DEVELOPMENT OF THE ADRENO-GENITAL SYSTEM

Female sex determination, ovarian and adrenal gland ontogeny regulated by *Wnt-4* in mice

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Abstract

Although the genetic sex of an embryo is determined at conception by the presence or absence of the Y chromosome, both females and males have bipotential, undifferentiated gonads early in their development. Genes and testicular hormones direct differentiation into either testes or ovaries. The first relevant gene to be identified was the Y-linked master regulatory gene, SRY, since when several other genes have been found to be of importance for sex determination.

The primary aim here was to identify the role of *Wnt-4* in the development of the gonad and adrenal gland. *Wnt-4* was found to be expressed in the developing gonad, the Müllerian duct and the adrenal gland, in addition to the kidney, pituitary gland and mammary gland as observed earlier. Expression in the gonad was found to be regulated in a sex-specific manner. After sex determination *Wnt-4* was downregulated in the testis, but the expression persisted until birth in the ovary. *Wnt-4*-deficient female mice demonstrated a partial female-to-male sex reversal and a reduction in the number of oocytes, while the Müllerian duct was absent from both sexes. Lack of *Wnt-4* in the adrenal gland led to reduced aldosterone production, indicating abnormal development of the *zona glomerulosa*. Flutamide administration to pregnant *Wnt-4* heterozygote females was shown to partially restore the sex reversal.

The results suggest that female development is not a default pathway but needs active signalling, in which *Wnt-4* plays an essential role.

Keywords: gonads; ovary, knock out mice

You can get anywhere,
take one step and
repeat it as many times as needed
(unknown)

To Toni and Venla

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Oulu, October 2002 Minna Heikkilä

Abbreviations

ACTH	adrenocorticotropic hormone
AHC	adrenal hypoplasia congenital
AMH	anti-Müllerian hormone
ARID	A-T-rich interaction domain
bp	base pair
CAH	congenital adrenal hypoplasia
CD	campomelic dysplasia
CRH	corticotrophin releasing hormone
Desrt	developmentally and sexually retarded with transient immune abnormalities
DHEA	dihydroepiandrosterone
DDS	Denysh-Drash syndrome
DHT	dihydrotestosterone
dpc	days post coitum
DSS	dosage-sensitive sex reversal
Dsx	doublesex gene
ERα	estrogen receptor alpha
ER	estrogen receptor beta
ERKO	estrogen receptor knock-out mouse
Fgf9	fibroblast growth factor 9
FSH	follicle-stimulating hormone
GCNA-1	germ cell nuclear antigen 1
GD	gonadal dysgenesis
GnRH	gonadotropin-releasing hormone
hh	hedgehog

HMG	high mobility group
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
Insl3	insulin-like factor 3
-KTS	without lysine-threonine-serine
+KTS	with lysine-threonine-serine
LDL	low density lipoproteins
LH	luteinizing hormone
MIS	Müllerian inhibitory substance
NHR	nuclear hormone receptor
Pax2	paired box-containing gene 2
PBS	phosphate buffered saline
PcG	polycomb group genes
PCR	polymerase chain reaction
PGC	primordial germ cells
Pref-1	preadipocyte factor 1
RT-PCR	reverse transcriptase polymerase chain reaction
Sf1	steroidogenic factor 1
Shh	sonic hedgehog
Sox	Sry-like homeobox containing gene
Sry	sex-determining region on the Y chromosome
TDF	testis determining factor
TGF	transforming growth factor
W	white spotting
wt	wild-type
Wt1	Wilms tumor suppressor gene 1

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Vainio S, Heikkilä M, Kispert A, Chin N & McMahon AP (1999) Female development in mammals is regulated by Wnt-4 signalling. Nature 397: 405-409.

II O'Shaughnessy PJ, Baker PJ, Heikkilä M, Vainio S & McMahon AP (2000) Localization of 17 -hydrozysteroid dehydrogenase/17-ketosteroid reductase isoform expression in the developing mouse testis – androstenedione is the major androgen secreted by fetal/neonatal Leydig cell. Endocrinology 141: 2631-2637.

III Heikkilä M, Peltoketo H & Vainio S (2002) Female-to-male sex reversal of Wnt-4 deficient mice can partially be rescued with flutamide treatment. *Manuscript.*

IV Heikkilä M, Peltoketo H, Leppäluoto J, Ilves M, Vuolteenaho O & Vainio S (2002) Wnt-4 deficiency alters mouse adrenal cortex function, reducing aldosterone production. Endocrinology, *in press*.

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1 Introduction

The sex determination process, in which the undifferentiated, “bipotential” embryonic gonads become either testes or ovaries is guided by genes and testicular hormones. So far the development and differentiation of the gonad is better known in males than in females. *Sry* was the first gene identified as playing a role in sex determination (Koopman *et al.* 1991), and since then several others, such as *Wilms tumor suppressor gene 1 (Wt1), steroidogenic factor 1 (Sf1), Sox9* and *Dax-1,* have been shown to be involved in this process (Swain & Lovell-Badge 1999). Traditionally, the female pathway of sexual differentiation has been considered a default alternative; occurring only due to absence of the Y-chromosome and testicular hormones, so that it lacks any active signalling.

Wnts are intercellular growth and differentiation factors, which are involved in a variety of developmental processes (Miller 2002). One member of this large gene family of secreted and soluble glycoproteins, Wnt-4, has been shown to be needed for the development of the kidney (Stark *et al.* 1994), pituitary gland (Treier *et al.* 1998), mammary gland (Brisken *et al.* 2000) and thymus (Mulroy *et al.* 2002) in the mouse. *Wnt-4*-deficient mice are not viable and die soon after birth, most likely due to dysfunction of the kidneys.

The present study was focused on the role of *Wnt-4* in the sex determination process and the development of the adrenal gland. *Wnt-4* was shown to be active in female development, so that its absence led to partial female-to-male sex reversal. In addition Wnt-4-deficient mice were shown to have problems in the development of the adrenal gland cortex.

2 Review of the literature

2.1 Gonadal development

Formation and differentiation of the sexually dimorphic genital system of the embryo is an important genetic cascade in mammalian development. The main phases are similar in mice and humans, and the main focus below will be on the situation in the mouse. Here gonadal development is divided into four main phases; formation of a bipotential gonad, migration of germ cells, sex determination and sex differentiation.

2.1.1 Formation of a bipotential gonad

Although the genetic sex of a mammalian embryo is determined at fertilization by the inheritance of an X or Y chromosome from the father, the gonads, associated sex ducts and external genitalia initially take the form of an identical primordium in all embryos. During this time, when the ovaries and testes are morphologically indistinguishable, they are called bipotential or indifferent gonads. (Reviewed in Parker *et al.* 1999, Capel 2000.)

In mice the gonads form between 10.5 and 11.5 dpc within the developing urogenital ridge, which actually comprises three segments (from anterior to posterior): the pronephros, mesonephros and metanephros. The pronephros is located at the anterior end and contains the adrenal primordium, while it is the central region, the mesonephros, from which the gonads arise. The extreme posterior region is the metanephros, in which the kidney forms. (Capel 2000.)

At first embryos of both sexes possess both a paramesonephric Müllerian duct and a mesonephric Wolffian duct, the former giving rise later to the oviduct, uterus, cervix and upper part of the vagina, while the latter differentiates into the epididymis, vas deferens and seminal vesicle (Behringer *et al.* 1994). Thus the Müllerian duct becomes female-specific after 14.5 dpc and the Wolffian duct male-specific, and the other duct starts to degenerate (Fig. 1).

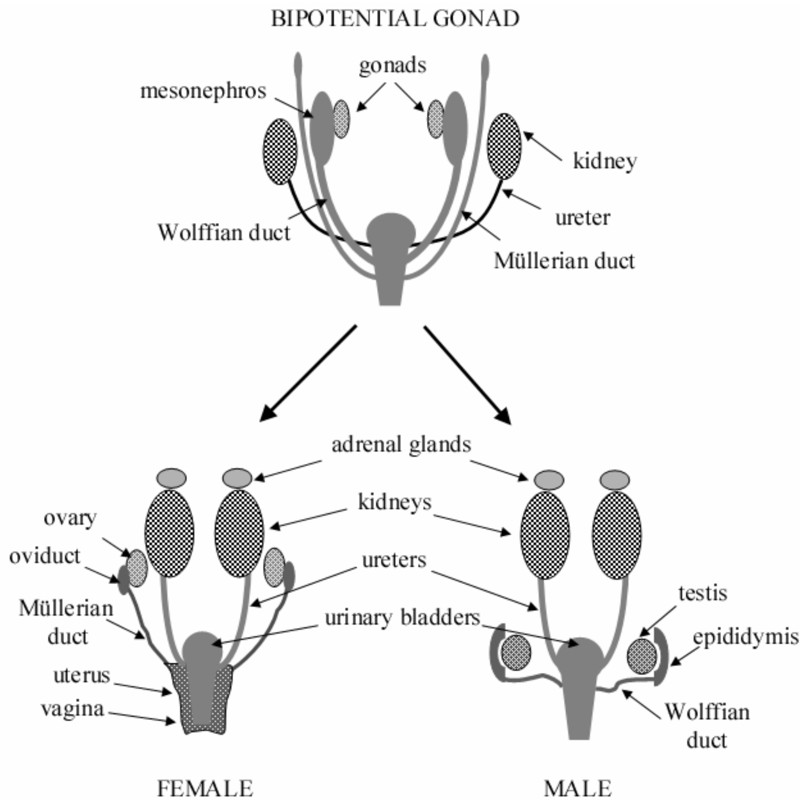

Fig. 1. Differentiation of the gonads and the gonad-associated sex ducts. Originally both Müllerian (female specific) and Wolffian ducts (male specific) are present in both sexes. Later in the females the Müllerian duct will differentiate into the oviduct, uterus and upper part of the vagina, while Wolffian duct regresses in the absence of testosterone. In turn, in males the Müllerian duct degenerates due the action of AMH and Wolffian duct will differentiate into epididymis and vas deferens.

The Wolffian duct plays a critical signalling role in the early development of the entire urogenital system, as the ureteric bud branches from its posterior end into the metanephric mesenchyme of the kidney and gonadal and adrenal development is induced at its anterior end (Fig. 2). Shortly after the appearance of the Wolffian duct, the mesonephric tubules form, and these then participate in the development of both the adrenal gland and the gonad, either through signalling to the surrounding regions or by contributing cells directly to the forming organs. The mesonephric tubules are not essential for gonadal development, however, as the gonads and adrenals still form in *Pax2*-deficient mice even though they lack mesonephric tubules and urogenital ducts (Torres *et al.* 1995, Capel 2000).

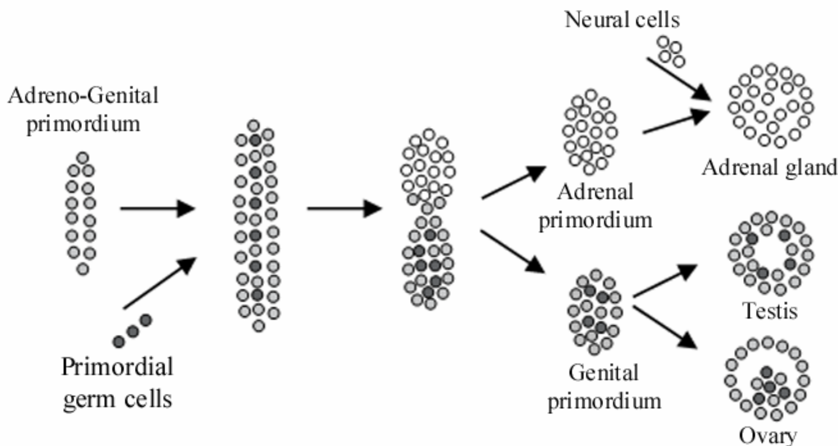

Fig. 2. Adrenal glands and gonads share a common primordium, from which adrenal gland, testis and ovary will be formed. (Modified from Morohashi 1997).

2.1.2 Germ cell migration

The founders of the germ line, the primordial germ cells (PGC), arise extragonadally, and in the mouse they migrate into the gonad through the gut mesentery and the mesonephros between 9.5 and 11.0 dpc (Ginsburg *et al.* 1990, Gomperts *et al.* 1994). These PGCs and their precursors are thought to be epiplastic in origin and to be totipotential at this and subsequent stages in development (Rohwedel *et al.* 1996, Yeom *et al.* 1996). The proliferation of germ cells is identical in the ovary and the testis until 12.5 dpc (Schmahl *et al.* 2000), when the germ cells in the testis arrest division for 1 week. Actual mitosis is resumed only after birth. The germ cells in the ovary undergo one final mitosis at 13.5 dpc and then enter meiosis (McLaren & Southee 1997). If germ cells, whether XX or XY, migrate into ectopic tissue by mistake, *e.g*. into adrenal tissue, they will go into meiotic arrest (McLaren 1995).

Germ cells are not needed for testis development/differentiation (McLaren 1988), but they are required for the initial organization of the ovary into the characteristic follicles and for the maintenance of these follicles thereafter. In the case of germ cell loss, the follicular structure of the ovary either never forms or rapidly degenerates (McLaren 1995).

Several genes have been shown to be important for controlling the migration or proliferation of germ cells in the mouse. Mutations in the *White spotting (W)* gene (Keshet *et al.* 1991), which encodes the c-kit receptor tyrosine kinase, *Steel* (Fleischman 1993), which encodes the c-kit ligand, and *TRIAR* (Beck *et al.* 1998), which encodes an RNA-recognition motif/ribonucleoprotein-type RNA-binding protein, lead to drastic reduction in the number of germ cells, while *Oct-4* is required to maintain the totipotency, so that downregulation of its expression correlates with loss of the potential to form germ cells (Pesce *et al.* 1998).

2.1.3 Sex determination

The decision as to whether the gonadal primordium forms into an ovary or a testis is the primary sex-determining step. Sex determination has generally been held to be synonymous with testis determination, so that the differentiation of Sertoli cells is the key event in this process.

The gonads are indistinguishable between the sexes until the genetic switch, *Sry,* is activated at 10.5-11 dpc in the mouse. There is no conservation of the sex determining switch between species, so that *Sry* exists only in mammals, while in other animals the sex is determined by the X chromosome - autosome ratio (Cline & Meyer 1996), environmental factors (Ferguson & Joanen 1982) or social cues (Francis *et al.* 1993). It is thought that the action of *Sry* triggers the differentiation of the Sertoli cell lineage in the testis and that the Sertoli cells in turn direct the differentiation of the rest of the cell types. Without the action of Sry the Sertoli cells would become follicle cells.

The chromosomal complement of the developing embryo, the genes, will determine the genetic sex and further decide the direction in which the gonads differentiate. The gonadal sex itself will nevertheless depend on hormones secreted by the gonads, and will in turn regulate the phenotypic sex. Germ cells evidently play no part in sex determination, as the sex is appropriately differentiated in *Steel*-deficient mice, which lack germ cells completely (Bendel-Stenzel *et al.* 1998, Wylie 2000).

