Anti-Müllerian Hormone:
Molecular Mechanism of Action

Anti-Müllerse Gang Hormoon:
Moleculair Werkingsmechanisme

PROEFSCHRIFT

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Abbreviations

ΔAMHRII dominant negative AMHRII
β-Gal β-galactosidase
ActRII activin type II receptor
AHC adrenal hypoplasia congenita
AIS androgen insensitivity syndrome
ALK activin receptor-like kinase
AMH anti-Müllerian hormone
AMHRI AMH type I receptor
AMHRII AMH type II receptor
ARE activin response element
ATP adenosine triphosphate
BMP bone morphogenetic protein
BMPRI BMP type I receptor
BMPRII BMP type II receptor
bp base pairs
BSA bovine serum albumin
C. elegans *Caenorhabditis elegans*
cAMP 3',5'-cyclic adenosine monophosphate
CD campomelic dysplasia
cDNA complementary DNA
CRE cAMP response element
C-terminus carboxyl-terminus
Dax-1 DSS-AHC critical region on the X chromosome, gene 1
dbcAMP dibutyryl cAMP
DDS Denys-Drash syndrome
DES diethylstilbestrol
DMEM Dulbecco's minimal essential medium
DNA deoxyribonucleic acid
DPP decapentaplegic
DSS dosage sensitive sex reversal
DTT dithiothreitol
e.g. for example (*exempli gratia*)
E# Embryonic day # (# days *post coitum*)
EGF epidermal growth factor
EGFR EGF receptor
EGR early growth response
Epo erythropoietin
EpoR Epo receptor
ERα estrogen receptor α
ERβ estrogen receptor β
ERE estrogen response element
ERK extracellular signal-regulated kinase
FCS fetal calf serum
FGF fibroblast growth factor
FSH follicle-stimulating hormone
FSHR FSH receptor
G protein GTP-binding protein
GAPD glyceraldehyde 3-phosphate dehydrogenase
GDF growth and differentiation factor
GFP green fluorescent protein
GTP guanosine triphosphate
h (prefix) human
HEK-293 human embryonic kidney 293 cell line
HHG hypogonadotropic hypogonadism
HMG high mobility group
Hox homeobox genes
i.e. in other words (*id est*)
IU international unit
JNK cJun N-terminal kinase
kb kilo base pairs

kDa	kilo Dalton
KO	knockout
LH	luteinizing hormone
LUC	luciferase
m (prefix)	mouse
Mad	mothers against dpp
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MH	Mad-homology domain
MIS	Müllerian-inhibiting substance
MRKH	Mayer-Rokitansky-Küster-Hauser syndrome
mRNA	messenger RNA
MT-1	metallothionein-1
Neo	neomycine
N-terminus	amino-terminus
oc (prefix)	*Oryctolagus cuniculus* (rabbit)
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate-buffered saline
PC5	prohormone convertase 5
PCR	polymerase chain reaction
PI	propidium iodide
PMDS	persistent Müllerian duct syndrome
r (prefix)	rat
rec (prefix)	recombinant
RNA	ribonucleic acid
RT	reverse transcription
S/T	serine/threonine
SAPK	stress-activated protein kinases
SEM	standard error of the mean
SF-1	steroidogenic factor 1
SHN	Shnurri
Sox	Sry-related HMG-box gene
SRY	sex determining region Y (human)
Sry	sex determining region Y (mouse)
SSCP	single-strand conformation polymorphism
TβRI	TGFβ type I receptor
TβRII	TGFβ type II receptor
TAB1	TAK1 binding protein 1
TAK1	TGFβ-activated kinase 1
TDF	testis determining factor (human)
TdT	terminal deoxynucleotidyl transferase
Tdy	testis determining factor (mouse)
TGFβ	transforming growth factor β
TUNEL	TdT-mediated dUTP nick end labeling
UTP	uridine triphosphate
WAGR	Wilms' tumor, aniridia, urogenital abnormalities and mental retardation syndrome
WT1	Wilm's tumor gene 1

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hwer't se net binne.

Chapter One

General Introduction

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General Introduction

1.1 Introduction

During early fetal development, the primitive urogenital system is bipotential and can develop into a male or female direction, depending on the chromosomal sex of the fetus. In this chapter, regulatory factors of sex determination and differentiation are described. The roles of two testicular hormones, testosterone and anti-Müllerian hormone (AMH), which are essential for proper differentiation of the internal genitalia, are discussed.

1.2 Formation of the urogenital system

The urogenital system develops from the intermediate mesoderm, which lies between the somites and the lateral plate (Figure 1.1). This mesoderm forms as a pair of thickenings on the coelome at the dorsal site of the embryo, running along the full length of the body cavity (Karl and Capel, 1995). At its most anterior region, the intermediate mesoderm forms the pronephros, which soon disappears in vertebrates. The mesodermal midsection forms the mesonephros that gives rise to the mesonephric ducts or Wolffian ducts. The Wolffian ducts extend continuously, ending in the cloaca at the posterior end of the embryo, and form the anlagen of the male reproductive tract. Within the mesonephric region a second pair of ducts is formed, the paramesonephric ducts or Müllerian ducts, which are the anlagen of the female reproductive tract. The Müllerian ducts are formed in a cranial to caudal direction, alongside the Wolffian ducts. The initial formation of the Müllerian ducts starts as an invagination of the coelomic epithelium, and their caudal tips are fused at the urogenital sinus. Conflicting data exist about the role of the Wolffian ducts in Müllerian duct formation. It has been suggested that the Wolffian ducts serve simply as a guidance for early growth of the Müllerian ducts (Dohr and Tarmann, 1984), whereas others have proposed that the Wolffian ducts release epithelial cells that contribute to the Müllerian ducts (Grünwald, 1941; Dyche, 1979).

