The gene is localised on chromosome 2p21 and spans over 90 kb, with a coding region divided into 11 exons (22, 23). Naturally occurring mutations within the LHR have been demonstrated to result in both loss as well as gain of function, depending
on their localisation (24–26). Inactivating mutations of the LHR are associated with gonadotrophin unresponsiveness and lead to Leydig cell agenesis and, subsequently, to defective sexual differentiation. More often, the result is a completely female phenotype, but incomplete virilisation due to partial receptor responsiveness with subnormal androgen synthesis has been described (21). These mutations are typically located in the transmembrane domain of the receptor; however, mutations within the extracellular domain have also been reported to result in loss of function. These molecular abnormalities imply an active role for the LHR in Leydig cell growth and differentiation. In contrast, constitutive activation of the LHR leads to normal male phenotype; however, precocious pseudo-puberty occurs due to mutation of the LHR with excessive secretion of testosterone by the Leydig cells (familial testotoxicosis). Microscopically, Leydig cell hyperplasia is evident in the testes of the respective patients. Activating mutations of the LHR are also located in the transmembrane domain; moreover, they are frequently located near the third intracellular loop of the receptor (21, 24, 26).

Early steps of androgen synthesis

The early steps of steroid synthesis are shared between glucocorticoids, mineralocorticoids, and sex steroids. Thus, defects within the first steps of steroid synthesis will affect either all or at least two of the final metabolites within the testes and the adrenals (27) (Fig. 3). Steroid hormones are synthesised from cholesterol within the mitochondria. The acute stimulation of steroid synthesis is mediated by the steroidogenic acute regulatory protein (StAR), which is an active transporter of cholesterol through the inner mitochondrial membrane. Mutations within StAR lead to severe lack of adrenal steroidogenesis, as well as lack of virilisation in 46,XY individuals, in lipid congenital adrenal hyperplasia (28). Intra-uterine survival of affected children is possible because placental steroidogenesis is not StAR dependent. Due to accumulation of cholesterol, both adrenals and testes are further damaged and the residual non-StAR dependent steroid synthesis is also diminished. Therefore, low levels of steroids may be measurable at birth, but these may be depleted later (27). The StAR gene has been cloned and several mutations have been characterised in patients with lipid congenital adrenal hyperplasia (28). StAR mutations, as with all other genetic defects of androgen biosynthesis, are inherited in an autosomal recessive fashion, and both genetic female and male individuals can be affected.

The first enzymatic step in steroid synthesis from cholesterol to pregnenolone is mediated by the mitochondrial cytochrome P450 enzyme which cleaves the cholesterol side chain (P450ccc). Until now, no naturally occurring mutations associated with human disease have been described in this enzyme (29). It has been postulated that such a defect would also, in contrast to defects in StAR, affect placental steroid synthesis and, therefore, these fetuses would not survive. This does not hold true for the other enzymes of early androgen biosynthesis. The P450c17 enzyme is a qualitative regulator of steroid synthesis with two distinct activities (27). Both the 17α-hydroxylase activity as well as the 17/20-lyase activity can be differentially regulated (30). While the 17α-hydroxylase catalyses the conversion of pregnenolone to 17-OH pregnenolone, and the conversion of progesterone to 17-OH progesterone, the 17/20-lyase activity is necessary for the enzymatic reaction from 17-OH pregnenolone to dehydroepiandrosterone, and from 17-OH progesterone to androstenedione (Fig. 3). P450c17 is

![Figure 3 Pathways and metabolites of androgen biosynthesis.](www.eje.org)
encoded by a single copy gene on chromosome 10q24.3 and mutations within this gene can inhibit either both functions or, selectively, the 17/20-lyase activity of the resulting protein. Only recently, patients with isolated 17/20-lyase deficiency have been described (31, 32). The underlying molecular abnormalities within the p450c17 protein result in a severely diminished 17/20-lyase activity, but only moderately inhibited 17α-hydroxylase function. Interestingly, 17/20-lyase activity depends largely on the phosphorylation of the protein, and dephosphorylation of P450c17 may be a major factor in isolated 17/20-lyase deficiency (27).

The third important enzyme of ubiquitous steroidogenesis which also plays a major role in androgen biosynthesis is 3β-hydroxysteroid dehydrogenase (3β-HSD). It catalyses the formation of Δ4 steroids, including that of androstendione which is the major precursor of testosterone. Two isoforms of this enzyme in humans have been cloned. The type 1 enzyme catalyses the formation from pregnenolone, dehydroepiandrosterenedione, and 17-OH pregnenolone of progesterone, androstenedione, and 17-OH progesterone (Fig. 3). The type II 3β-HSD shares 90% sequence homology, but has a lower catalytic efficiency. Both genes are located on chromosome 1p13.1 and consist of four exons. In 3β-HSD deficiency, mutations within the gene encoding for the type II enzyme have been found. 3β-HSD type II is predominantly expressed in the adrenals and gonads; hence, its blockade results in congenital adrenal hyperplasia and, in males, in defective virilisation (33) (Fig. 3).