The main actors in mammalian sex determination can be divided into at least three groups (Swain & Lovell-Badge 1999): *general transcription factors*, which are involved at several stages from early genital ridge development through to differentiation of specific cell types in the gonads and include *Lim1, Sf1, Wt1* and *GATA4* (see section 2.1.6), *specific promoters of testis development,* such as *Sry* and *Sox9* (see section 2.1.4), and *promoters of ovarian development*, such as *Dax-1* and *Wnt-4* (see section 2.1.5).

After sex determination several genes are also active in sex differentiation. Mutations in these genes generally lead to male-to-female sex reversals of different degrees, as in the case of mutations in genes that are active in testosterone biosynthesis (Imperato-McGinley *et al.* 1974, Kagimoto *et al.* 1988, Yanase *et al.* 1990, Yanase *et al.* 1991, Rheaume *et al.* 1994, Russell *et al.* 1994a, Russell *et al.* 1994b) or the genes encoding the *androgen receptor (AR)* (Quigley *et al.* 1995), anti-Müllerian hormone (AMH) (Imbeaud *et al.* 1994) and its receptor (Imbeaud *et al.* 1995).

2.1.3.1 Differentiation of the ovary and testis

The founding cell populations in the gonads of mammals have the capacity to differentiate into ovarian or testicular cell types (Fig. 3). In addition to germ cells, there are three cell lineages present in the gonad, and the supporting cell lineage will differentiate into follicular cells in the ovary and Sertoli cells in the testis. The function of these cells is to provide an appropriate growth environment for the germ cells that are surrounded by them. Sex hormones are produced by steroidogenic cells, which are theca and granulosa cells in females and

Leydig cells in males. The connective cell lineage, comprising peritubular myoid and stroma cells, will participate in the formation of the organ as a whole.

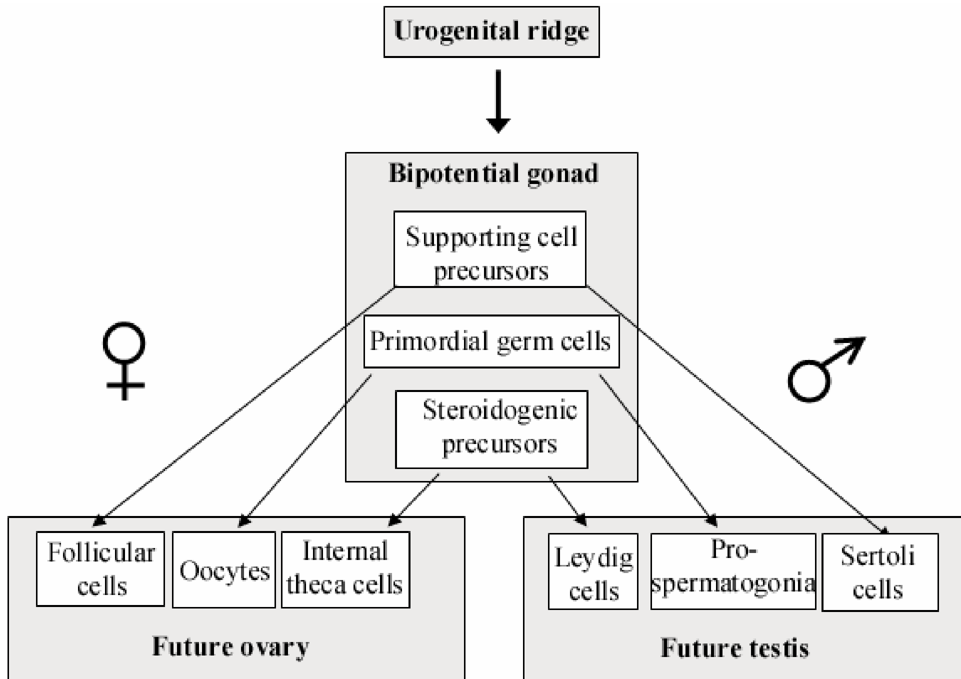

Fig. 3. Differentiation of the bipotential gonad into the ovary and the testis. (Modified from (Modified from Vaiman & Pailhoux 2000.)

During the indifferent stage, the superficial coelomic mesothelium of the genital ridge continues to proliferate into loose connective mesenchymal tissue and forms the cortical cords (the primary sex cords). In females the cortical sex cords split into clusters and differentiate into granulosa cells without penetrating deeply into the mesenchyme, while the mesenchymal cells differentiate into theca cells. Together, the theca and granulosa cells form the follicles that envelope the germ cells and secrete steroid hormones. In females, steroid production occurs in a two-cell system (Fig. 4). Theca cells first produce androgens that then diffuse into the granulosa cells, where they will further be converted to estrogens. Trophic hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) regulates this system.

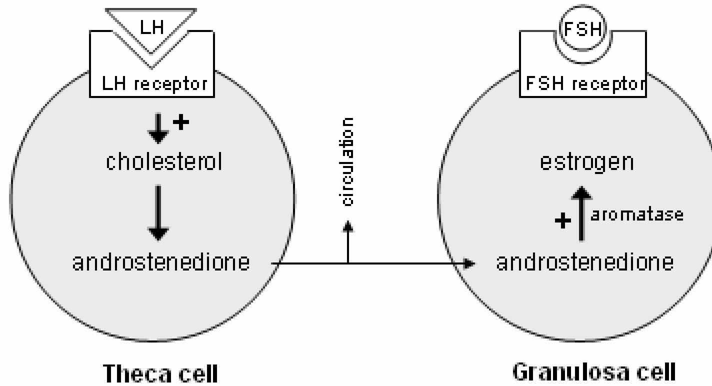

Fig. 4. Two-cell, two-gonadotropin model of steroidogenesis in the ovary. Theca cells produce androstenedione under the control of LH, which increases the number of LDL-receptors and hence cholesterol entry into the cells. Then androstenedione diffuse into the granulosa cells, where it will be converted to estrogen. Granulosa cells respond to FSH by inducing aromatase activity.

In males the testicular cords contain Sertoli cells, which surround the primordial germ cells. Sertoli cells are the first cell type known to differentiate in the gonad, and are thought to direct the differentiation of the rest of the testis. The deeper layer of the mesenchyme forms the interstitial mesenchymal cells of the testes, further differentiating into testosterone-producing Leydig cells. In addition to the Sertoli and Leydig cells, peritubular myoid cells and endothelial cells also exist among the other, as yet uncharacterized cell types in the interstitial space of the early testis. These cells move into the gonad from the mesonephros (Buehr *et al.* 1993), a male-specific development mechanism that is dependent on Sry (Martineau *et al.* 1997, Capel *et al.* 1999, Capel 2000). This migration of cells from the mesonephros occurs at least until 16.5 dpc in male mouse gonads, but not at any stage during this period in the case of XX gonads (Martineau *et al.* 1997). The migrating cells contribute to the vasculature and are positive for endothelial markers. Even though no migration occurs into the XX gonad between 11.5-16.5 dpc, the vasculature continues to expand from its inception at 11.5 dpc. (Gilbert 1997, Kaufman & Bard 1999.)

2.1.3.2 Differentiation of the sex ducts and external genitalia

The gonad-associated ducts, the Müllerian and Wolffian ducts, serve as tubular passageways for the exit of gametes produced by the gonads. Initially the embryo has both sex duct pairs, but normally only one of these systems will develop any further. The Müllerian duct regresses in males under the influence of AMH, and testosterone promotes the Wolffian duct differentiation into the epididymis and vas deferens, while in females, in the absence of AMH,

the Müllerian ductal system differentiates further into the oviduct, uterus and upper part of the vagina, while the Wolffian ductal system regresses. (Gilbert 1997, Kaufman & Bard 1999.)

The external genitalia develop the female phenotype in the absence of the Y chromosome, while the differentiation that occurs in males is dependent solely on androgen production by the testes, dihydrotestosterone (DHT) being specifically required for full virilization. The genital tubercle of the female will become the clitoris, the adjacent genital swellings the labia majora and the genital folds the labia minora. If androgen levels are elevated, as in human congenital adrenal hypoplasia (CAH), the external genitalia may become masculinized (Wiener *et al.* 1997). Mice that are deficient in *Wnt-5a* have only a stunted genital tubercle, leading to a lack of external genitalia (Yamaguchi *et al.* 1999).

Conversion of testosterone to DHT is catalyzed by steroid 5α-reductase. Surprisingly, mice lacking steroid 5α-reductase 1 and 2 had fully formed internal and external genitalia and were fertile, so that only their prostates and seminal vesicles were smaller than in controls and the expression of androgen-responsive genes was decreased. This suggests the probable existence of a third 5α-reductase (Mahendroo *et al.* 2001).

2.1.3.3 Hermaphroditism

Hermaphroditism, the existence of testicular and ovarian tissue at the same time, marks one exception to the model of Sry-induced sex determination. True hermaphroditism requires the explanation of either the existence of ovarian tissue in the presence of Sry or the existence of testicular tissue in its absence. Hermaphrodite humans more commonly have the ovaries located on the left side and the testes/ovotestes on the right, while hermaphrodite mice also show bilateral asymmetry, but in the opposite direction, the ovaries being on the right and the testes/ovotestes on the left. (Reviewed in Mittwoch 2000.)

2.1.3.4 Hormonal control of sex differentiation

After the sex is determined, further differentiation is almost exclusively hormone dependent. This is a sexually unimorphic process which is active only in males.

When the testes are formed, they will produce two major hormonal effectors, testosterone and AMH, which will guide sexual differentiation further. Testosterone, produced by the Leydig cells, is responsible for the development of the epididymis, vas deferens, seminal vesicles and, after conversion to 5α-dihydrotestosterone, the penis and scrotum. AMH, in turn, causes regression of the Müllerian ducts, which would otherwise give rise to specific female structures. (Kaufman & Bard 1999 and references therein.)

Knock-out mouse models have shown that estrogens appear not to be necessary for the normal differentiation of either sex (for a review, see Couse & Korach 1999a). The action of estrogens is normally mediated through two receptors; estrogen receptor α (ERα) and estrogen receptor (ER). ERα deficient female mice (αERKO) have hypoplastic uteri and hyperaemic ovaries without any corpora lutea and are infertile (Lubahn *et al.* 1993), while

male αERKO mice are also infertile, showing that a functional receptor is essential for normal fertility in both sexes. In contrast, ER -deficient mice (ERKO) develop normally and are histologically indistinguishable from their wild-type littermates (Krege *et al.* 1998). Only the older male mice show signs of prostate and bladder hyperplasia. In addition to these, double mutant mice (α ERKO) are also generated, lacking both receptors α and (Couse & Korach 1999b). These mice show partial gonadal female-to-male sex reversal, which is not detectable at birth but appears later in adulthood. The ovaries of α ERKO female mice contain structures resembling seminiferous tubules, and oocytes are either totally absent or in a degenerative state.

AMH (also known as Müllerian inhibitory substance, MIS) belongs to the transforming growth factor β (TGFβ) family and acts through two receptors, type I, which binds the ligand, and type II, which conveys the signal (Baarends *et al.* 1994). AMH signalling via the membrane-bound serine/threonine kinase type II receptor also requires recruitment and phosphorylation of a type I receptor (Hughes 2001). Many years after the type II receptor was identified it was revealed that AMH uses activin receptor-like kinase2 (ALK2) as its type 1 receptor (Clarke *et al.* 2001, Visser *et al.* 2001) AMH plays a role only in sex differentiation and not in determination, as shown by the fact that disruption of the AMH and AMH type II receptor genes does not prevent testis descent (Behringer *et al.* 1994, Mishina *et al.* 1996). Likewise, an addition of exogenous AMH leads to partial sex reversal in fetal XX rats (Vigier *et al.* 1987), while male mice lacking AMH develop as pseudohermaphrodites (Behringer *et al.* 1994). AMH receptor defect or a non-functional AMH molecule can also leads to the persistent Müllerian duct syndrome (PMDS), which is characterized by the persistence of Müllerian derivatives,uterus and tubes, in otherwise normally virilized males (Knebelmann *et al.* 1991).

2.1.4 Specific promoters of testis development

Sry (sex-determining region of the Y chromosome), the most intensively studied gene involved in sex determination and the one that encodes for the testis-determining factor (TDF), is a Y-linked gene expressed in the developing male gonad of the mouse between 10.5 and 12.5 dpc, initiating testis development (Gubbay *et al.* 1990b, Sinclair *et al.* 1990). Interestingly, it is also expressed in a variety of brain structures (Vilain 2000). Sry is a transcription factor (Werner *et al.* 1996, Capel 2000) and is both necessary and sufficient to drive development of the undifferentiated gonad towards a testis outcome. It is thought to activate other genes involved in defining and maintaining Sertoli cell identity, and one of its main roles is upregulation of *Sox9* expression in Sertoli cell precursors (Swain & Lovell-Badge 1999). It has been demonstrated that both mice and XX humans with *Sry* translocation develop as males (Koopman *et al.* 1991). On the other hand, overexpression of *Sry* does not have any further effect on sex determination in males (Jacobs *et al.* 1965, Gubbay *et al.* 1990a, Hawkins 1993).

The most critical part of the Sry protein is the high mobility group (HMG) box type of DNA-binding domain, similar to that found in other transcription factors. All mutations in this area, with one exception, lead to XY male to female sex reversal (Harley *et al.* 1992), and in

addition, the HMG domain is the only well conserved area in the protein (Tucker & Lundrigan 1993, Whitfield *et al.* 1993).

Among sex-reversed humans, *SRY* mutations are found only in 25% of XY females with pure gonadal dysgenesis (McElreavy *et al.* 1992). SRY is present in 10% of XX males or true hermaphrodites, and in 90% of XX males without sexual ambiguities (McElreavey *et al.* 1995). It is possible for an XX individual with no Y chromosomal sequences that include *SRY* to have a completely normal male phenotype (Vilain *et al.* 1994), which indicates that *SRY* is not the only sex-determining gene (for a review, see Vilain & McCabe 1998).

One possibility is that Sry directly activates a testis-determining cascade. First, it has a DNA-binding and bending domain and acts as a transcription factor (Goodfellow & Lovell-Badge 1993), second, mouse Sry acts as a transcriptional activator *in vitro*, although human SRY cannot (Dubin & Ostrer 1994), and third, Sry induces (although not directly) the expression of *AMH*, when transfected into a rat gonadal ridge-derived cell line (Haqq *et al.* 1994).

Sox (Sry-related HMG box) genes show a high sequence similarity to Sry in their conserved DNA-binding domains, or HMG boxes. It is specifically Sox9 that is important for testis development in many species, being one of the downstream Sry candidates and therefore thought to be involved in determining the fate of the Sertoli cells. Unlike Sry, Sox9 is well conserved throughout vertebrate evolution (Sudbeck *et al.* 1996, Veitia *et al.* 2001).

Sox9 is expressed at low levels in the genital ridges of male and female mice early in their development and is upregulated in males and shut down in females at the time of sex determination, i.e. at 11.5 dpc. Thereafter its expression is restricted to the Sertoli cells (Kent *et al.* 1996, Morais da Silva *et al.* 1996). *Sox9* has also been shown to be sufficient to induce testis formation in transgenic female mice when expressed under the control of regulatory regions of *Wt1*. In addition, the male reproductive ducts developed normally in these transgenic females, indicating that some cells in mutant ovary adopted Seroli cell-like and Leydig cell-like functions (Vidal *et al.* 2001).