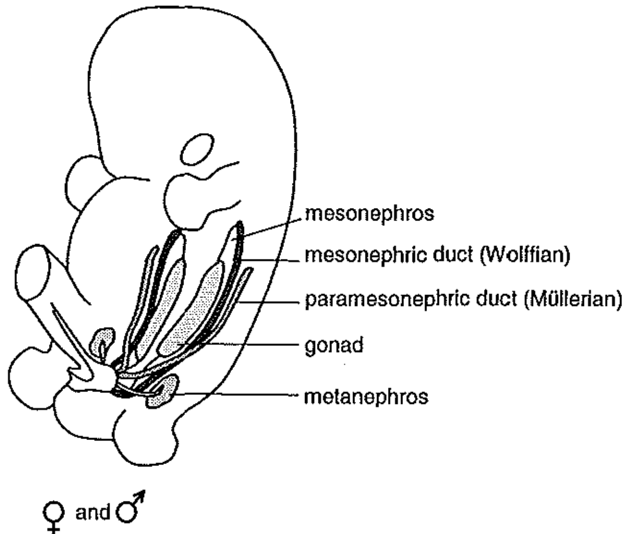


Figure 1.1. Schematic representation of the mouse E11.5 urogenital ridge.
The gonads are formed in association with the mesonephros and are morphologically indistinguishable between male and female fetuses. Two complete ductal systems form in both male and female fetuses: mesonephric (Wolffian) ducts and paramesonephric (Müllerian) ducts [modified from Larssen (1997)].

Both anlagen of the reproductive tracts, Wolffian and Müllerian ducts, are present in male as well as in female embryos, and their ability to differentiate and develop is dependent on the gonadal sex (Figure 1.2). In the male, the Wolffian ducts differentiate into the epididymides, vasa deferentia and seminal vesicles, due to the action of the testicular hormone testosterone. Testicular secretion of AMH causes regression of the Müllerian ducts (Josso and Picard, 1986). In the female, the ovaries do not produce AMH and the Müllerian ducts differentiate into oviducts, uterus and upper part of the vagina, whereas the Wolffian ducts degenerate in the absence of testosterone. Apparently, differentiation and development of the female reproductive tract is independent of ovarian hormones.

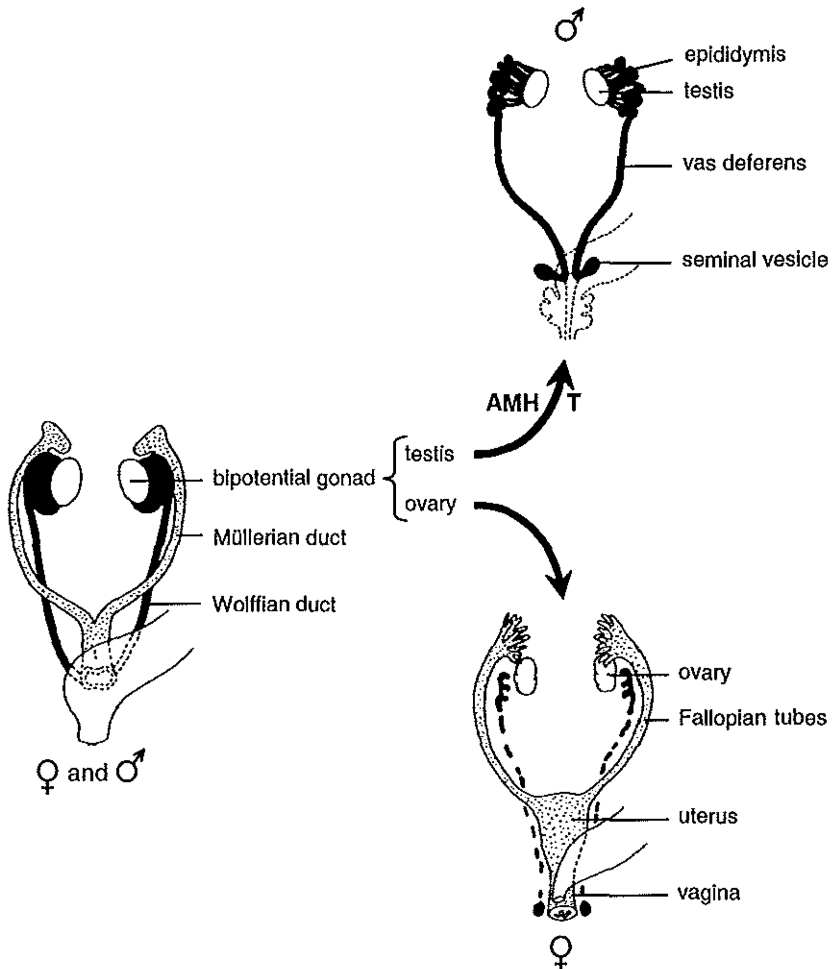


Figure1.2. **Schematic representation of mammalian sex differentiation.**
The indifferent stage is characterized by the presence of the bipotential gonads and Wolffian and Müllerian ducts, which form the anlagen of the male and female internal genitalia, respectively. In the male fetus, the gonads differentiate into testes, the Müllerian ducts regress due to the action of AMH, and the Wolffian ducts differentiate into the epididymides, vasa deferentia, and seminal vesicles due to the action testosterone. In the female fetus, the gonads differentiate into ovaries, the Wolffian ducts degenerate, and the Müllerian ducts differentiate into the Fallopian tubes, uterus, and upper part of the vagina [modified from Wilson *et al.* (1981a)].