**Late steps of androgen biosynthesis**

These enzymatic reactions are solely confined to androgen synthesis and do not inhibit glucocorticoid and mineralocorticoid formation. While 17β-hydroxysteroid dehydrogenase (17β-HSD) converts androstendione to testosterone within the testes, 5α-reductase (5α-R) catalyzes the conversion of testosterone to dihydrotestosterone (DHT) in the peripheral target tissues (34). At least five different isoenzymes of 17β-HSD exist (35). Only mutations in the type 3 enzyme have been demonstrated to be responsible for defective sex differentiation in patients with 17β-HSD deficiency (36–39). This disorder is characterised by a severe virilisation defect in 46,XY individuals who, however, show strong signs of virilisation during puberty with marked phallic enlargement (36). The 17β-HSD 3-gene is located on chromosome 9p22, spanning over 11 exons and encoding a protein of 310 amino acids. To date, 17 different mutations have been described in the 17β-HSD 3-gene, in association with the clinical findings of 17β-HSD deficiency (36–42). This enzyme is expressed only in the testes, compatible with its important role in testicular androgen formation. However, no strict genotype–phenotype correlation could be demonstrated in 17β-HSD deficiency. Whether the other 17β-HSD isoenzymes play a critical role in the phenotypic expression of 17β-HSD deficiency due to decreased peripheral conversion of testosterone to androstenedione, remains to be investigated (35).

In contrast, further conversion of testosterone to DHT is catalysed in the peripheral target tissues and not within the gonads. The two isoenzymes of 5α-reductase are expressed in diverse tissues; however, in genital structures the type 2 5α-reductase is more abundant (34). The type 1 enzyme is necessary for the reduction of androgens which inhibits excess formation of oestrogens, and thus mice lacking this enzyme fail to uphold normal pregnancies (43). A specific role of the 5α-reductase type 1 enzyme in male sexual differentiation has not been demonstrated. In contrast, several mutations have been described in the type 2 enzyme in patients with defective virilisation (34, 42, 44). The underlying gene has been localised on chromosome 2p23 and is divided into 5 exons. In 5α-reductase deficiency, DHT formation is severely diminished (44). However, testosterone levels are normal or even elevated. Affected 46,XY individuals are usually born with ambiguous external genitalia, but the phenotype may be highly variable (44–46). The differentiation of Wolffian structures, which is largely dependent on testosterone, is not obstructed. At the time of puberty, strong virilisation may occur due to high endogenous testosterone levels. However, gynaecomastia due to oestrogen excess has rarely been described (45).

**Molecular mechanisms of androgen action**

Androgen action on target tissues is dependent on normal expression of a functionally intact androgen receptor (AR). The AR is a hormone-activated DNA-binding transcription factor of androgen-regulated target genes. Transcriptional regulation of target genes by steroid receptors is a complex and, so far, not completely understood molecular mechanism involving hormone binding, receptor phosphorylation, dissociation of heat-shock proteins, dimersisation, intracellular trafficking and nuclear translocation, DNA binding and transcription activation, resulting in a variety of biological effects in various tissues (Fig. 4) (47, 48). Several groups have cloned and sequenced the gene for the human AR (49–53). This gene has been mapped to Xq11–12 (53). It spans about 90 kilobases (kb) and comprises 8 exons, named 1–8 or A–H. The predominantly used open reading frame encodes for a protein migrating at about 110 kDa consisting of between 910 and 919 amino acids. However, in various genital and non-genital tissues, a different 87 kDa isoform of the AR has been detected, comprising about 4–26% of the total AR protein level (55–57). With respect to similar observations on the progesterone receptor (PR), these isoforms have been termed AR-B (110 kDa) and AR-A (87 kDa) respectively. AR-A appears to use the first internal methionine start site at codon

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188 for initiation of translation. The physiological significance of AR-A is not clear as yet. Androgen insensitivity patients, bearing premature stop codon mutations upstream of codon position 188, have been described as expressing AR-A as the only AR isoform. However, these patients did not show any significant degree of virilisation due to expression of AR-A (58, 59).

With respect to similar observations on the PR, as well as co-expression studies on N-terminal truncated rat AR, AR-A may act as a dominant inhibitor of transactivation on the full-length AR-B (60, 61).

The AR is divided into three major functional domains, which are similar to those identified within other members of the steroid receptor superfamily. A large N-terminal domain precedes the DNA binding domain, followed by the C-terminal hormone-binding domain (62). Additional functional subdomains have been identified by in vitro investigation of specifically truncated, deleted or point mutated ARs.