Point mutations in human *SOX9* are associated with campomelic dysplasia (CD), a disorder involving skeletal malformation and cartilage formation defects (Foster *et al.* 1994, Wagner *et al.* 1994). In addition, most 46XY patients are females with partial or complete CD and gonadal dysgenesis, GD (Houston *et al.* 1983, Vidal *et al.* 2001). A mutation in only one allele of *SOX9* generally leads to male to female sex reversal (Foster *et al.* 1994, Wagner *et al.* 1994), but as these mutations can be located in different regions of the protein, several domains are implicated as being active in sexual development (Kwok *et al.* 1996, Meyer *et al.* 1997). One case of female to male sex reversal has also been reported with duplication of a genomic region containing Sox9 (Huang *et al.* 1999).

It has been hypothesized that a specific "*Z-gene*" exists which acts as a repressor of male development and/or activates female development, and that *Sry* may inhibit *Z*, which is in turn an inhibitor of the male pathway (McElreavey *et al.* 1993a, McElreavey *et al.* 1993b, Jimenez *et al.* 1996). Inactivation of *Z* in females could lead to male pathway activation even in the absence of Sry, while over-expression of *Z* in males could block male development in the presence of Sry. One candidate proposed for this *Z-gene* is *Dax-1* (see section 2.1.5.), in the light of data indicating that it is cloned from the Xp21region in humans, the same region that is responsible for a syndrome called dosage-sensitive sex reversal (DSS), since duplication of this region leads to male to female sex reversal. A comparable sex reversal was seen in transgenic mice when *Dax-1* was expressed in combination with weak or late-acting alleles of

Sry, whereas the null mutation in mouse *Dax-1* did not affect ovary or testis development. *Sox3* is another X-linked gene that could be Z. There is no clear evidence so far for its involvement in sex determination, but it has been thought to be an X-linked antagonist of Sry. Sox3 belongs to the Sry-like HMG protein family and is X-linked in all mammals, although Z could also be linked to an autosomal chromosome. Sox3-deficient mice die *in utero* early in their development, and no evidence exists before a conditional mouse model is generated (Capel 2000).

2.1.5 Specific promoters of ovarian development

Dax-1 is an X-linked gene that belongs to the nuclear hormone receptor (NHR) superfamily. In addition to being linked with a DSS in humans, its expression is upregulated during sexual differentiation in mice. These facts led to proposal that *Dax-1* may play a role in the female developmental pathway (Parker & Schimmer 1998), possibly as an ovarian determination gene (Swain *et al.* 1996). Since overexpression of *Dax-1* in transgenic mice also generates XY female sex reversal (Swain *et al.* 1998), it was named the "antitestis" gene, acting antagonistically to *Sry* (Swain & Lovell-Badge 1999).

Dax-1 expression persists in the developing ovaries after sex determination, but decreases rapidly in males, paralleling the curve of decreasing *Sry* expression, but lagging just slightly behind it (Swain *et al.* 1996). Genetic data also suggest that Dax-1 is part of the sex determination process but is not required for testis formation.

Surprisingly, a deficiency in Dax-1 does not impair mouse ovarian development or any other aspects of female sexual differentiation, but it does affect spermatogenesis in males. The testes are relatively normal in appearance at birth, but subsequently exhibit progressive epithelial dysgenesis. An unusual proliferation of Sertoli cells blocks the rete testis and prevents the route for normal sperm flow into the epididymis (Yu *et al.* 1998, Jeffs *et al.* 2001).

It is interesting that mutations in *DAX-1* do not affect male development in humans (Bardoni *et al.* 1994, Muscatelli *et al.* 1994, Zanaria *et al.* 1994), but instead either mutation or deletion causes a disorder called adrenal hypoplasia congenita, AHC, in which development of the steroid-producing zones in the adrenal gland is impaired. Patients also have defects in both the hypothalamus and the pituitary, which impairs gonadotropin production and leads to decreased gonadal function at puberty (Kletter *et al.* 1991). Because of the completely different phenotypes involved in mice and humans, further research will be required to clarify the role of Dax-1.

2.1.6 Autosomal genes involved in early gonad formation

There are several gene mutations that can cause abnormal gonad development or interfere with the sex determination process (Table 1), and the consequences can be so severe, that the mouse model suggests that it is impossible to address the functions which the products of

these genes are involved in, as in the case of *Sf1*. The genes involved in the differentiation of the intermediate mesoderm as a whole, e.g. *Lim-1*, generally also play an important role in early gonad development and these mice usually show defects in both early kidney and gonad development. In addition, there is a group of genes expressed in the developing gonad, which may have an important role, e.g. *GATA-4*, but these need, some further analysis.

Table 1. Mutations in genes involved in early gonad development

	Mouse phenotype	Human phenotype	References
Lack of gonads			
Sf1	- lack of gonads and adrenals - feminized internal and external genitalia	- XY individual with adrenal insufficiency and complete sex-reversal	(Luo *et al.* 1994, Achermann *et al.* 2001)
Wt1	- lack of kidneys, gonads and adrenals	- XY female - Denys-Drash and Frasier syndromes	(Kreidberg *et al.* 1993, Hammes *et al.* 2001)
Lim-1	- lack of kidneys and gonads	- gene 97% identical with mouse	(Shawlot & Behringer 1995, Dong *et al.* 1997)
Lhx9	- lack of gonads	- gene 98% identical with mouse	(Birk *et al.* 2000, Ottolenghi *et al.* 2001)
Emx-2	- impaired gonad and kidney development	- gene known	(Miyamoto *et al.* 1997, Noonan *et al.* 2001)
Impaired gonad development			
M33	- retarded gonad development - •: different degrees of sex reversal		(Katoh-Fukui *et al.* 1998)
Fgf9	- •: varies from testicular hypoplasia to complete sex reversal	- gene known	(Mattei *et al.* 1995, Colvin *et al.* 2001)
Insl3	- bilaterally cryptochid, cubernacular bulbs fail to develop and resemble female structure	- associated with cryptorchidism	(Nef & Parada 1999, Zimmermann *et al.* 1999, Tomboc *et al.* 2000, Marin *et al.* 2001)
Destr	- •: uni/bilaterally cryptorchid - •: uterine horns and uterine glands smaller in size - both: defect in adrenals		(Lahoud *et al.* 2001)

Table I. continues

	Mouse phenotype	Human phenotype	References
Genes possibly playing a role			
GATA-4	- expressed in adrenal cortex and in developing gonad, later testis specific - knock-outs die in utero 8.5 – 11.5 dpc	- gene known, expressed in adrenal cortex	(Molkentin *et al.* 1997, Kiiveri *et al.* 2002)
Dmrt1	- expressed in the developing gonad, later testis specific - adult testes hypoplastic and seminiferous tubules are disorganized		(Raymond *et al.* 2000)
tescalcin	- expressed early in testis cords	- not yet cloned - may be implicated in XY gonadal dysgenesis	(Mailander *et al.* 2001, Perera *et al.* 2001)
SET	- in rat; expressed in gonad, steroidogenic cells and germ cells		(Zhang *et al.* 2001)

2.1.6.1 Gene mutations leading to total absence of the gonads

So far deficiencies in five genes, *Sf1, WT, Lim-1, Lhx9* and *Emx-2*, are known to lead to a complete lack of gonads. Steroidofenic factor 1 (Sf1) is a transcription factor (Lala *et al.* 1992, Morohashi *et al.* 1992, Honda *et al.* 1993) belonging to the subfamily of nuclear receptors, the orphan receptors, for which no clear activating ligand is known. It is expressed in tissues with endocrine function, such as the gonads, adrenals, pituitary and hypothalamus (Hatano *et al.* 1994, Ikeda *et al.* 1994, Ingraham *et al.* 1994, Morohashi *et al.* 1994).

Analysis of mutant *Sf1* mice indicates that this gene has a role throughout gonadal development. Mice deficient in *Sf1* lack gonads and adrenals, suggesting an essential role for the gene in the early development of the precursors of these, and both the internal and external genitalia are male-to-female sex reversed. In addition, *Sf1* mutant mice have impaired expression of a number of genes regulating gonadal steroidogenesis, including LH, FSH and gonadotropin-releasing hormone (GnRH), and they lack any ventromedial hypothalamic structure (Ingraham *et al.* 1994, Luo *et al.* 1994, Ikeda *et al.* 1995, Sadovsky *et al.* 1995, Shinoda *et al.* 1995).

The genital ridges of *Sf1*-deficient mice begin to form and the gonads are colonised by germ cells, but the gonads and adrenal glands degenerate via apoptosis around 11-11.5 dpc (Luo *et al.* 1994). Sf1 therefore seems to be necessary for the differentiation and/or maintenance and growth of the somatic cells and not for specifying the initial development. No mutation in the human *SF1* gene has yet been demonstrated, even though the human gene shares extensive homology with its mouse counterpart, suggesting a similar function (Oba *et al.* 1996, Wong *et al.* 1996, Ramayya *et al.* 1997).

The *Wilms tumor suppressor gene (Wt1),* an important transcription factor which is critical for kidney and gonad formation, is expressed during the early development of the urogenital ridge. Expression becomes restricted to the Sertoli and granulosa cells after sex determination (Sharma *et al.* 1992, Armstrong *et al.* 1993).

WT1 mutations are associated with Wilm's tumor and several human conditions involved in developmental abnormalities of the kidneys and gonads (Pelletier *et al.* 1991, Bowles & Koopman 2001). At least 24 isoforms exist, the best known being $WT1^{+KTS}$ and $WT1^{-KTS}$, which differ only by the presence or absence of three amino acids. Denys-Drash syndrome (DDS) involves mutations in the zinc finger region of the protein, leading to urogenital abnormalities and degenerated gonads, while in Frasier syndrome production of the $WT1^{+KTS}$ isoform is prevented, causing male-to-female sex reversal, male pseudohermaphroditism and gonadal dysgenesis (Bowles & Koopman 2001).

Wt1-deficient mice die *in utero* with a complete absence of kidneys, gonads and adrenal glands, and have defective heart and spleen formation (Kreidberg *et al.* 1993, Moore *et al.* 1999). Gonadal development is initiated in the mutant mice, but is then arrested at a very early stage, indicating a role for Wt1 in the early establishment of the genital ridge. Isoform-specific $Wt1^{+KTS}$ and $Wt1^{-KTS}$ deletions have been performed in mice by gene targeting methods in order to gain an understanding of the distinct functions of these two isoforms (Hammes *et al.* 2001). Mice lacking the +KTS isoform serve as a model for Frasier syndrome and mice lacking the –KTS isoform are generally referred to as KTS mice. In contrast to the human Frasier and DDS patients, neither of the mutant mouse models showed gonadal abnormalities as heterozygotes, although the Frasier mice had similar kidney pathology. In addition, the overall level of *Wt1* expression was not altered in either mouse model, because deletion of one isoform led to overexpression of the other (Bowles & Koopman 2001, Hammes *et al.* 2001).

Homozygous Frasier mice show complete male-to-female sex reversal, due to a dramatic reduction in *Sry* expression levels. The gonads develop normally in the females, however (Hammes *et al.* 2001). Homozygous KTS mice have been found to die within 24 hours of birth. Both the XX and XY mice had small, poorly differentiated gonads and abnormal genital ducts at birth, indicating that $Wt1^{-KTS}$ is required for the differentiation and survival of the gonadal cells in both sexes. Interestingly, neither mouse model showed complete blockage of the genital ridge as in *Wt1*-null mice, which suggests that some isoform of Wt1 is essential for early genital ridge development, irrespective of the presence or absence of KTS (Bowles & Koopman 2001).

Lim-1, which belongs to the LIM class of homeobox proteins, is expressed in the mesonephric duct and tubules and in the ureteric bud of the metanephros during early urogenital development (Fujii *et al.* 1994). Mice with homozygous disruption of the *Lim*-1 gene lack kidneys, gonads and anterior head structures and die early *in utero* (Shawlot & Behringer 1995). Interestingly, *Pax2*, a member of the paired-box family of transcription factors, is expressed in similar regions to *Lim-1* and is also found in the mesenchyme, but although a deficiency leads to a lack of kidneys and urogenital ducts, the gonads and adrenal glands do form (Torres *et al.* 1995).

Lhx9 is another LIM homeobox gene family member having a role in early gonadal development. It is expressed first in the presumptive gonadal area of both sexes, but by 13.5 dpc, expression is mainly restricted to the cortical region of the ovary in females and to the outer part (developing tunica) of the testis in males. In *Lhx9*-deficient mice the urogenital ridge first forms in a similar manner to that in wild-type littermates, but no discrete gonad

evolves. Since there is no testosterone or anti-Müllerian hormone present in mutant mice, the genetically male ones are phenotypically female. Expression of *Sf1* is reduced to minimal levels in the urogenital ridge, indicating that *Lhx9* lies upstream of *Sf1* in the developmental cascade. Lhx9 may not regulate Sf1 directly, but it may permit the proliferation of *Sf1* positive cells. No change in the expression pattern of *Wt1* was observed, however, and exceptionally, the *Lhx9* mutants did not exhibit any other major developmental defects. Heterozygotes appeared to be normal and fertile. (Birk *et al.* 2000.)

Loss of expression of the homeobox gene *Emx-2*, which is normally expressed in the urogenital ridge, Wolffian duct, mesonephric tubule and coelomic epithelia, leads to the complete absence of gonads and genital tracts, although the adrenal glands develop normally (Miyamoto *et al.* 1997).

2.1.6.2 Gene mutations leading to abnormalities in gonad development

Several genes, which are normally expressed in the gonads, can cause abnormalities in gonadal development/differentiation if they undergo mutation. The mouse *M33* gene is related to the *Drosophila polycomb group (PcG)* of genes, which are thought to be part of a system that maintains the expression patterns of homeotic genes. M33 is also involved in early development of the gonad, and deficient mice show retarded gonad development and the males varying degrees of sex reversal (Katoh-Fukui *et al.* 1998). They may also have a defect in positional information during gonad development, in that some cells of the mesonephros may have acquired partial metanephric identity (Dolle *et al.* 1991).

Fibroblast growth factor 9 (Fgf9) is active in testicular embryogenesis and plays a role in the development of the lung, limb and anterior pituitary. In the gonads it is expressed in the developing testis from 11.5 dpc onwards, expression being restricted to the testicular cords at 12.5dpc. The reproductive system of *Fgf9*-deficient mice varies from testicular hypoplasia to complete male-to-female sex reversal. *Fgf9* acts downstream of *Sry* to stimulate mesenchymal proliferation, mesonephric cell migration and Sertoli cell differentiation in the embryonic testis (Colvin *et al.* 2001).