Through a process of mesenchymal-epithelial induction, the indifferent gonads are formed along the length of the mesonephros (Karl and Capel, 1995; Capel, 1996) (Figure 1.1). The cells of the indifferent gonads are bipotential, since they have the capacity to differentiate into cell types of an ovary or a testis. In the developing gonads several cell lineages can be identified: germ cells and at least three somatic cell types. The origin of the somatic cell types is not completely understood. It is suggested that cells from the mesonephric tubules contribute to the population of cells that form the gonads (Wartenberg, 1982; Satoh, 1985). Antibody staining of laminin, which is present in the basement membrane of the mesonephric tubules, revealed cellular bridges between the mesonephric tubules and the gonad (Karl and Capel, 1995). However, it is also suggested that cells from the coelomic epithelium invade the interior of the developing gonad (Smith and MacKay, 1991). Scanning electron microscopy of mouse embryos showed pores in the surface epithelium of the gonad, through which cells could invade (Capel and Lovell-Badge, 1993). The somatic cell lineages are defined in the adult gonad as: (i) the supporting cells, differentiating into Sertoli cells in the testis or granulosa cells in the ovary; (ii) the steroidogenic cells, which are the Leydig cells in the testis and theca cells in the ovary; (iii) and the connective tissue cells that constitute the testis tunica, blood vessels and peritubular myoid cells in males, and stromal cells in females (Byskov and Hoyer, 1994). An important cell lineage of the gonads is contributed by the primordial germ cells. In the early embryo, before gonadal sex determination, these primordial germ cells are localized at the posterior end of the primitive streak, and then migrate along the hindgut and dorsal mesentery into the developing genital ridge (Ginsburg *et al.*, 1990). The presence of primordial germ cells in the gonad is not essential for testis differentiation. Mutations of the genes encoding the receptor Kit or its ligand Stem cell factor, prevent germ cell proliferation and survival, resulting in gonads devoid of germ cells, but these mutations do not inhibit formation of a normal testicular structure (McLaren, 1985). In contrast, the presence of germ cells is a prerequisite for a proper organization of the ovary. The absence of germ cells in female gonads leads to the formation of so-called "streak ovaries" (Burgoyne and Palmer, 1993). The development of the germ cells is dependent on the genetic sex

of the germ cells, but also on the surrounding environment (McLaren, 1995; Whitworth, 1998). Germ cells enclosed in the testicular tubules enter mitotic arrest, whereas in an ovarian environment germ cells enter the prophase of meiosis, independent of the genetic sex of the germ cells (McLaren, 1995).

1.3 Molecular genetic aspects of sex determination and differentiation

1.3.1 Mechanism of sex determination

Proper sex differentiation depends on the mechanism by which the sex of the bipotential gonad is determined. The mechanism of sex determination varies among species. In many reptiles, the temperature at which the eggs are incubated determines sex (temperature-dependent sex determination) (Crews *et al.*, 1994). In a variety of other species, sex determination is regulated at a genetic basis, either by the number of X chromosomes or by the presence of the Y chromosome.

In *Drosophila* and *C. elegans* the primary determinant of sex is the ratio between the number of X chromosomes and the number of sets of autosomes (the X:A ratio). In XX females (*Drosophila*) or hermaphrodites (*C. elegans*), the X:A ratio is 1, whereas in XY males (*Drosophila*) or XO males (*C. elegans*) the X:A ratio is 0.5 (Cline, 1993; Parkhurst and Meneely, 1994). Chromosomal based sex determination is coupled to a mechanism of dosage compensation, the process by which the total level of X-linked gene expression is equalized between the sexes. The strategies used to accomplish dosage compensation vary. In *Drosophila* the level of transcripts produced by the single X chromosome in males is elevated to a level produced by two X chromosomes in females (Baker *et al.*, 1994). In *C. elegans* the expression of the X-linked genes in XX hermaphrodites is reduced (Chuang *et al.*, 1994).

The sex chromosomes in birds are designated Z and W, and the male is the homomorphic sex (ZZ) and the female heteromorphic (ZW). It is suggested that sex determination in birds is by a genic balance mechanism, in which the ratio of autosomes to Z chromosomes is the crucial factor (Stevens, 1997).

Sex determination in mammals is based on the presence or absence of the Y chromosome, irrespective or the number of X chromosomes

present. However, a dosage-dependent sex determination mechanism, in which the number of active copies of *DAX-1* versus *SRY* plays a role (see 1.3.3), may still be present in mammals. X-chromosomal dosage compensation, an event that also occurs in mammals, is accomplished by inactivation of one of the two X chromosomes in female somatic cell types (Migeon, 1994).