Upon entering their target cells, androgens bind specifically to the inactive AR, which is usually located within the cytoplasm before ligand binding (63, 64). This results in dissociation of different proteins from the AR which are initially associated with steroid receptors in a heteromeric complex (e.g. heat shock proteins) (65) and thus promotes the activation and nuclear translocation of the AR. An important further step in the transactivation cascade prior to receptor binding to target DNA consists in homodimerisation of two AR proteins. This androgen-dependent process is mediated by distinct sequences within the second zinc finger of the DNA-binding domain as well as through specific structural N-C-terminal interactions (66). The AR homodimer consequently binds to hormone responsive elements (HRE) which usually consist of two palindromic (half-site) sequences within the promoter region of androgen regulated target genes. At this stage, a complex interaction of the receptor with other, not completely known, transcription factors is initiated, modulating the assembly of the basal transcription machinery. This results in up- or downregulation of gene transcription, eliciting specific biological effects (48, 66) (Fig. 4).

Inhibition of androgen action due to end-organ resistance to androgens is the essential pathophysiological mechanism underlying androgen insensitivity syndrome (AIS). The first experimental evidence supporting this assumption came from the demonstration of reduced specific androgen binding to cultured genital skin fibroblasts of affected individuals (67). With the cloning of the AR-gene, it rapidly became clear that mutations of the AR-gene resulting in defective function of the AR represented the molecular genetic basis of the disease (54, 68, 69). Due to the X-chromosomal recessive inheritance, only genetic male individuals (46,XY) are affected by AIS, while female carriers may be conductors. The general pattern of clinical symptoms observed in AIS patients results from the combination of defective androgen action in androgen dependent target tissues, despite normal or even elevated testicular androgen secretion, and the normal ability of the fetal testes to produce AMH. Thus, AIS patients...
show defective masculinisation of the external genitalia with defective Wolffian duct development, in conjunction with usually absent Müllerian duct derivatives (70).

The partial androgen insensitivity syndrome (PAIS) is based on partial impairment of AR function (71, 72). The considerable variability in the degree of impaired AR activity accounts for the wide clinical spectrum of external undervirilisation observed in PAIS (73). Wolffian duct structures may be partly or entirely present. In the complete androgen insensitivity syndrome (CAIS), any in vivo androgen action is totally abolished due to complete inactivation of the AR. Therefore, these patients have a normal female external genitalia with a short and blind-ending vagina. At puberty, CAIS patients acquire a normal female body shape and they show normal breast development. This is caused by increasing oestradiol levels due to elevated testosterone biosynthesis during puberty and its conversion to oestradiol by aromatisation. Usually, any pubic or axillary hair is absent.

Ten years after cloning the AR-gene, more than 300 different mutations have been identified in AIS. No obvious mutational hotspots exist. Large structural alterations of the AR may result from complete or partial deletions of the AR-gene (74, 75). Such extensive molecular events usually result in severe functional defects of the AR being associated with a CAIS phenotype. Intriguingly, a deletion of exon 4 coding for the N-terminal part of the ligand binding domain was reported in an infertile male (76). Smaller deletions may also result in substantial structural changes of the AR due to the introduction of a frame shift into the open reading frame, initiating a premature translation termination codon further downstream (77). A similar effect results from the direct introduction of a premature translation termination codon. Both the indirect and the direct formation of premature stop codons due to germ-line mutations have usually been associated with a CAIS phenotype (72). Extensive disruption of the AR protein structure with a severe loss of AR activity can also be due to mutations leading to aberrant splicing of the AR mRNA. However, as aberrant splicing can be partial, enabling expression of the wild type receptor, the AIS phenotype is not necessarily CAIS but might also present with PAIS (78–80). The most commonly observed molecular alterations of the AR-gene are missense mutations predicting for an isolated amino acid exchange. Most frequently, point mutations have been detected in exons 2–8 while in exon 1 only a few mutations altering the amino acid sequence have been described (81). Missense mutations may result in either CAIS or in PAIS because of complete or only partial loss of AR function. Depending on the localisation of the amino acid exchange within the AR, various molecular mechanisms altering AR activity have been elucidated. Mutations within the ligand binding domain may alter androgen binding but may, in addition, influence dimerisation due to disruption of N-C-terminal structural interactions (82–84). Mutations within the DNA binding domain have been demonstrated to affect receptor binding to target DNA (85). Mutations may also impair AR mRNA stability, leading to additional dysfunction of androgen action (86). Stronger virilisation than expected from the function of the mutant AR may result if de novo mutations occur at the post-zygotic stage leading to somatic mosaicism, thus enabling expression of the wild type receptor in a subpopulation of somatic cells (59, 87, 88). The functional role of structural alterations of the N-terminal transactivation domain in male sexual differentiation is not yet completely understood. Extreme elongation of the polyglutamine stretch (48–75 repeats) has been demonstrated in spinal and bulbar muscular dystrophy with a proposed selective gain-of-function mechanism accounting for increasing neurotoxicity with increasing length (89–91). However, moderate expansion of this trinucleotide segment (>30) may also lead to moderate inhibition of transcriptional activity and, therefore, result in mild symptoms of androgen insensitivity such as gynaecomastia or impaired spermatogenesis (92, 93).

In the future, the identification of specific androgen regulated genes as well as the consideration of genetic factors other than the AR or the androgen level alone that may affect AR signalling (94–96), will lead to further understanding of the physiology and pathophysiology of androgen action in male sexual differentiation.

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