Two new genes, *insulin-like factor 3 (Insl3)* and *developmentally and sexually retarded with transient immune abnormalities (Desrt)*, have recently been identified, the mutations in which cause abnormal development of the reproductive organs. *Insl3* is expressed in fetal Leydig cells, and deficient male mice are bilaterally cryptochid, the cubernacular bulbs failing to develop, so that they resemble normal female cubernacular structures (Nef & Parada 1999, Zimmermann *et al.* 1999). Most of the mutant male mice also have some degree of testis maldescent at birth, but this rectifies itself by adult life. *Insulin like 3* (INSL3) gene is also associated with cryptorchidism in humans (Tomboc *et al.* 2000, Marin *et al.* 2001). *Desrt*, a member of novel AT-rich interaction domain (ARID) family, is expressed in the testis, but no expression was detected in the ovaries. Mutant *Desrt* mice display abnormalities in the development of the reproductive organs in addition to other defects, the males being found to be unilaterally or bilaterally cryptorchid, while the females appeared to have smaller uterine horns and uterine glands than their wild-type littermates. In addition, both males and females

had abnormalities in the adrenal gland, with a reduction in the size of the *zona reticularis.* (Lahoud *et al.* 2001.)

Dazla and *factor in germline alpha (FIG)* affect gametogenesis and ovarian follicle formation. *Dazla* is the autosomal homologue of the Y-chromosomal *DAZ* gene, which has been found to be deleted in azoospermic males. Mouse *Dazla* gene has high similarity to human *DAZLA* gene expression. Gene disruption in mouse leads to loss of germ cells and complete absence of gamete production, indicating that Dazla is essential for the differentiation of germ cells (Ruggiu *et al.* 1997). Also in humans the DAZLA mutation causes male and female sterility (Brekhman *et al.* 2000). *FIG* is basic helix-loop-helix transcription factor that is expressed in early oocytes. Mutant *FIG* mice had normal embryonic gonadogenesis, but primordial follicles were not formed at birth and massive depletion of oocytes resulted in shrunken ovaries and female sterility. *FIG* also plays a key regulatory role in the expression of multiple oocyte-specific genes, including folliculogenesis initiating genes and zona pellucida genes, which are needed for fertilization and early embryonic survival. (Soyal *et al.* 2000, Dean 2002).

2.1.6.3 Genes possibly playing a role in gonad development

The expression patterns of some genes or homologues in other species may predict a role in the development or differentiation of the gonad. *GATA-4, Dmrt1*, tescalsin and *SET* are all expressed in the area of the developing gonad at the time of sex determination.

The zinc finger transcription factor *GATA-4* is expressed in the genital ridge of developing mouse embryos of both sexes at 11.5 dpc, and later in the Sertoli and interstitial cells of the developing testis and in the developing ovary (Viger *et al.* 1998). The early function of GATA-4 is not known, and conditional knock-out would be required to clarify whether it is active in gonad development, because *GATA-4*-deficient mice die *in utero* before any such development has even taken place (Molkentin *et al.* 1997).

Dmrt1 is the mouse homologue of the *C. elegans* sex-determining gene *Mab-3* and the Drosophila sex-determining gene *doublesex (dsx)*. It is expressed in the genital ridge of both females and males during sex determination. Later *Dmrt1* has a punctate expression pattern throughout the ovary and marks newly forming cords in the testis. Expression is then reduced to a hardly detectable level in females but remains strong in the later stages of sex differentiation in males, indicating that Dmrt1 may have a role in sex determination/differentiation (De Grandi *et al.* 2000). Dmrt1 is essential for postnatal testis differentiation in the mouse, as the testes of deficient mice are severely hypoplastic, the seminiferous tubules are disorganized and the germ cells are missing. No transformation of a testis to an ovary has been detected (Raymond *et al.* 2000).

The recently identified regulatory gene *tescalcin* is expressed specifically in early fetal mouse testis cords, suggesting that it may play a role in sex differentiation (Perera *et al.* 2001). The human homologue has also been cloned but is not connected with sex differentiation, being observed instead to play a role in the development of cardiac tissue (Mailander *et al.* 2001).

In addition, a novel nuclear phosphoprotein *SET* has recently been characterized as a transcriptional regulator that binds to the rat *Cyp17* gene. Its expression precedes that of *Cyp17* in the testis and ovary during development, and the suggestion has been advanced that it may play a multiple role in gonadal development and in the maintenance of differentiated gonadal function. During fetal life it is expressed in steroidogenic cells within the gonad and within the mature oocytes and spermatocytes. (Zhang *et al.* 2001.)

2.1.7 Genetic interactions in gonadal development

In addition to Y-chromosomal *Sry* and X-linked genes (*e.g. Dax-1*) autosomal genes (*e.g. Sf1*, *Wt1*) also play a critical role in the processes of sex determination and differentiation (Fig. 5).

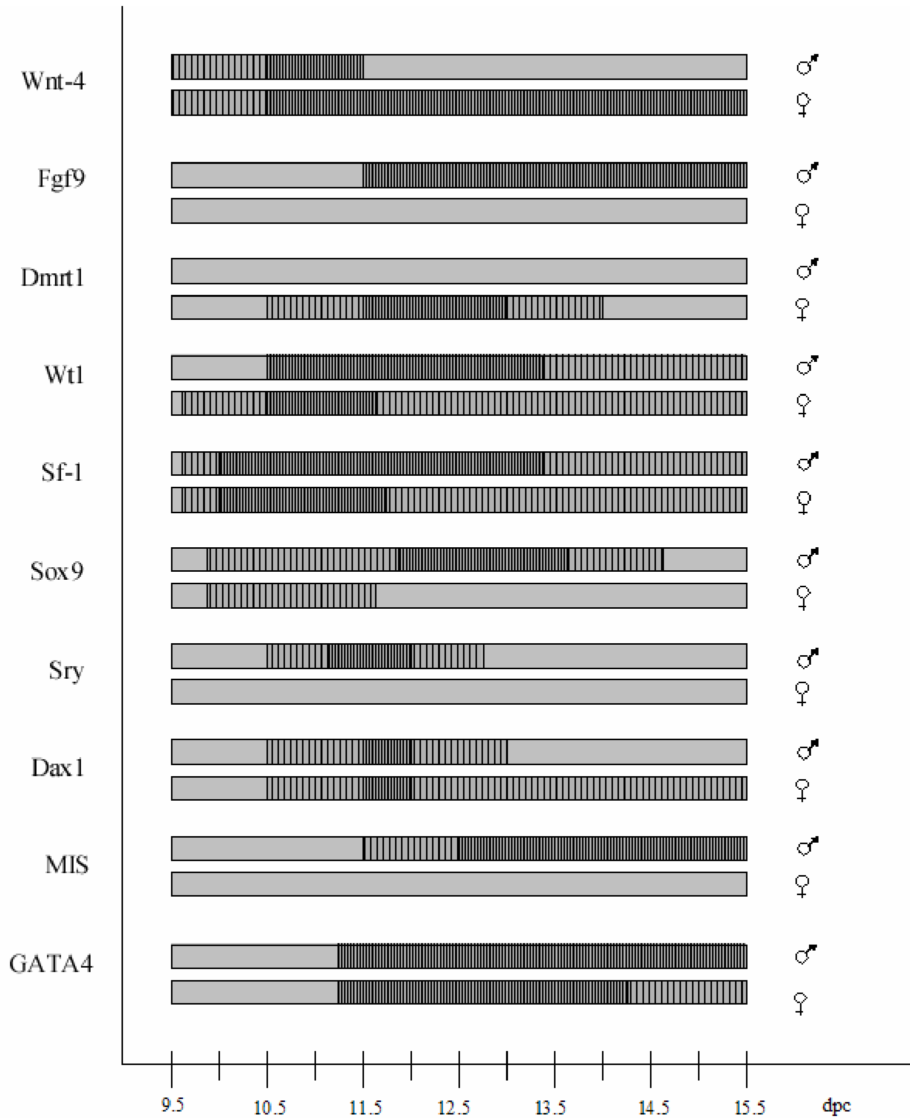

Fig. 5. Transcription patterns in gonads of the genes involved in sex determination and differentiation. The level of the transcription is illustrated with black stripes; the more dense stripes the higher level of transcription. Gray area indicates no transcription. The ages up to 12 dpc represent the transcription in the whole gonad and thereafter only in the supporting cells. (Modified from Swain & Lovell-Badge 1999.)

A simplified model for genetic interactions in gonadal development is presented in Fig. 6.

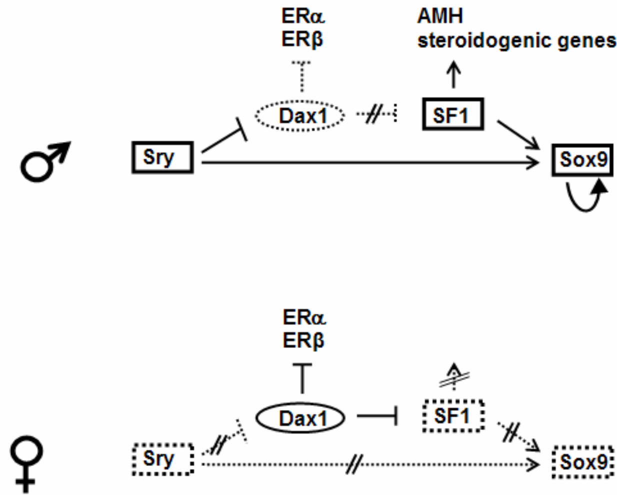

Fig. 6. Main gene interactions in the sex determination. In males *Sry* is thought to repress *Dax-1,* which would otherwise inhibit the activation of *estrogen receptor (ER)* α and . When Dax-1 is not present steroidogenic factor 1 (Sf1) is fully functional and can further participate into the activation of anti-Müllerian hormone (AMH), steroidogenic genes and *Sox9*. In addition, *Sox9* could maintain its own expression through an autoregulatory loop. Furthermore, *Sry* can probably contribute also directly to the *Sox9* activation. In the absence of Sry in females, the Dax-1 remains functional leading to inhibition of the genes active in male specific development.

Expression of *Sf1* precedes that of *Sox9* in both sexes, the latter being upregulated immediately after the onset of Sry expression in males. By contrast, the extinction of *Sox9* in the XX gonad is consistent with the onset of *Dax-1* expression, which probably prevents *Sox9* transcription through the repression of Sf1 action. Dax-1 cannot be the only repressor, however, as deficient mice have normal ovaries and female reproductive tracts. Sry may prevent this repression in males, or contribute directly to *Sox9* activation. In addition, *Sox9* could maintain its own expression through an autoregulatory loop after reaching the critical threshold. (Swain & Lovell-Badge 1999.)

Apart from being required for gonadal development, Sfl could also play a critical role in sex determination. It may be involved in the determination and differentiation of Sertoli cells by serving as the first upstream regulator of either *Sry* or *Sox9* or both, but it may equally well be located downstream of these genes. *Sf1* and *Sox9* could be mutually dependent on each other for their continued expression at a high level, in which case *Sf1* could participate in subsequent Sertoli cell-specific gene regulation (Swain & Lovell-Badge 1999).

Dax-1 is able to inhibit the transcriptional activation of *Sf1 in vitro* (Ito *et al.* 1997, Lalli *et al.* 1997, Zazopoulos *et al.* 1997, Crawford *et al.* 1998), the latter in turn being essential for adrenal and gonadal development and, in addition to the steroid hydroxylases, regulating the expression of other adrenal and gonadal genes involved in steroidogenesis, including *3β-hydroxysteroid dehydrogenase (Hsd3b1)*, *adrenocortical tropic hormone (ACTH) receptor* and the *steroidogenic acute regulatory protein (StAR)* (Parker & Schimmer 1997). Along with *Sox9, Wt1, GATA-4* and *Dax-1, Sf1* also participates in regulation of the expression of the

AMH gene (Hatano *et al.* 1994, Shen *et al.* 1994, Giuili *et al.* 1997, Tremblay *et al.* 2001)**.** It has similarly been reported that there is an Sf1 response element present in the Dax-1 promoter (Burris *et al.* 1995, Guo *et al.* 1996) and that Sf1 is capable of upregulating Dax-1 (Vilain *et al.* 1997). Protein-protein interaction has been observed between SF1 and Dax-1, but so far with no known physiological significance (Ito *et al.* 1997).

Dax-1 can interact with ERα and ER and inhibit their activation (Zhang *et al.* 2000). *Dax-1* and the *ERs* are coexpressed in reproductive tissues during embryogenesis and in adulthood (Ikeda *et al.* 1996, Lemmen *et al.* 1999), and the sterility of *ERα*-deficient male mice showed that a functional receptor is essential for normal male fertility. These mice exhibited atrophy of the testes, a low sperm concentration and abnormal rete testes and efferent ducts (Jeffs *et al.* 2001).

Sf1 and Wt1 can form heterodimers *in vitro*, and Wt1 can also increase the expression of reporter genes driven by the Sf1 promoter when they are cotransfected. Wt1 is not vital for *Sf1* expression, however, because the latter can still be detected in *Wt1*-deficient mice. In turn, Wt1 can antagonize Dax-1 inhibition of Sf1 mediated transactivation (Nachtigal *et al.* 1998).

AMH is first expressed in in the developing Sertoli cells of the mouse embryo at 12 dpc, and its expression closely follows the upregulation of *Sox9* (Munsterberg & Lovell-Badge 1991, Morais da Silva *et al.* 1996). Four proteins are known to interact with the AMH promoter and to regulate its expression: Sf1, Wt1, Sox9 and GATA-4. The AMH promoter region contains an Sf1 binding site, and mutation of this site led to an absence of expression in the embryonic testes of transgenic mice, suggesting that Sf1 can bind and activate the AMH promoter (Shen *et al.* 1994). The AMH promoter region also contains a binding site for proteins with a HMG box domain. The most likely candidate for binding to this site being Sox9, which is present in the male genital ridge at the same time as AMH is activated and is turned off in the female genital ridge at 11.5 dpc. Sox9 is able to synergise Sf1 activation of the AMH promoter, suggesting that Sf1 and Sox9 can act together to create the tissue-specific expression of AMH. *In vitro* studies have shown that Wt1 (-KTS isoform) can also potentiate the activation of AMH by Sf1, and that Dax-1 is capable of repressing the synergistic action of Sf1 and Wt1 on the AMH promoter (Nachtigal *et al.* 1998). The AMH promoter element also contains a binding site for the GATA transcription factors, and GATA-4 protein has been shown *in vitro* to bind to this consensus site and activate expression of a reporter construct. (Viger *et al.* 1998.)

2.2 Adrenal gland development

The urogenital ridge, from which the gonads form, also contains an adrenal primordium. The adrenal gland is actually composed of two distinct functional units, which are of different embryological origins: a mesodermal cortex and a neural crest ectoderm-derived medulla. In addition, each gland is surrounded by a connective tissue capsule. (Nyska & Maronpot 1999 and references therein.)