1.3.2 The role of Sry in sex determination and differentiation

Studies by Jost on rabbit fetal development, demonstrated that ovariectomy had no effect on female differentiation, whereas removal of the testes led to feminization of the male embryo (Jost, 1947; Jost, 1953). Therefore, it was suggested that female differentiation follows a default pathway, whereas the male pathway requires the action of a testis-determining factor (TDF in humans or *Tdy* for testis-determining Y gene in mice). Loss of TDF would result in sex-reversal in males. Indeed, approximately 80% of XX human males are sex-reversed due to an aberrant X-Y translocation during meiosis of the father (Petit *et al.*, 1987). Analysis of these XX males revealed that the testis-determining factor gene was localized within a 35 kb Y-chromosomal region. Through a positional cloning strategy the gene that is functionally equivalent to TDF was isolated (Gubbay *et al.*, 1990a; Sinclair *et al.*, 1990). This gene, *SRY* (sex-determining region of the Y) in human and *Sry* in mouse, was shown to be conserved on the Y chromosome of all mammals, and the presence or absence of the gene correlates with male versus female sex determination (Foster *et al.*, 1992). A number of mutations have been found in the *SRY* gene of XY individuals that develop as females (Table 1.1) (Berta *et al.*, 1990; Jäger *et al.*, 1990; Hawkins *et al.*, 1992a; Hawkins *et al.*, 1992b; McElreavy *et al.*, 1992; Hawkins, 1993; Hawkins, 1995). Furthermore, XX *Sry*-transgenic mice carrying a 14 kb genomic DNA containing the *Sry* locus, develop as male mice with testes and male accessory sex organs, and male mating behavior (Koopman *et al.*, 1991).

Table 1.1. **Disorders in male sex determination and differentiation**

Gene	Disorder	Phenotype			Role
		Gonads	**External genitalia**	**Internal genitalia**	
WT1	Denys-Drash syndrome	streak	female/ambiguous	MD present WD present	kidney/gonad development
SF-1	? (knockout)	absent	female	MD present WD present	adrenal/gonad development testis differentiation
SRY	46,XY gonadal dysgenesis	streak	female	MD present WD absent	testis determination
DAX-1	Adrenal hypoplasia congenita and hypogonadotropic hypogonadism	hypogonadal	male	MD absent WD present	adrenal/gonad development ovarian determination?
	(DSS duplication) 46,XY gonadal dysgenesis	streak/ovaries	female/ambiguous	MD present WD absent	ovarian determination?
SOX9	Campomelic dysplasia	streak/testes	female/ambiguous	MD present WD absent	testis differentiation
AMH/AMHRII	Persistent Müllerian duct syndrome	testes	male	MD present WD present	MD regression
Androgen receptor	Androgen insensitivity syndrome	testes	female/ambiguous	MD absent WD absent	WD differentiation and differentiation external genitalia

Genes involved in early regulatory pathways of sex determination and differentiation are shown. Some of the gene mutations result in ambiguous genitalia. In these cases, the degree of differentiation of the internal genitalia also varies. MD, Müllerian ducts; WD, Wolffian ducts.

Analysis of the SRY amino acid sequence revealed the presence of a 79 amino acid HMG (high mobility group) box type DNA binding domain. The HMG domain of SRY is conserved among other species, while the regions outside the domain show no homology (Whitfield *et al.*, 1993). The HMG domain of SRY resembles a class of transcription factors with sequence specific binding properties (Travis *et al.*, 1991; Van de Wetering *et al.*, 1991). *In vitro* experiments have demonstrated that the HMG domain of SRY binds preferentially to AACAA(A/T)(G/C) and induces a sharp bend in the DNA (Ferrari *et al.*, 1992; Van de Wetering and Clevers, 1992). In 20% of the patients with gonadal dysgenesis, mutations have been found in the HMG box, while only a few mutations were identified outside the HMG box region, indicating that the functional domain of SRY is located in the HMG domain (McElreavy *et al.*, 1992; Hawkins, 1993; Poulat *et al.*, 1994; Tajima *et al.*, 1994).

Consistent with a role for Sry in testis determination, Sry mRNA is only expressed by cells within the genital ridge. In the mouse, expression of Sry mRNA in the genital ridge is first detected at E10.5 (days *post coitum*), peaks at E11.5 and declines rapidly within 24 hrs (Figures 1.3 and 1.4). This small period of Sry mRNA expression (E10.5–12.5) coincides with the onset of morphological changes of testicular cells to form testis cords (Koopman *et al.*, 1990; Hacker *et al.*, 1995). The absence of Sry mRNA expression in germ cells suggests that this gene is expressed in a testicular somatic cell type. Since the Sertoli cells are the first morphological distinguishable cell type of the testis, it is suggested that Sry triggers the onset of Sertoli cell differentiation. Indeed, in a XX/XY mosaic mouse, 95% of the Sertoli cells are XY, whereas for the other somatic cell types an even distribution of XX and XY cells are found (Burgoyne *et al.*, 1988; Palmer and Burgoyne, 1991). Since it is only expressed for a short period of time, Sry is not required to maintain the differentiated state of the testis, suggesting the involvement of other genes in testis differentiation, possibly induced by Sry. However, thus far no physiological target genes of Sry are known.