The mouse adrenal gland starts its development at 11.5 dpc, when the anlage of the adrenal cortex is first formed (Sass 1996). The cortex develops by budding from the coelomic epithelium between the mesogastrium and the urogenital fold. At this stage the cortical cells

have the appearance of a focus of dark cells located medially relative to the gonads. The cortical anlage is further pushed between the mesonephros and the aorta. The cortical cells are found close to the adrenal medulla sympathoblasts at 12.5 dpc, and the capsule surrounding the adrenal gland and cortical capillaries is formed at 14.5 dpc. Both the cortex and the capsule have completed their development by 15 dpc. (Nyska & Maronpot 1999 and references therein.)

The medulla also starts its development at 11.5 dpc, when the two chains of sympathoblasts are observed on either side of the aorta. These sympathoblasts then migrate along the sympathetic nerves towards the cortical anlage, where they can be found at 12 dpc. Two days later, at 14 dpc, the medullary cells are located in the centre of the adrenal gland, which is fully functional at birth. (Nyska & Maronpot 1999 and references therein.)

The mammalian cortex is generally divided into three layers: the *zona glomerulosa, zona fasciculata* and *zona reticularis* (Fig. 7). The *zona glomerulosa* is located immediately beneath the capsule and is very narrow, while the *zona fasciculata* is the thickest one. In contrast to most mammals, there is no *zona reticularis* in the mouse, but instead it is replaced by an *X-zone*, which is unique to this species. The function of the X-zone is not known, but it appears few days after birth and is fully developed by weaning. It begins to degenerate at pubertal maturity in males and early in the first pregnancy in females. (Deacon *et al.* 1986, Nyska & Maronpot 1999 and references therein.)

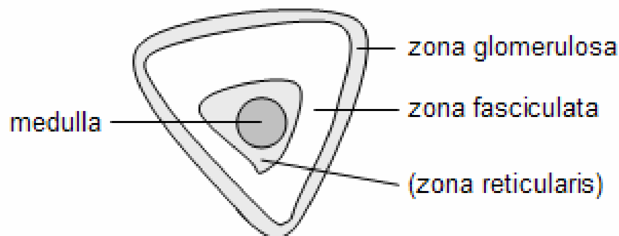

Fig. 7. Composition of the adrenal gland. In humans the adrenal gland is composed of medulla and three cortical layers; zona glomerulosa, zona fasciculate and zona reticularis. In mouse there exist no zona reticularis, but instead it is replaced with so called "X-zone".

The cortex forms the major part of the gland. It is essential for life, as its hormones influence essential somatic processes. The corticosteroids synthesized in it can be divided into mineralocorticoids, which control electrolyte and water balance, glucocorticoids, which influence carbohydrate metabolism, and sex steroids. Their production is regulated by the renin-angiotensin system and the hypothalamic-pituitary-adrenal (HPA) axis. The main mineralocorticoid, aldosterone, is synthesised within the *zona glomerulosa*, while the *zona fasciculata* and *zona reticularis* produce glucocorticoids such as corticosterone and cortisol.

The adrenal medulla makes up a smaller volume of the adrenal gland and is composed of catecholamine-producing chromaffin cells, ganglion cells, venules and capillaries. The chromaffin cells produce norepinephrine, epinephrine, chromogranins and neuropeptides, which are components of the autonomic nervous system (Ehrhart-Bornstein *et al.* 1998, Nyska & Maronpot 1999). The adrenocortical and chromaffin cells of the medulla are intermingled

and interact which each other (Ehrhart-Bornstein *et al.* 1998). The adrenal glands constitute one of the most highly vascularized organs in the primate fetus, and nearly every adrenocortical cell is directly adjacent to a vascular endothelial cell (Mesiano & Jaffe 1997).

Dysfunction of the adrenal gland results in life-threatening diseases such as CAH and Addison's disease, and may otherwise have severe consequences for the individual, as in Cushing's syndrome, Conn's syndrome, pheochromocytoma and stress-related and metabolic disorders (Böttner & Bornstein 2001).

2.3 The hypothalamus-pituitary-adrenal /gonadal axis (HPA/HPG)

In addition to gonadal differentiation, normal sexual development also requires normal development of the entire HPA/HPG axis, also referred as the reproductive axis (Vilain & McCabe 1998). HPA and HPG form a functional endocrine axis with hormonal regulation and feedback loops (Fig. 8). In contrast to the testes and adrenals, which produce hormones during fetal life, the ovaries start to release steroid hormones when they are first stimulated by gonadotropins at the onset of puberty (Miller & Strauss 1999).

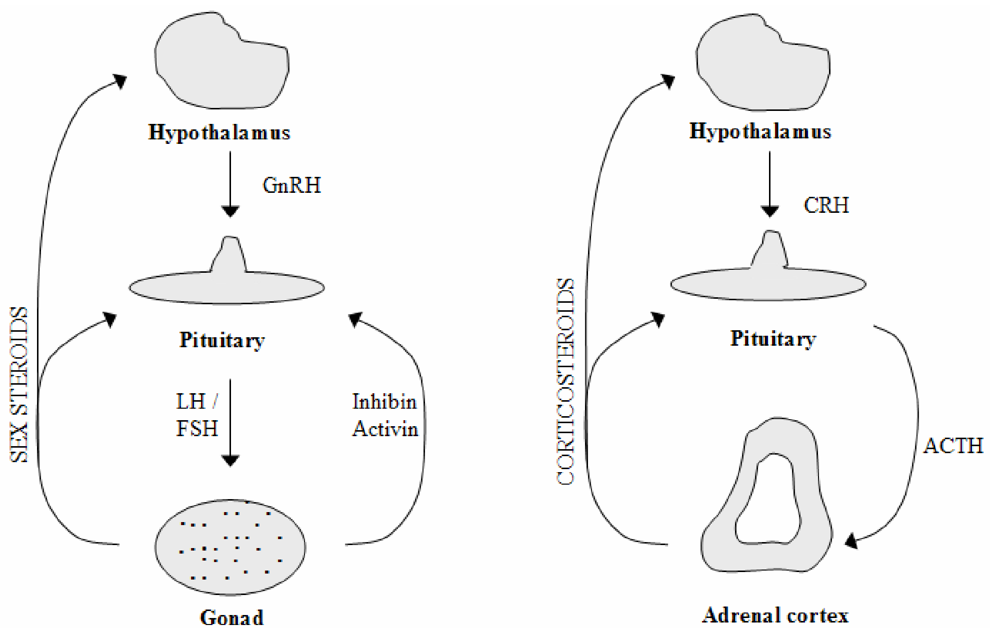

Fig. 8. The function of hypothalamus-pituitary-adrenal (HPA) and hypothalamus-pituitary-gonadal (HPG) axes. Gonadotropin releasing hormone (GnRH) and adrenocorticotropin releasing hormone (CRH) are discharged from hypothalamic central nervous system to stimulate the function of gonadotrophs and corticotrophs in pituitary gland. In response to gonadotropins (FSH, LH) and adrenocortical tropic hormone (ACTH) the gonads and the adrenal cortex synthesize and secrete sex steroids and corticosteroids. (Modified from Morohashi 1997.)

These two axes, HPA and HPG, show significant similarity in terms of their regulation mechanisms. The hypothalamic central nervous system discharges GnRH and adrenocorticotropin releasing hormone (CRH), which are transported to the anterior pituitary, where they stimulate the gonadotrophs and corticotrophs, respectively. In response to stimulation, these cells in turn secrete the gonadotropins FSH, LH and ACTH. Furthermore, these tropic hormones stimulate the gonads and adrenal cortex to synthesise and secrete sex steroids and corticosteroids. This stimulation take two forms: chronic stimulation, lasting for several hours or days and occurring through increased levels of P450 side chain cleavage (P450scc) protein and a consequent enhancement in steroidogenic capacity, and acute regulation, occurring within minutes and mediated by steroidogenic active regulatory protein (StAR), which facilitates the movement of cholesterol into the mitochondria for conversion to pregnenolone. The steroid hormones have an inhibitory feedback effect on secretion of the tropic peptide hormones (as reviewed in Miller 1988, Morohashi 1997, Miller & Strauss 1999, Edwards & Burnham 2001).

2.3.1 Steroidogenesis

One of the main functions of the adrenal cortex and the gonads is steroid hormone synthesis. The process in which specialized cells in specific tissues synthesize steroid hormones is generally referred to as steroidogenesis. Steroid hormones can be roughly divided into five groups according to their physiological behaviour: adrenal mineralocorticoids, which regulate the salt balance and maintain blood pressure, glucocorticoids, which regulate carbohydrate metabolism and manage stress, progestogens and estrogens, which are mainly produced by the ovaries and regulate reproductive function and secondary sex characteristics in the female, and androgens, which are mainly testicular in origin and are essential for fertility and secondary sex characteristics in the male (Fig. 9). These all share certain structural similarities and arise from a common series of pathways. Cholesterol is the precursor for all the steroid hormones, and although it can be synthesised *de novo* from acetate, most of the cholesterol needed is derived from plasma low density lipoproteins (LDL). Tropic hormones also stimulate the uptake of LDL cholesterol in addition to stimulating steroidogenesis.

Most of the steroidogenic enzymes belong to the cytochrome P450 oxidation group, among which five enzymes are involved in adrenal steroidogenesis: P450scc (side chain cleavage), P450c11 (11- -hydroxylase), P450c17 (17-α-hydroxylase), P450c21 (21-hydroxylase) and P450aldo (aldosterone synthase). The first two of these are located in the mitochondria and the last two in the endoplasmic reticulum. In addition, P450aro mediates the aromatisation of androgens to estrogens in the gonads.

All steroid hormones are synthesised from the same precursor, cholesterol, but the end product depends on the complement of enzymes present in the tissues (Miller 1998). Adrenal glomerulosa cells mainly synthesise and secrete the mineralocorticoid aldosterone, while the adrenal fasciculata cells synthesise and secrete the glucocorticoids cortisol (in humans) and corticosterone (in rodents). The theca cells in the ovaries synthesise and secrete androgens, while the granulosa cells convert these androgens to estradiol and the *corpora lutea* together

with the placental syncytiotrophoblasts synthesise and secrete progesterone. The Leydig cells in the testis synthesise and secrete the androgen testosterone (for a review, see Stocco 2001).

The first, rate-limiting step in the synthesis of all steroid hormones is the conversion of cholesterol to pregnenolone. In mitochondria this involves three distinct reactions, which are mediated by a single enzyme, P450scc, the activity of which is not the critical factor, but rather the supply of the substrate cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane where the P450scc enzyme is located (Black et al. 1994). Absence of P450scc activity results in an inability to synthesise any steroid hormone, and leads to death due to mineralocorticoid deficiency. Patients with a lack of P450scc can be treated with glucocorticoid and mineralocorticoid steroid hormone replacement if diagnosed early enough.

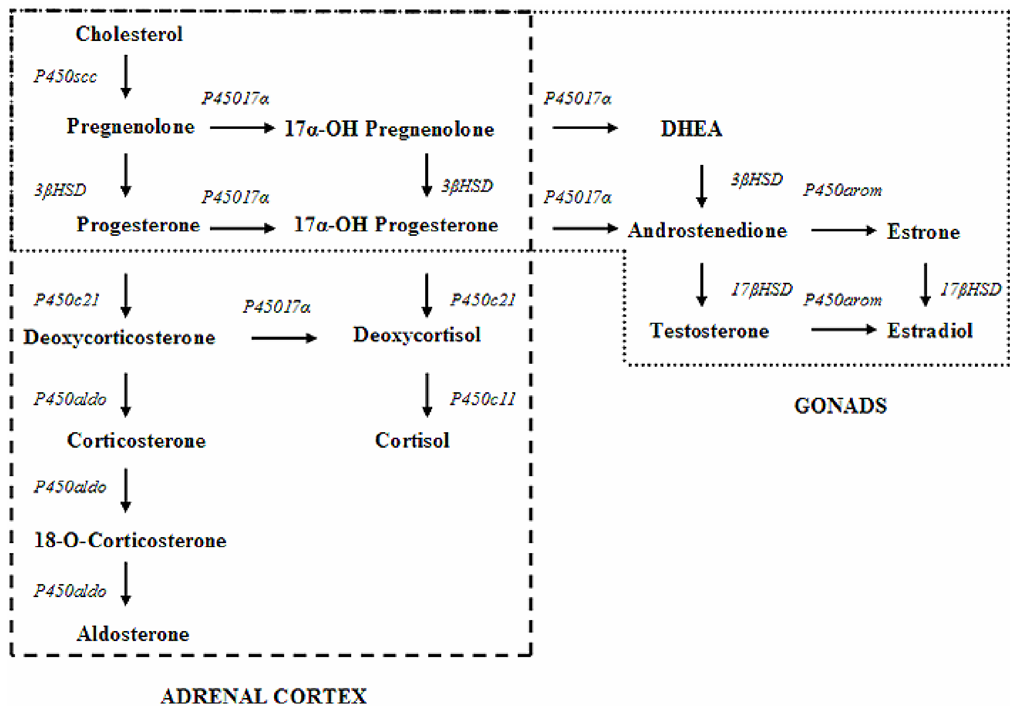

Fig. 9. Steroid hormone synthesis pathways. All steroid hormones will be synthesized from cholesterol and the end products can be classified according to their principal effects; mineralocorticoids (aldosterone), glucocorticoids (cortisol in human, corticosterone in rodents), progestins, androgens and estrogens.

Pregnenolone may subsequently undergo one of two conversions. It may be converted by 3 HSD to progesterone, which is the first biologically important steroid in the pathway, or else it may undergo 17α-hydroxylation mediated by P450c17 to yield 17α-hydroxypregnenolone. Progesterone can also be hydroxylated by P450c17, resulting in 17α-hydroxyprogesterone. P450c17 has two activities, that of a 17α-hydroxylase and that of a C-17,20 lyase capable of breaking up the C-17,20 carbon bond of 17α-hydroxypregnenolone or

17α-hydroxyprogesterone, yielding dehydroepiandrosterone (DHEA) or androstenedione, respectively. P450c17 is a key branching point in steroid hormone synthesis, directing pregnenolone towards the sex steroids (both hydroxylation and cleavage activities of the enzyme), the glucocorticoids (only hydroxylation) or the mineralocorticoids, if neither of the enzyme activities is participating.

After the synthesis of progesterone and 17-hydroxyprogesterone P450c21 can hydroxylate these steroids at the 21 position, resulting in deoxycorticosterone and 11-deoxycortisol, respectively.

The final step in the synthesis of adrenal mineralocorticoids and glucocorticoids is mediated by P450c11, which also mediates the final steps in the synthesis of aldosterone from deoxycorticosterone.

Conversion of androstenedione to testosterone is mediated by 17 -hydroxysteroid dehydrogenase type III (Geissler *et al.* 1994). Androgens are precursors for estrogens in the female and the aromatisation of estrogenic steroids from them is mediated by P450aro. In peripheral target tissues, the genital tubercle, labial swellings and labioscrotal folds, testosterone can further be converted to dihydrotestosterone by 5α-reductase (non-P450 enzyme).