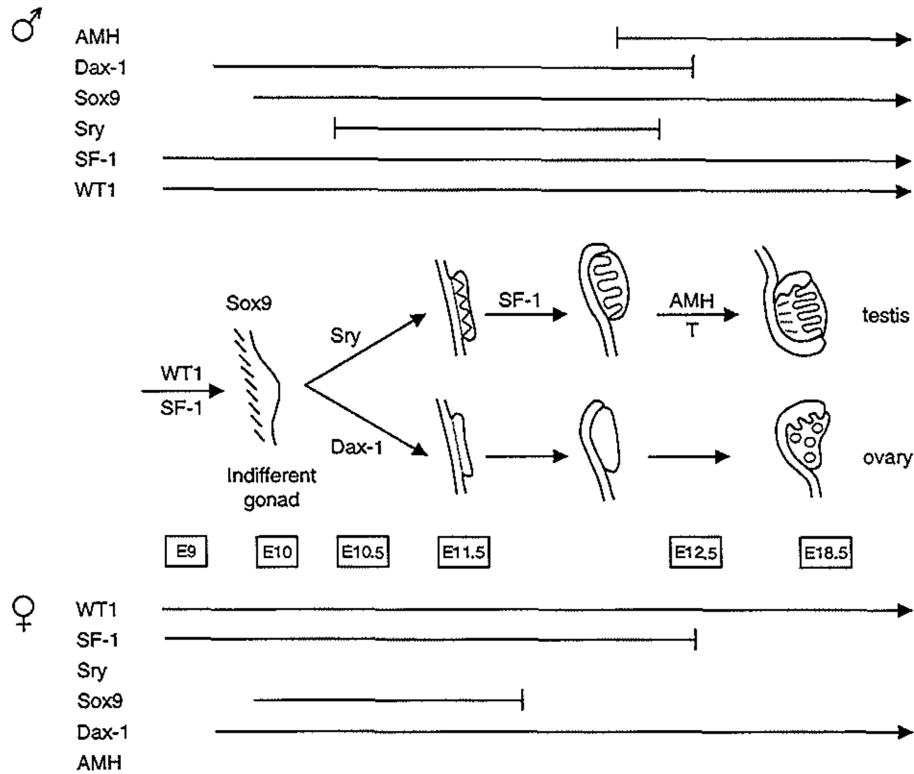


Figure 1.3. **Genetic regulatory pathway involved in mammalian sex determination and differentiation.**
The diagram illustrates the development of the gonads and the genes involved, at the approximate number of days *post coitum* (E) in the mouse. The time course of expression of these genes is shown at the top (male) and the bottom (female).

In the adult testis, Sry mRNA is expressed by the postmeiotic germ cells, and the onset of this mRNA expression coincides with the first wave of spermatogenesis and the development of round spermatids (Hacker *et al.*, 1995). Nevertheless, it is believed that Sry has no critical function in spermatogenesis, since XY germ cells deleted for *Sry*, within a founder male chimera, were able to give rise to offspring (Gubbay *et al.*, 1990b; Lovell-Badge and Robertson, 1990). In the adult testis, a 1.23 kb circular *Sry* transcript is found, which may not be transcribed. In contrast, in the developing gonad a linear transcript of 4.8 kb is expressed. This suggest the presence of two promoters for the *Sry* gene, a proximal promoter that gives rise to functional transcripts in the genital ridge, and a distal promoter used in germ cells of adult testis resulting in circular Sry mRNA that may not be functional (Capel *et al.*, 1993; Hacker *et al.*, 1995; Dolci *et al.*, 1997).

1.3.3 Other genes involved in sex determination and differentiation

It was expected that *Sry* could function as the master regulatory gene in a cascade of events leading to testis differentiation (Figure 1.3). However, in the majority of XY patients with gonadal dysgenesis the *SRY* gene is not affected. Furthermore, some XX males lack *SRY*. This has led to the understanding that other sex-determining genes are involved in a network of interactions leading to gonadal determination. In addition to *Sry*, several autosomal genes and one X-linked gene have been identified in relation to failure in testis development (Table 1.1). These genes will be discussed below.

WT1

The Wilms' tumor gene, *WT1*, encodes a transcription factor with homology to members of the EGR (early growth response) family (Call *et al.*, 1990; Gessler *et al.*, 1990). The WT1 protein contains a zinc finger DNA binding domain at the C-terminus and a Pro/Glu rich N-terminus. This latter domain can function as a transcriptional repressor or activator depending on the promoter sequence of the target gene (Madden *et al.*, 1991; Drummond *et al.*, 1992; Wang *et al.*, 1992; Drummond *et al.*, 1994). Four different WT1 isoforms are recognized, based on the presence or absence of two

alternatively spliced exons. The presence or absence of the exons determine the transcriptional repression or the DNA binding specificity, and subnuclear location of the WT1 isoforms. It has been shown that one of the WT1 isoforms (-KTS) can affect expression of genes involved in cellular proliferation and differentiation (Haber *et al.*, 1990; Madden *et al.*, 1991; Drummond *et al.*, 1992).

The *WT1* gene was first identified in relation to Wilms' tumor, a malignant tumor of the embryonic kidney. However, mutations in *WT1* also affect other organs. Chromosomal deletions that include *WT1* cause the WAGR syndrome (Wilms' tumor, aniridia, urogenital abnormalities, and mental retardation). The Denys-Drash syndrome (DDS), which results from point mutations in the *WT1* gene (Pelletier *et al.*, 1991a), is characterized by the presence of Wilms' tumor, more severe urogenital abnormalities and varying degrees of abnormal gonadal development. Patients, that are heterozygous for certain point mutations display features of XY-sex reversed females (Hastie, 1994), suggesting a role for WT1 in sex determination and/or sex differentiation.