When the P450c21 step is impaired, cortisol synthesis decreases, leading to overproduction of ACTH. When this occurs adrenal steroid synthesis is stimulated and 17-hydroxyprogesterone is converted to androstenedione and further to testosterone, leading to severe virilisation of the female fetus. This disorder is known as CAH. Mutations in StAR cause lipoid CAH, which disrupts the synthesis of all adrenal and gonadal steroids. Affected genetic males are born with normal female external genitalia. (Miller & Strauss 1999, Stocco 2001.)

2.3.2 Mutations in biosynthetic enzymes

Some autosomal recessive mutations have been identified in biosynthetic enzymes responsible for converting cholesterol to androgens. These mutations generally lead to partial male-to-female sex reversal (reviewed in MacLean *et al.* 1997). Mutations in human *HSD3b1* are reported to lead to either ambiguous or female external genitalia in 46XY patients, so that the conversion of DHEA to androstenedione is not possible (Rheaume *et al.* 1994, Russell *et al.* 1994a). Likewise, mutations in *Cyp17* lead to a female phenotype in 46XY males, if they are unable to convert either 17α-hydroxypregnenolone to DHEA or 17α-hydroxyprogesterone to androstenedione (Kagimoto *et al.* 1988, Yanase *et al.* 1990, Yanase *et al.* 1991). The lack of *HSD17b3* results in a 46XY phenotype with male internal genitalia but female external ones. These patients lack the enzyme that converts androstenedione to testosterone (Geissler *et al.* 1994). Mutations in the *5α-reductase type 2* gene lead to an inability to convert testosterone to DHT. These 46XY patients have feminised external genitalia and failed prostate development (Imperato-McGinley *et al.* 1974).

2.4 Wnts

The Wnt pathway plays an important role in development. Several key steps, such as gastrulation, anterior-posterior patterning, neurulation and organogenesis of the mid-brain, central nervous system, kidney, limbs and genitals, are regulated by locally acting *Wnts*. (Reviewed in Nusse & Varmus 1992, Cadigan & Nusse 1997, Uusitalo *et al.* 1999.)

Wnts usually serve as growth and differentiation factors during development. This large gene family of secreted and soluble glycoproteins consists of 19 members (Table 2), most of which are composed of 350-400 amino acids and have 23-24 residues of conserved cysteines. In addition Wnt proteins are also N-glycosylated at several sites.

Table 2. Known phenotypes of Wnt gene knock-out mice

Gene	Phenotype	Reference
Wnt-1	Loss of midbrain and cerebellum Defects in neural crest derivatives	(McMahon & Bradley 1990, Thomas & Capecchi 1990)
Wnt-2	Defects in placentation	(Monkley *et al.* 1996)
Wnt-2b/13	–	–
Wnt-3	Defects in axis formation, lack of primitive streak, mesoderm, and node, Defects in hair growth	(Liu *et al.* 1999, Kishimoto *et al.* 2000)
Wnt-3a	Lack of caudal somites, disruption of notochord, CNS dysmorphology and failure in tailbud formation Loss of hippocampus	(Takada *et al.* 1994, Yoshikawa *et al.* 1997, Lee *et al.* 2000)
Wnt-4	Failure in kidney tubule formation Failure in Müllerian duct formation, partial sex-reversal in females and loss of oocytes Defects in side-branching in mammary gland Decrease in number of thymocytes	(Stark *et al.* 1994, Kispert *et al.* 1998, Vainio *et al.* 1999, Brisken *et al.* 2000, Mulroy *et al.* 2002)
Wnt-5a	Truncated A-P axis, reduced number of proliferating cells, outgrowth defects in developing ears, face and genitals Distal lung morphogenesis	(Yamaguchi *et al.* 1999, Li *et al.* 2002)
Wnt-5b	–	–
Wnt-6	–	–
Wnt-7a	Limb polarity, A-P patterning, failure in regression of Müllerian duct in males, abnormalities in uterus and oviduct Delayed maturation of synapses in cerebellum	(Parr & McMahon 1995, Miller & Sassoon 1998, Parr & McMahon 1998, Hall *et al.* 2000)

Table II. continues

Gene	Phenotype	Reference
Wnt-7b	Defects in placentation	(Parr *et al.* 2001)
Wnt-8a	–	–
Wnt-8b	–	–
Wnt-10a	–	–
Wnt-10b	Inhibition of adipogenesis	(Ross *et al.* 2000)
Wnt-11	Needed for cardiogenesis	(Pandur *et al.* 2002)
Wnt-14	–	–
Wnt-15	–	–
Wnt-16	–	–

See also http://www.stanford.edu/~rnusse/wntgenes/mousewnt.html (03.10.2002)

When Wnts are secreted, they can have either a paracrine and/or an autocrine effect, accumulating in the recipient cell or in the donor cell, respectively, by a binding process that involves extracellular matrix molecules such as proteoglycans.

In the Wnt signalling cascade frizzled (Fz) receptors are required for relaying the signal inside the cell (Wang *et al.* 1996). These are seven-fold transmembrane receptors having a cysteine-rich domain (CDR), which is essential for Wnt binding and signalling (Bhanot *et al.* 1996). Actually, Wnts have a dual-receptor complex, in which LRP5/6 (Low-density-lipoprotein, LDL-receptor-like protein 5 or 6) act as a co-receptor (Tamai *et al.* 2000). Wnt signalling can be inhibited by secreted frizzled-like proteins (sFRPs), which also have a CDR domain (Rattner *et al.* 1997), by Dickkopf (Dkk) (Niehrs 1999) or Cerberus (Piccolo *et al.* 1999). At least three pathways exist for Wnt signalling: the canonical Wnt or dishevelled-dependent β-catenin pathway, which contributes to the establishment of the dorsal-ventral axis (Willert & Nusse 1998), the planar cell polarity pathway, which is essential for cell polarisation (Tada & Smith 2000, Wallingford *et al.* 2000), and the dishevelled-independent protein kinase C-pathway (Fz/PKC), which controls cell-sorting behaviour in the mesoderm (Winklbauer *et al.* 2001).

In the dishevelled-dependent β-catenin pathway (Fig. 10) the signalling cascade is initiated at the cell membrane by interaction between the Fz receptor and the Wnt protein. The signal is then mediated inside the cell and further transduced to *dishevelled (dsh)* (Noordermeer *et al.* 1994), which becomes activated. This activation is followed by the inactivation of *zeste-white 3/glycogen synthase kinase 3β (zw3/GSK3)* (Siegfried *et al.* 1994) leading to the accumulation of β–catenin/Armadillo protein in the cytoplasm (van Leeuwen *et al.* 1994). β–catenin/Arm enters the nucleus and modulates gene expression together with TCFs (T-cell factors) (van de Wetering *et al.* 1997).

The planar cell polarity (PCP) pathway does not involve the components usually placed on Dsh, such as zw3/GSK3, Arm (β–catenin) or Tcf-3. Instead, it consists of small GTPases such as RhoA and Rac, followed by the activation of JNK/SAPK-like kinases (Strutt *et al.* 1997, Boutros *et al.* 1998).

The Fz/PKC pathway differs from the other two in being independent of Dishevelled function. Instead, the activation of heterotrimeric G proteins of the G_i class is involved in signal transduction (Winklbauer *et al.* 2001).

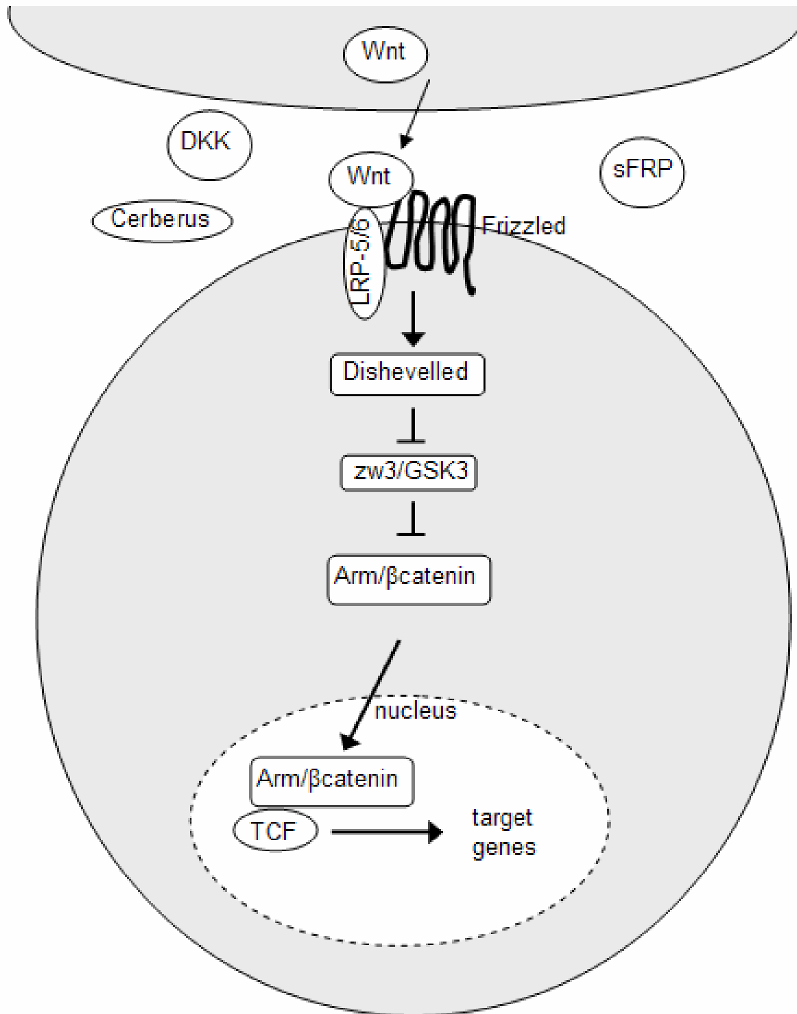

Fig. 10. Simplified model of Wnt/β-catenin signalling pathway. Wnt ligand activates Frizzled/LRP-5/6 receptor complex, which is followed by signalling cascade leading to accumulation of β-catenin in the cytoplasm. Then β-catenin enters the nucleus, where it modulates gene expression together with TCFs.

2.4.1 Wnts in sex differentiation

Of the known Wnts, at least *Wnt-4, Wnt-5a* and *Wnt-7a* are involved in the development of the female reproductive tract (reviewed in Heikkila *et al.* 2001). In *Wnt-7a* mutant mice the Müllerian ducts fail to regress, most likely because of the absence of the AMH receptor. This

leads to sterility, as the ectopic Müllerian ducts in males prevent the vas deferens from connecting properly at its distal end, thus blocking the passage of sperm, while in females the oviduct and uterus develop abnormally. Adult female mutants lack visible coiled oviducts, so that the uterus is intermediate between the normal uterus and the vagina. (Miller & Sassoon 1998, Parr *et al.* 1998.)

Wnt-5a is strongly expressed in the genital primordial and genital tubercle, so that mutant mice have a stunted genital tubercle and lack external genitalia (Yamaguchi *et al.* 1999). No member of the Wnt family has been reported to be definitively needed for development of the male reproductive tract in mice, whereas *DWnt-2* is required for growth of the male-specific pigment cells of the reproductive tract sheath in *Drosophila*, while conversely, misexpression of *DWnt-2* in females leads to the appearance of these cells (Kozopas *et al.* 1998).

2.4.2 Wnt-4

Wnt-4 is expressed in the kidney mesenchyme and its derivatives (Stark *et al.* 1994), in pituitary gland (Treier *et al.* 1998), in the mammary gland (Brisken *et al.* 2000) and in thymus (Mulroy *et al.* 2002). Mutant mice have a severe, complex phenotype. First, since *Wnt-4* is essential for kidney development, the pretubular cell aggregates fail to form, although mesenchymal and ureteric development is otherwise unaffected. Probably due to this kidney dysfunction, *Wnt-4* deficient mice are not viable and die within 24 hours of birth (Stark *et al.* 1994). Secondly, since Wnt-4 is required at least for the expansion of some pituitary-cell-type precursors, the ventral α-GSU, TSHβ and GH cell populations are drastically reduced in mutant mice, although the ACTH-expressing corticotropes are unaffected. Thus the pituitary glands of these mice are hypocellular, even though all the cell types are correctly determined (Treier *et al.* 1998). Thirdly, as Wnt-4 is needed for ductal branching in the mammary gland during the early pregnancy, in addition to which progesterone signalling is required for the induction of *Wnt-4* expression, the mammary glands of mutant mice resemble those of their wild-type littermates more later in the pregnancy than they do in early to mid-pregnancy. It could be that the actions of other members of the Wnt family of factors that are known to be expressed late in pregnancy, such as *Wnt-5a, Wnt-5b,* and *Wnt-6*, can compensate for the lack of *Wnt-4*. (Brisken *et al.* 2000.)

3 Aims of the research

Sex is determined genetically at the time of fertilisation, by the presence or absence of the Y chromosome. Thereafter various genes guide the formation of indifferent gonads, determine the gonadal sex and control differentiation into testes or ovaries.

The female development pathway has traditionally been regarded as a default alternative, resulting primarily from the lack of a Y chromosome and of active signalling, while the development of the male phenotype is an active genetic cascade initiated by the Y-chromosomal gene *Sry*. Despite extensive research, the complexity of gonadal sex determination is still poorly appreciated.

Early in embryonic development, the gonad and the adrenal gland arise from the common primordia in where *Wnt-4* is expressed. *Wnt-4* belongs to a large gene family of secreted growth and differentiation factors that are involved in a variety of phenomenon during development.

The specific aims of the present work were:

1) to characterise the role of *Wnt-4* in sex determination,

2) to characterise the mechanisms of the female-to-male sex reversal that takes place in Wnt-4 mutant female mice, and

3) to establish the function of *Wnt-4* in adrenal gland development.

4 Materials and methods

4.1 Mouse strains (I, III-IV)

Wnt-4 knock-out (Stark *et al.* 1994), *Pax2lacZ* (Kispert *et al.* 1996) and respective wild-type CD-1 mouse lines were used in the experiments. The day when a vaginal plug was detected in the mated female was designated as E0.5 of gestation.

4.1.1 Genotyping and sex typing of embryos

Embryos were genotyped as published previously (Stark *et al.* 1994, Kispert *et al.* 1996). The sex typing of embryos at age E12.5 and younger was performed by PCR, using the following primers to amplify a 353 bp region in the last intron of the mouse *Zfy* gene: 5'-GGA AAT GCT GTT ACA TGT TGA CC-3' (corresponding to positions 47-70 as defined by Chang *et al.* 1994) and 5'-CAG CTT GAC CTG CAA AGG AAG-3' (corresponding to positions 378-399). The PCR reactions were performed in a total volume of 15 µl, containing 18 ng template DNA, 1x AmpliTaq reaction buffer (Perkin Elmer), 0.2 mM of each nucleotide, 50 ng of primers and 2.5 U of AmpliTaq Gold DNA Polymerase (Perkin Elmer). The reactions were carried out for 25 cycles of denaturation at 95°C for 1 min, annealing at 59°C for 2 min and extension at 69°C for 3 min. Denaturation was carried out for 12 min in the first cycle and extension for 10 min in the last. Embryos older than E12.5 were sex-typed based on the morphology of the gonads.