Expression of WT1 mRNA is found in the developing kidney, the genital ridge and the fetal gonads, coinciding with their common origin, i.e. intermediate mesoderm (Pritchard-Jones *et al.*, 1990; Pelletier *et al.*, 1991b). Expression was detected as early as E9, prior to the mesenchyme-to-epithelial transition (Figure 1.3) (Armstrong *et al.*, 1993). Generation of *WT1* null mice revealed the absence of kidneys and gonads in both sexes, due to a failure in mesenchymal differentiation (Kreidberg *et al.*, 1993). Although the onset of gonadal development was detected at E11, the thickenings of the epithelium were reduced, and at E14 no gonads were visible. These results suggest that WT1 is required for early establishment of the bipotential gonads (Kreidberg *et al.*, 1993). Furthermore, these results indicate that WT1 acts upstream of Sry. In addition, WT1 remains expressed in the developing gonad, and a role of WT1 in gonadal differentiation can therefore not be excluded (see also 1.4.2) (Pelletier *et al.*, 1991b).

SF-1

The gene *steroidogenic factor 1* (*SF-1*) encodes an orphan nuclear receptor, which was found to regulate the expression of steroidogenic enzymes in the adrenal and gonads (Lala *et al.*, 1992; Morohashi *et al.*, 1992; Honda *et al.*, 1993; Ikeda *et al.*, 1993). Analysis of the prenatal expression pattern revealed that SF-1 mRNA is expressed in the developing adrenals, gonads, ventromedial hypothalamic nucleus and pituitary (Hatano *et al.*, 1994; Ikeda *et al.*, 1994; Ingraham *et al.*, 1994). In the urogenital ridge, SF-1 mRNA expression is found in both sexes during the sexually undifferentiated stage (E9-10) (Figure 1.3). At later developmental stages, SF-1 mRNA shows a sex dimorphic expression pattern. In the developing testes, SF-1 mRNA remains expressed at a relatively high level in both the interstitial Leydig cells and in Sertoli cells. In the ovary, however, expression of SF-1 mRNA declines at E12, the time point of morphological differentiation (Figures 1.3 and 1.4) (Ikeda *et al.*, 1994). Due to the temporal difference in peak expression of Sry (E11.5-12) and SF-1 mRNA (E16-18), it is most likely that there is no direct transcriptional regulation of *SF-1* by Sry.

Besides its regulatory role in the establishment of the steroidogenic pathway, SF-1 was shown to regulate expression of AMH mRNA, indicating an important role of SF-1 in Sertoli cell differentiation (see 1.4.2) (Ingraham *et al.*, 1994; Shen *et al.*, 1994; Giuili *et al.*, 1997). Furthermore, SF-1 proved to be essential for the development of adrenals and gonads, since SF-1-deficient mice lack adrenals and gonads (Luo *et al.*, 1994). Detailed analysis of SF-1-deficient fetuses revealed that the initiation of urogenital ridge development is not hampered. However, the gonads degenerate due to apoptosis around the period of normal testis determination, resulting in a complete absence of gonads at E12.5 in both sexes (Luo *et al.*, 1994). These results indicate that SF-1 plays an essential role in gonadal development before the onset of Sry mRNA expression and, based on the sexual dimorphic expression pattern, also has a role in testis differentiation.

SOX9

Genes encoding proteins containing an HMG box with more than 60% similarity to the Sry HMG box region, are named *SOX* genes (*Sry-related HMG box* gene) (Wright *et al.*, 1993). One of these *SOX* genes, *SOX9*, is

involved in XY sex reversal. SOX9 contains the HMG box domain and a Pro/Glu rich region characteristic for transcription factors (Wright *et al.*, 1995). Mutations in *SOX9* in human lead to the severe dwarfism syndrome, campomelic dysplasia (CD) (Foster *et al.*, 1994; Wagner *et al.*, 1994). Defects in cartilage and bone development, abnormalities of the olfactory system, central nervous system, heart and kidney were observed. In addition, in 75% of the XY CD patients sex reversal occurs. The gonads of CD sex reversed patients display a range of phenotypes, varying from some testicular differentiation to ovary-like gonads with some primary follicles (Foster *et al.*, 1994; Wagner *et al.*, 1994).

In the mouse, Sox9 mRNA is expressed at all sites of chondrogenesis, in line with the skeletal abnormalities in CD patients (Wright *et al.*, 1995). Furthermore, Sox9 mRNA expression was found in genital ridges of both XX and XY fetuses at E10.5 (Figure 1.3) (Kent *et al.*, 1996; Morais da Silva *et al.*, 1996). At this time point the ridges are indistinguishable and male fetuses just start to express Sry mRNA, suggesting that the initiation of Sox9 mRNA expression requires other factors than Sry. At E11.5, Sox9 mRNA is abundantly expressed in the genital ridge of male fetuses and expression remains high in the developing testis, whereas, from E11.5 onwards, expression declines in the fetal ovary and is absent in the adult ovary (Figure 1.3) (Kent *et al.*, 1996; Morais da Silva *et al.*, 1996). These observations indicate that Sry may be required for sex-specific expression of Sox9. In the developing and adult testis, Sox9 mRNA expression is restricted to Sertoli cells and is independent of the presence of germ cells (Kent *et al.*, 1996; Morais da Silva *et al.*, 1996), suggesting a role of Sox9 in Sertoli cell differentiation and in maintenance of Sertoli cell function. The sex reversal in CD patients is consistent with a failure in Sertoli cell differentiation. All CD patients identified so far are heterozygous for the mutations, indicating that one copy of *SOX9* is insufficient for testis differentiation (Foster *et al.*, 1994; Wagner *et al.*, 1994).

One can conclude that Sertoli cell differentiation is controlled by a precise balance between several genes, including *Sry*, *SF-1*, *Sox9* and *Dax-1*.