4.1.2 Tissue samples

Tissues were microdissected in Dulbecco's PBS and fixed immediately in 4% paraformaldehyde (PFA) overnight (o/n). Fixed samples for hematoxylin-eosin or antibody stainings, section *in situ* hybridization and morphometric studies were dehydrated in a graded

ethanol series, washed with xylenes, embedded in paraffin and sectioned at 5 µm, while those for whole mount *in situ* hybridization were dehydrated in a graded methanol series and stored in 100% methanol at -20°C.

4.2 Histology (I,III-IV), -galactosidase (I) and antibody staining (I)

Hematoxylin-eosin stainings performed according to Hogan and coworkers (Hogan *et al.* 1994) were used to study the histology of the gonads and adrenal glands, and -galactosidase staining (Hogan *et al.* 1994) to identify the Wolffian duct in the *Wnt-4/Pax2lacZ* transgenic mice. GCNA-1 (germ cell nuclear antigen 1) antibody at a concentration of 1:1000 was used for germ cell identification (Enders & May 1994) and the Vectastain ABC kit (Vector Laboratories) to visualise the antibody binding.

4.3 Whole mount and section *in situ* hybridization (I-IV)

Even a single cell that is positive for gene expression can be detected by whole mount *in situ* hybridization, and the cellular localisation can be determined by section *in situ* hybridization. The probes used here are summarized in Table 3.

After linearization of the plasmids, the cDNAs were transcribed in vitro to prepare digoxigenin-labelled (Digoxigenin RNA labelling kit; Boehringer, Mannheim) or 35S radiolabelled (Amersham, Pharmacia) riboprobes for the whole mount and section in situ hybridizations, respectively. The in situ hybridizations were performed according to standard procedures (Wilkinson 1992).

Table 3. Probes used for whole mount and section in situ hybridization

Gene	Reference
Cyp11B2	Keith Parker, University of Texas Southwestern Medical Center, Dallas, USA
Cyp17	Expressed sequence tag AA822113, NCBI
Cyp21	Keith Parker, University of Texas Southwestern Medical Center, Dallas, USA
Dhh	Andy McMahon, Department of Molecular and Cellular Biology, Harvard University, USA
Follistatin	Olli Ritvos, Haartman Institute, Helsinki, Finland
Hsd3bI	Robin Lovell-Badge, National Institute for Medical Research, London, UK
Hsd17bIII	(Mustonen *et al.* 1997)
Mis	Patricia Donahoe, Department of Surgery, Pediatric, Massachusetts General Hospital, Boston, USA
Oct-4	(Scholer *et al.* 1990)
Pax-8	Peter Gruss, Max Planck Institute for Biophysical Chemistry, Goettingen, Germany
Shh	(Bellusci *et al.* 1997)
Wnt-4	(Stark *et al.* 1994)
Wnt-6	Andy McMahon, Department of Molecular and Cellular Biology, Harvard University, USA
Wnt-7a	Andy McMahon, Department of Molecular and Cellular Biology, Harvard University, USA

4.4 Morphometric studies (IV)

The morphometric studies were performed to compare the volumes of the adrenal glands in the *Wnt-4* mutant and wild-type mice. Adrenal glands were collected from four groups of E14.5 and newborn mice: wt females, wt males, *Wnt-4* mutant females, and *Wnt-4* mutant males. Two pairs of glands from each group were fixed o/n in 4% PFA, dehydrated in an ascending series of ethanol solutions and xylenes and the tissues embedded in paraffin. Five-micrometre serial sections were cut, placed on microscope slides and stained with eosin. The total area of the adrenal gland in each section was measured with a MCID-M4 digital image analysis system (Imaging Research Inc., Brock University). The images were acquired with a Sony DXC-930P colour video camera attached to a Nikon Optiphot II microscope fitted with a 4x objective (Nikon). Tissue volume was determined by integrating section areas with section thicknesses.

4.5 Quantitative reverse transcription-PCR (IV)

Cyp11B2, Cyp21 and *Cyp17* mRNA concentrations in the mouse adrenal gland samples were measured by quantitative reverse transcription-PCR analysis (Majalahti-Palviainen *et al.* 2000). The adrenal glands of newborn mice were dissected and pooled by sex and phenotype (5-8 adrenals in each group). Total RNA was isolated using the Quick Prep kit from Pharmacia, and the reverse transcriptase reaction was carried out using the First Strand cDNA synthesis kit from MBI Fermentas.

4.6 Hormone analysis (III-IV)

Concentrations of aldosterone (ICN Pharmaceuticals), corticosterone (DRG Instruments GmbH), cortisol (Orion DG), ACTH, (beta-endorphin radioimmunoassay, (Vuolteenaho *et al.* 1981), estradiol, DHEA, androstenedione and testosterone were measured in plasma samples. In addition, testosterone, androstenedione and DHEA concentrations were measured in gonadal adrenal gland tissue samples.

Blood from decapitated mice was collected into heparinized glass tubes, centrifuged for 10 min at 3000 rpm and the plasma samples were stored at -20°C. These samples were pooled, 6 per tube, to provide a sufficient volume. Gonadal and adrenal tissues were collected from newborn mice, frozen quickly and stored at -20°C. The tissues were sonicated for 15 seconds in 100 µl of distilled water and washed twice with 300 µl of a solution containing 9 volumes of diethyl ether and one volume of ethyl acetate. After centrifugation, the water phase was taken for protein assays and the organic phases were combined, evaporated, reconstituted with 500 µl of radioimmunoassay buffer (DRG Instruments GmbH, Germany) and double aliquots assayed for steroids. Protein concentrations in the tissue samples were measured using the

Bio-Rad protein assay kit. The hormone analyses were performed by Olli Vuolteenaho and Juhani Leppäluoto at the Department of the Physiology, University of Oulu.

4.7 Flutamide treatment (III)

Flutamide is a non-steroidal antiandrogen, which can be used to block the effect of androgens *in vivo*. It was used here in the form of subcutaneous injections of 100 mg/kg of flutamide (F9397, Sigma) per day to treat 23 pregnant Wnt-4 heterozygote females, while 9 mice received the vehicle, turnip oil, daily from E10.5 until delivery. Anogenital distances in the newborn mice were measured using a dissecting microscope with a micrometer lens, after which the urogenital systems were microdissected and used for *in situ* hybridizations or histological analysis.

5 Results

5.1 Expression of *Wnt-4* in the developing urogenital system (I, IV)

The whole mount and section *in situ* hybridizations used to study the expression of *Wnt-4* in the urogenital region during fetal life pointed to expression in the mesonephric mesenchyme, the gonads, the Müllerian ducts and the adrenal glands, but none in the mesonephric tubules. *Wnt-4* is also known to be expressed in the developing kidney (Stark *et al.* 1994).

Wnt-4 expression starts in the mesonephric mesenchyme before gonad formation, at 9.5 dpc (I, Fig. 1a-c), and continues in the gonadal mesenchyme of both sexes after the formation of the indifferent gonad at E11 (I, Fig. 1e,f). It is down-regulated in the testes after the beginning of differentiation, at 11-5-12.5 dpc, but continues in the ovaries throughout their development (I, Fig. 1g-i). This sexually dimorphic expression pattern in the gonads continues throughout fetal life, and the expression in the mesonephric mesenchyme is maintained in both sexes.

Wnt-4 expression is observed in the Müllerian duct immediately after it is formed by in-folding of the coelomic epithelium at E12 (I, Fig. 1k), whereas none is present in the developing mesonephric tubules or in the Wolffian duct (I, Fig. 1e,f).

Wnt-4 is also expressed in the future adrenal gland as soon as it is formed at the anterior site of the mouse mesonephros at E11.5 (IV, Fig. 1a), and expression is detected later in the cortical region of the developing adrenal gland. This expression is maintained in both sexes throughout development (IV, Fig. 1c), but none is detected in the medulla (IV, Fig. 1e).

5.2 Lack of *Wnt-4* leads to masculinization of the female reproductive system (I, III)

The male and female reproductive tracts are morphologically different at birth. The ovaries are normally elongated in shape and covered by suspensory ligaments, but those of *Wnt-4* mutant females are round and “naked” and have certain characteristics of testes (I, Fig. 2i-l). The proximal region of the sex duct in mutant females is highly coiled, like the epididymal

region of the male Wolffian duct, whereas only two or three large coils are visible in the Müllerian duct in wild-type females (I, Fig. 2 e-h). In addition, the gonads of mutant females develop in close association with a fat body that is typical only of males (I, Fig. 2l). The position of the mutant ovaries is not altered compared with that in wild-type females, however, and they are located closer to the kidney than are the testes. No expression of the pre-Sertoli cell markers *Dhh, AMH* and *Sox9* was detected in the gonads of *Wnt-4* mutant females before the normal loss of the Wolffian duct (I, Fig. 3a-d and data not shown), indicating that no primary sex reversal had occurred, but expression of *Dhh* and *AMH* was detected at birth. In addition, expression of the female specific genes *follistatin* (Fig. 9) and *Msx-1* (data not shown) was lost from the ovaries of the *Wnt-4* mutant females.

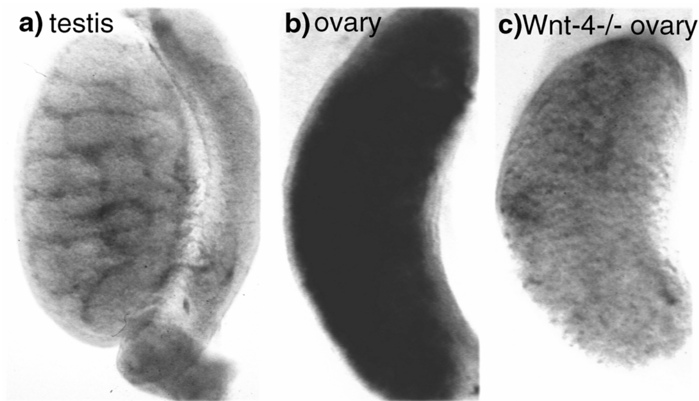

Fig. 11. The transcription of female specific gene, *follistatin* is lost from the ovary of *Wnt-4* deficient mouse. *In situ* hybridization results show that *Follistatin* is not transcribed in the testis (a), but strongly in the 14.5 dpc ovary of the wild-type female shown as black reaction product in unstained specimen (b), while no transcription can be detected in the one of *Wnt-4* mutant female (c).

Further evidence for masculinization in mutant females is the identity of the single sex-associated duct, which proved to be a Wolffian duct, when the *Wnt-4* mutant mouse line was crossed with *Pax2lacZ* mice that express a transgene in the Wolffian duct and its derivatives, in the collecting duct system and in the ureter of the kidney. This transgene was expressed in the single duct of the *Wnt-4* mutant female, as in the Wolffian duct of mutant and wild-type males, while no expression was detected in the wild-type females after regression of the Wolffian duct (I, Fig. 2m-p). The results were verified by *in situ* hybridization of *Sonic hedgehog (Shh),* which also marks the Wolffian duct (data not shown).

The development of the Müllerian duct was analyzed by means of *Wnt-7a* and *Pax-2* whole mount *in situ* hybridizations. The Müllerian duct is normally present in both sexes until 14.5 dpc, after which it degenerates in males due to activation of AMH. No expression was detected in the Müllerian duct of either sex at 12.5 dpc, however, indicating that *Wnt-4* signalling is required for the initial stages of duct formation and that the duct does not form in either sex in the absence of Wnt-4 (I, Fig. 3 a-l). *Wnt-7a* and *Pax-2* expression was clearly seen in the developing Müllerian ducts of wild-type littermates.

Measurement of the anogenital distances, to find out whether the external genitalia of *Wnt-4* mutant females are also masculinized, showed no significant difference between the wild-type and *Wnt-4* mutant females (III, Table I).

Wnt-4 was also found to be involved in gametogenesis, as indicated by the fact that the number of germ cells had been dramatically reduced in the *Wnt-4* mutant females at birth. Analysis of *Oct-4* by *in situ* hybridization indicated that germ cell migration has not been affected and that the number of germ cells at E14.5 was normal (data not shown), but it is evident that most of the germ cells die later, so that less than 10% of the normal number of oocytes is present in *Wnt-4* mutant ovaries at birth and can be recognized with the GCNA-1 antibody (I, Fig. 5e-h).

5.3 Steroidogenesis is initiated in the ovaries of *Wnt-4* mutant females (I-IV)

To find out whether the ovaries of *Wnt-4* mutant female are also masculinized at the functional level, steroidogenesis was studied. This function is dependent on the expression of several enzymes, which are expressed strongly in the Leydig cells of the testes during normal fetal development (I, Fig. 4a,d,g), but not in the ovaries (I, Fig. 4b,e,h). In contrast, *Hsd3b1* and *Cyp17,* both of which are needed for testosterone biosynthesis, are ectopically expressed in the ovaries of *Wnt-4* mutant females (I, Fig. 4c,f,i), indicating that a lack of *Wnt-4* leads to the initiation of steroidogenesis in the ovary.

In addition, expression of *Hsd17b3*, which is testis-specific and has been demonstrated earlier in the Leydig cells of the adult mouse (Baker *et al.* 1997), was not detected in the Leydig cells during fetal life, but was expressed in the Sertoli cells of the normal testes of newborn mice, unlike *Hsd3b1* and *Cyp17. Hsd17b3* was also present in the *Wnt-4* mutant ovary (II, Fig. 4 and data not shown).

To determine whether androgens are produced in the ovaries of *Wnt-4* mutant females, the concentrations of testosterone, DHEA and androstenedione were measured in samples of both plasma and gonadal tissue. In agreement with the ectopic expression of *Hsd3b1*, *Cyp17* and *Hsd17b3,* testosterone was indeed found to be produced in the *Wnt-4* mutant ovaries (III, Table II, Fig. 4).

5.4 Flutamide can rescue partially the *Wnt-4* mutant female phenotype (III)

Pregnant *Wnt-4* heterozygote females were treated from E10.5 until delivery with daily injections of the anti-androgen flutamide to determine the effects of androgens on the *Wnt-4* mutant female phenotype. After the treatment the structure of the newborn *Wnt-4* mutant female gonads was normalized compared with that of the untreated animal, i.e. the gonads were more elongated in shape and almost indistinguishable from those of their wild-type

littermates (see Table 4). In addition the sex duct, which was earlier shown to be a Wolffian duct, was absent. In view of the lack of *Wnt-4* and the action of the flutamide, the treated mutant females did not have any sex duct at all, but instead the gonads were attached to the urogenital system by thin ligaments. This indicates that androgens will be produced in females in the absence of *Wnt-4,* leading to masculinization, and that the situation can be restored with anti-androgens.

Normal male development was also affected by flutamide treatment, in that the testes of treated mice were smaller in size than those of their untreated littermates and the Wolffian duct was less coiled in the epididymal region.