Dax-1

Duplication of a defined segment of the short arm of the X chromosome causes sex reversal in XY individuals (Bernstein *et al.*, 1980). The presumptive gene at this locus has been termed *DSS* (*dosage-sensitive sex reversal*), and is suggested to play a female-specific function (Bardoni *et al.*, 1994). In males, DSS would be negatively down-regulated by SRY, whereas this repression fails when DSS is present in a double dose. In contrast, XY individuals with deletions of the *DSS* region remain phenotypically male. This suggests that DSS is not involved in testicular differentiation, but that a double dose will interfere.

From the DSS region, the gene *DAX-1* has been identified, which encodes an unusual member of the nuclear hormone receptor family. Mutations of this gene have been found in patients with adrenal hypoplasia congenita (AHC) (Muscatelli *et al.*, 1994; Zanaria *et al.*, 1994). DAX-1 (DSS-AHC critical region on X) contains a ligand-binding domain similar to other family members, but lacks the characteristic zinc fingers. Instead, three repeats and a fourth incomplete repeat at the N-terminus encode a new structural motif with a DNA-binding function (Zanaria *et al.*, 1994; Lalli *et al.*, 1997). Deletions or mutations in *DAX*-1 cause AHC as well as hypogonadotropic hypogonadism (HHG). AHC-HHG is characterized by impaired adrenal differentiation and arrested development of the gonads (Muscatelli *et al.*, 1994).

In the mouse, Dax-1 mRNA is expressed in the same tissues as SF-1, such as the adrenals, genital ridges, gonads, hypothalamus and pituitary (Guo *et al.*, 1995; Swain *et al.*, 1996). In the urogenital ridge, the timing and localization of Dax-1 mRNA expression corresponds to those of SF-1, although the earliest time point of SF-1 mRNA expression precedes Dax-1, indicating that SF-1 might function upstream of Dax-1 (Figure 1.3) (Guo *et al.*, 1995; Ikeda *et al.*, 1996; Swain *et al.*, 1996). In contrast to SF-1 mRNA expression, Dax-1 mRNA remains expressed in the fetal ovary and expression declines in the fetal testes. Furthermore, *DAX-1* mutations in human do not interfere with the initial stages of gonadal differentiation, in contrast to the effect of SF-1 deficiency, and a normal mRNA expression pattern of Dax-1is found in SF-1-deficient mice. Taken together, these

observations suggest that Dax-1 mRNA expression is regulated independently of SF-1 (Ikeda *et al.*, 1996).

DAX-1 has been proposed to be responsible for dosage-sensitive sex reversal. In a recent study, Swain *et al.* (1998) showed that XY mice carrying extra copies of *Dax-1* have delayed testis development, while complete sex reversal occurs in *Dax-1* transgenic mice with weak alleles of *Sry*. These results strongly suggest that Dax-1 is responsible for dosage-sensitive sex reversal, and that there is a rather direct interplay with Sry. Furthermore, the level and timing of Dax-1 mRNA expression are critical in sex determination and differentiation.

1.4 Anti-Müllerian hormone

In fetal rabbit castration experiments, Jost demonstrated that testosterone alone was not sufficient for normal male reproductive development. An additional factor proved to be necessary for Müllerian duct regression in male fetuses (Jost, 1947; Jost, 1953). This factor is known as anti-Müllerian hormone (AMH) or Müllerian-inhibiting substance (MIS) (Josso *et al.*, 1993; Lee and Donahoe, 1993). Isolation and characterization of the human *AMH* gene revealed that the gene contains five exons and maps to chromosome 19p13 (Cate *et al.*, 1986; Picard *et al.*, 1986; Cohen-Haguenauer *et al.*, 1987). Furthermore, sequence similarity between the C-terminal region of AMH and members of the TGFβ superfamily of growth and differentiation factors was found (Cate *et al.*, 1986; Massagué, 1990). AMH is synthesized as a precursor protein that requires proteolytic cleavage to generate the bioactive C-terminal domain, like other members of the TGFβ superfamily (see Chapter 2) (Pepinsky *et al.*, 1988). Members of the TGFβ superfamily mediate their actions through a heteromeric receptor complex composed of type I and type II transmembrane serine/threonine kinase receptors (see Chapter 2). A receptor for AMH was cloned which has structural characteristics of a type II serine/threonine kinase receptor (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994b; Grootegoed *et al.*, 1994).

1.4.1 Expression of AMH and its receptor

AMH is one of the earliest products known to be secreted by the fetal Sertoli cells (Tran *et al.*, 1977; Tran and Josso, 1982; Vigier *et al.*, 1983; Hayashi *et al.*, 1984). Expression of AMH mRNA is detected in the fetal testis before the seminiferous tubules are morphologically apparent (E12.5) (Münsterberg and Lovell-Badge, 1991). During later developmental stages AMH mRNA expression is restricted to Sertoli cells, and a high expression level is maintained during the period of Müllerian duct regression (Figure 1.3; see also 1.4.3.1) (Münsterberg and Lovell-Badge, 1991). The fetal Sertoli cells also express the AMH type II receptor (AMHRII) (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994b). Furthermore, mRNA expression of AMHRII is found in the target cells of AMH, the mesenchymal cells surrounding the Müllerian ducts (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994b). In male rat fetuses, mRNA expression is detected at E13 and disappears during later developmental stages, correlating with regression of the Müllerian ducts. Expression of AMHRII mRNA remains high in the Müllerian duct cells of female fetuses (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994b).