Table 4. Comparison of the gonad phenotype of Wnt-4 wild-type and mutant mice before and after the flutamide treatment

	untreated				flutamide treated			
	wt ♂	Wnt-4$^{-/-}$ ♂	wt ♀	Wnt-4$^{-/-}$ ♀	wt ♂	Wnt-4$^{-/-}$ ♂	wt ♀	Wnt-4$^{-/-}$ ♀
Shape of the gonads	round	round	elongated	round	round	round	elongated	elongated
fat body	present	present	absent	present	present	present	absent	nd
germ cells	present	present	present	reduced	present	present	present	nd
sex duct	Wolffian	Wolffian	Müllerian	Wolffian	Wolffian	Wolffian	Müllerian	none

wt = wild type

-/- = Wnt-4 deficient mice

nd = not determined

5.5 Wnt-4 deficiency alters the function of the adrenal cortex (IV)

Since the fact that *Wnt-4* is expressed in the cortical region of the adrenal gland may indicate some kind of developmental role for it, we studied the histology and function of the adrenal gland in Wnt-4 mutant mice.

Histological analysis by means of hematoxylin-eosin staining showed no apparent differences between the adrenal glands of *Wnt-4* mutant mice and their wild-type littermates (IV, Fig. 2a-d), while morphological studies correspondingly indicated that there was no significant difference in volume (IV, Table II). Thus the main structure of the *Wnt-4* mutant adrenal gland had not been affected.

In contrast, analysis of the expression of *Cyp11B2,* coding for P450aldo, by *in situ* hybridization demonstrated that the number of cells expressing this gene had decreased (IV, Fig. 3c,d). This result was verified by quantitative reverse transcription PCR, which showed a significant reduction in the expression of *Cyp11B2* (IV, Table II). In agreement with this, the concentration of aldosterone in the plasma of the *Wnt-4* mutant mice was also reduced relative to that of their wild-type littermates (IV, Fig. 5a). Likewise, the number of cells expressing *preadipocyte factor 1 (Pref-1)*, which specifically marks the *zona glomerulosa* in the cortex, had also decreased in the cortex of the Wnt-4-deficient mice (IV, Fig. 3e,f). All these data

together indicate that the *zona glomerulosa*, the outermost layer of the adrenal gland, had not developed properly in the cortex of the Wnt-4 mutant mice.

In addition, analysis of the expression of *Cyp21*, which encodes P450c21-hydroxylase, in the adrenal glands of the *Wnt-4* mutants by *in situ* hybridization and quantitative reverse transcription PCR failed to reveal any changes (IV, Fig. 3a,b, Table II).

In contrast to *Cyp11B2* and *Cyp21*, *Cyp17* showed sexually dimorphic expression in the adrenal gland, being abundantly expressed in the wild-type females, beginning at E12.5, peaking at E14.5 and continuing until birth (data not shown and IV, Fig. 4 c,g), but markedly lower in the wild-type and mutant males and the mutant females, in which it is downregulated at E16.5-E18.5 (II, Fig. 4e,f,h). Thus *Cyp17* expression in the adrenal glands of *Wnt-4* mutant females can be considered to be "masculinized" in this respect. These results were also verified by rt-PCR (IV, Fig. 4i-k).

In addition, no differences were found in the concentrations of DHEA measured in the adrenal gland tissues of wild-type and *Wnt-4* mutant mice. The concentrations of androstenedione and testosterone were also measured, but remained below the detection limits (data not shown). Altogether, these results indicate that the main endocrine routes in the adrenal glands of *Wnt-4* mutant mice are not altered.

5.5.1 Elevated function of the HPA-axis in Wnt-4 mutant mice (IV)

Since ACTH is secreted from the pituitary gland to stimulate the functioning of the adrenal gland, we measured the concentration of -endorphin, which reflects that of ACTH, in the plasma of newborn *Wnt-4* mutants and their wild-type littermates. The data revealed a statistically significant elevation of -endorphin concentrations in the blood of both male and female mutant mice relative to their wild-type littermates (IV, Fig. 5b). In response to this stimulation, the adrenal glands in turn secrete corticosteroids, the concentrations of which were also found to be significantly higher in the mutants than in their wild-type counterparts, both male and female (IV, Fig. 5c).

5.6 *Wnt-4* may control the migration/sorting of adrenal and gonadal cells (IV)

Although *Cyp21* is expressed specifically in the adrenal gland during development, some positive cells were also detected in the gonads of both *Wnt-4* mutant males and females between E11.5 and E13.5 (IV, Fig. 6b,d,f), but never outside the adrenal gland in their wild-type littermates (IV, Fig. 6a,c,e).

Also, although both *Hsd3b1* and *Cyp17* are normally expressed in the adrenal glands and testes but not in the developing ovary (IV, Fig. 7a-d, 4 a-h), both of them were expressed in the gonads of *Wnt-4* mutant females, where their expression was generally restricted to the anterior tip (IV, Fig. 4d,h), the part located closest to the adrenal gland. These results further

support the hypothesis that the cells that are positive for steroidogenic genes such as *Hsd3b1* and *Cyp17* in the *Wnt-4* mutant ovary may have originated from the adrenal gland.

6 Discussion

6.1 *Wnt-4* expression in the gonad is regulated sex-specifically, while that in the adrenal gland is independent of sex

Although Wnt molecules are involved in the development of a number of tissues, the main purpose here was to analyse the function of *Wnt-4* in the development of the adreno-genital system, especially in females.

The *Wnt-4* expression pattern in the gonad mimics that of other genes such as *Dax-1*, which participates in sex determination, in that expression can be detected in both sexes at first and is then shut down in one sex. *Wnt-4* is present in the mesenchyme of the mesonephros and in the developing gonads of both sexes until E11.5, when sex determination commences and expression is shut down in the male gonad but persists in the ovary at least until birth. Expression is also retained in the Müllerian duct in males, however, until the duct regresses in response to the action of AMH after E14.5. The fact that Wnt-4 is shut down in males at the time when *Sry* expression begins may indicate that it could be inhibited by *Sry*, especially since it has been shown in *Xenopus* that Sox3, the closest homologue of Sry (Graves 1998), is able to inhibit Wnt signaling (Zorn *et al.* 1999).

In contrast to the situation in the gonads, the expression of *Wnt-4* in the adrenal gland is independent of sex, being detectable from the very beginning of the formation of the adrenal cortex and later being restricted to the outermost cortical region.

The nuclear localization of beta-catenin suggests activation of the canonical Wnt signalling pathway. Surprisingly, significant nuclear localization of beta-catenin in humans has been observed in the fetal lung, cartilage, placenta, kidney, capillaries, skin and adrenal, but not in the ovary or testis (Eberhart & Argani 2001).

6.2 Lack of Wnt-4 leads to female-to-male sex reversal

Of the genes identified so far, *Wnt-4* is the only mouse gene causing female-to-male sex reversal during fetal life when deleted. Thus the *Wnt-4* mouse model proves that female differentiation is not a default pathway in development but requires active signalling.

The morphology of the *Wnt-4* mutant female ovary closely resembles the structure of the testis. The mutant ovary is round, associated with a fat body and lack the suspensory ligament of the oviduct, all of which features are typical only of the male sex. In addition to the *Wnt-4* mouse model, mice lacking *estrogen receptors* α and (α ERKO) go through a partial female-to-male sex reversal in the gonads, but not before adulthood (Couse & Korach 1999b). Interestingly, *Wnt-4* could also, at least partially, lie behind this phenotype if the estrogens regulate *Wnt-4* expression in the ovaries as they do in the uterus of adult female mice (Miller *et al.* 1998). The lack of estrogen regulation could then lead to abnormal expression of *Wnt-4* and partial sex reversal.

The lack of a Müllerian duct in both male and female *Wnt-4*-deficient mice has no consequences in the male case, where development is unaffected, but in females it means that the Müllerian duct derivatives, the oviducts, uterus and upper part of the vagina, are not able to form. Furthermore, as the uppermost part of the Müllerian duct forms the suspensory ligament of the oviduct, the gonads of Wnt-4$^{-/-}$ females lack it. Since Wnt signalling is also required after the formation of the duct, it is *Wnt-7a* that regulates the sex-specific development of the Müllerian duct and its differentiation into derivatives (Miller & Sassoon 1998, Parr & McMahon 1998).

The gonads of *Wnt-4*-deficient female mice have not undergone any primary sex reversal, however, because no morphologically identifiable sex cords are present and no expression of the pre-Sertoli cell markers *Dhh, AMH* and *Sox9* is detected before the germ cells are lost, at which point their expression is most likely a secondary effect of germ cell loss and some supporting cells adopt the characteristics of Sertoli cells, as in other reported cases (Vigier *et al.* 1987, Behringer *et al.* 1990, Lyet *et al.* 1995). In contrast, expression of the Leydig cell markers *Hsd3b1* and *Cyp17* is detectable very early in the development of the gonads of *Wnt-4* mutant females. In addition to the ectopic activation of these testosterone biosynthesis pathway genes, testosterone itself can also be measured in the plasma of these females, although not in that of wild-type females. Steroidogenesis must therefore also be activated in females in the absence of Wnt-4. The external genitalia of *Wnt-4* mutant females are normal, indicating that the testosterone and DHT levels are too low to masculinize them.

Wnt-4-deficient female mice lack most of their oocytes at birth. As Wnt-4 expression can also be detected in the ovaries of germ cell-deficient *Steele (S1)* mice (Fleischman 1993), it must exist either partly or completely within somatic cell lineages. Thus it is not known whether the function of Wnt-4 is vital for oocyte survival on account of its presence in the oocytes themselves or in the supporting cells.

Thus Wnt-4 is needed for three aspects of female development, formation of the Müllerian duct, suppression of Leydig cell formation and oocyte development.

Structural masculinization in *Wnt-4* mutant females can be prevented by treatment with the anti-androgen flutamide, whereupon the genital systems resemble to those of their wild-type littermates. This indicates that the sex reversal that takes a place in these mutants is due to the action of androgens. The normal function of Wnt-4 may be to suppress the action of Leydig

cell differentiation and the initiation of steroidogenesis in wild-type females, and after flutamide treatment the expression of the markers of the testosterone biosynthesis pathway genes in *Wnt-4* mutant females has not been changed relative to untreated ones. These results are congruent with earlier flutamide studies, in which the concentration of testosterone in the plasma was unaffected in wild-type males (Silversides *et al.* 1995).

Thus, anti-androgens block the action of androgens and can partially rescue the structure of the ovary of *Wnt-4* mutant females. *Wnt-4* itself is needed to suppress Leydig cell differentiation, however, and despite of the action of the anti-androgens, the steroidogenic genes in the females are still activated in the absence of *Wnt-4*.

6.3 Wnt-4 and the adrenal gland

Aldosterone production, as reflected in P450c11aldo expression, is reduced in *Wnt-4* mutants. The section *in situ* hybridization results suggest that the number of aldosterone-producing cells decreases. Aldosterone is synthesized exclusively within the *zona glomerulosa*, indicating that this layer of the adrenal gland has not developed properly. The enzyme P450c11aldo is required both for corticosterone and aldosterone production, although corticosterone biosynthesis is not affected by its reduced amount, whereas aldosterone production is more severely affected.

Wnt-4 may also be involved in sorting of the gonadal and adrenal cells, which arise from the same progenitor cells. This means that several genes must affect both gonadal and adrenal development. The strongest evidence for mismigration is the presence of *Cyp21*-positive cells in the gonads of *Wnt-4* mutant embryos, where the gene is not normally expressed. The location of *Hsd3b1* and *Cyp17*-positive cells in the anterior end of the *Wnt-4* mutant ovary further supports the notion of mismigration.

6.4 Relationship of *Wnt-4* to other sex-determining genes

Wnt-4 is the only gene identified so far, the deletion of which causes female-to-male sex reversal during fetal life. This overrules the earlier assumption that female development is a default pathway, resulting from a lack of any active signalling. As *Wnt-4* is first expressed in the gonads of both sexes, it must be down-regulated in the future testis by some male-specific genes. *Sry* has not been shown to inhibit *Wnt-4*, but this might be possible in the light of their expression patterns. In addition the closest homologue of *Sry*, *Sox3*, is able to inhibit *Wnt* signalling in *Xenopus* (Zorn *et al.* 1999).

The observations that the expression of *Dax-1* is reduced in *Wnt-4* mutant mice (Jordan *et al.* 2001) and that *Wnt-4* is capable of up-regulating this expression in Leydig and Sertoli cell lines suggest that Wnt-4 acts upstream of Dax-1.

Sry is the main testis-determining gene and *Dax-1* is thought to have an “anti-testis” role. It has been hypothesized (Jordan *et al.* 2001) that *Wnt-4* could be the molecular signal linking these two transcription factors together. Sry may down-regulate Wnt-4, which leads to a

reduction in Dax-1 and allows testis formation. On the other hand, *Wnt-4* may induce *Dax-1* expression in females and the action of these two genes may prevent testis formation. There is one reported human case in which the duplication of *Wnt-4* led to overproduction of *Dax-1* and a feminized XY male (Jordan *et al.* 2001).

Interestingly, human WNT-4 is mapped to the 1p35 region, which shows similarity to the mouse chromosome 4q, in which the mouse Wnt-4 is also located (based on to http://www.stanford.edu/~rnusse/wntgenes/mousewnt.html, 20.02.2002). Mouse chromosome 4q is also thought to contain the tda-1 locus and to be involved in the XY sex reversal phenotype in mice. Linkage analysis of sex-reversed mice revealed that the tda-1 locus prevented normal male development and suggested that it could serve as a promoter of ovarian development. *Wnt-4* is a strong candidate for *tda-1* in the mouse (Eicher *et al.* 1996, Jordan *et al.* 2001).

Finally, *Wnt-4* basically fulfils the criteria for the "Z-gene". Its absence leads to activation of the male pathway in female mice in the absence of *Sry* (I), while its over-expression in humans leads to male feminization, indicating that it is able to block male development in the presence of *Sry* (Jordan *et al.* 2001).

7 Conclusions

The present results demonstrate a role for *Wnt-4* in female development. It is expressed in a sexually dimorphic manner in the developing urogenital system, in the gonads of both sexes at first but only in the developing ovary after sex determination while its expression in the Müllerian duct and adrenal gland is independent of sex. The lack of *Wnt*-4 leads to partial female-to-male sex reversal, which is the first female sex reversal to be identified, as all the other reported cases are male-to-female changes. In addition, Wnt-4 is needed for Müllerian duct development in both sexes. Absence of the Müllerian duct admittedly has no essential consequences in males, but in females the oviduct, uterus and upper part of the vagina will not form. Furthermore, the lack of *Wnt-4* leads to a dramatic reduction in the number of oocytes, indicating that it is needed either for the survival of the oocyte itself or for the development of the supporting cells.

Wnt-4 also plays a role in adrenal gland development. The number of cells producing aldosterone is reduced in *Wnt-4* mutant mice, and as aldosterone is produced only within the *zona glomerulosa,* this layer evidently does not develop normally in *Wnt-4* mutant mice.

Wnt-4 is the first signalling factor to be identified as playing a role in mammalian sex determination and in the female developmental pathway. Identification of its essential function allows the researcher to set out on a search for the molecular mechanisms, which control female sex determination.

The conditional *Wnt-4* knock-out mouse model should enable the precise roles of Wnt-4 in specific tissue types to be determined at some time in the future.

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