After birth, AMH mRNA expression strongly decreases and becomes hardly detectable in the Sertoli cells (Münsterberg and Lovell-Badge, 1991; Baarends *et al.*, 1995a). Although expression of AMH mRNA is low in the adult testis, the mRNA expression pattern was found to be stage-dependent during the rat spermatogenic cycle: a maximum level of AMH mRNA was found at stage VII of the spermatogenic cycle, whereas this expression is much lower at other stages of this cycle (Baarends *et al.*, 1995a). In contrast to AMH, Sertoli cells of postnatal rat testis express a high level of AMHRII mRNA, although in the rabbit a low level of mRNA expression was found (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994b). In the rat, it was observed that AMHRII mRNA expression markedly increases during postnatal testis development coinciding with the initiation of spermatogenesis. In parallel with AMH, AMHRII shows a stage-dependent mRNA expression pattern during the spermatogenic cycle: expression increases from stage XIII, becomes maximal during the stages VI and VII and declines to undetectable levels at stages IX-XII (Baarends *et al.*, 1995a).

Fetal ovaries express AMHRII mRNA (Baarends *et al.*, 1994), while AMH mRNA expression is absent. Obviously, this inhibition of fetal ovarian

AMH gene expression is essential to prevent Müllerian duct regression in the female. Surprisingly, after birth, ovarian expression of AMH mRNA is detected in granulosa cells, first in preantral follicles in the peripheral area (Münsterberg and Lovell-Badge, 1991; Baarends *et al.*, 1995b). After initiation of follicular growth, expression becomes also evident in preantral and small antral follicles. AMH mRNA expression is low or absent in large antral follicles, corpora lutea and atretic follicles (Ueno *et al.*, 1989a; Ueno *et al.*, 1989b; Hirobe *et al.*, 1992; Hirobe *et al.*, 1994; Baarends *et al.*, 1995b). During the estrous cycle no evident change in the mRNA expression pattern of AMH in follicles of the same size classes was observed, except at estrous when mRNA expression in the large preantral follicles was decreased (Baarends *et al.*, 1995b). AMHRII mRNA is expressed at a higher level than AMH, and in all small follicles. From day 15 of postnatal development in the rat, AMHRII mRNA expression colocalizes with AMH in the granulosa cells of preantral and small antral follicles (Baarends *et al.*, 1995b).

1.4.2 Regulation of AMH expression

The spatial and sex-specific expression pattern of AMH mRNA during fetal development suggests that *AMH* might be a target gene for one of the genes involved in sex determination and differentiation. AMH mRNA expression is initiated in the fetal Sertoli cells 20 hours after onset of Sry mRNA expression (Figure 1.4A) (Hacker *et al.*, 1995). Transfection studies using a cell line derived from differentiating gonadal ridges of male rat fetuses, showed that Sry expression causes an increase in *AMH* promoter activity (Haqq *et al.*, 1994). In addition, using DNA footprinting, a 24 bp Sry binding region was identified in the *AMH* promoter, which contains 6 bp with strong resemblance to a consensus Sry binding sequence. Mutation of this footprint region reduced binding of Sry to this mutated sequence but had no effect on the Sry-induced transcriptional regulation of the *AMH* promoter (Haqq *et al.*, 1994), suggesting that the Sry dependent activation of *AMH* is indirect. Moreover, other factors may be important, since postnatally, AMH mRNA is also expressed in the ovary.

A

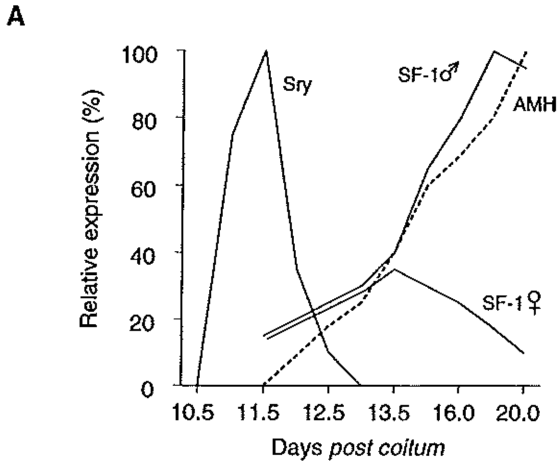
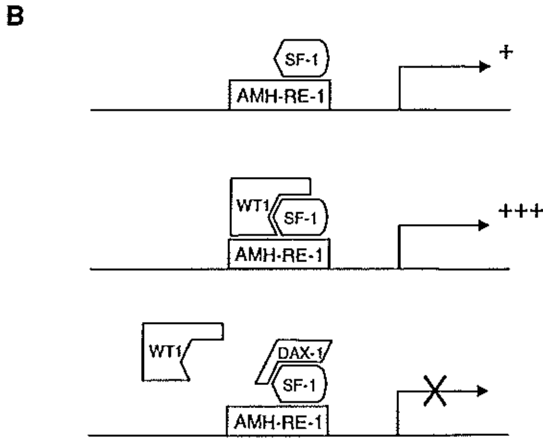


Figure 1.4. Regulation of AMH mRNA expression.
A. Relative mRNA expression levels of Sry, SF-1 and AMH during mouse gonadal development. These levels are depicted taking E11.5 Sry, E18 SF-1 and E20 AMH as 100% of signal (E: days *post coitum*). AMH mRNA expression starts 24 hrs after the peak expression of Sry mRNA, and the mRNA expression pattern runs parallel with that of SF-1 in the male gonad. In the fetal ovary, SF-1 mRNA expression declines and AMH mRNA is not expressed [data adopted from (Shen *et al.*, 1994; Hacker *et al.*, 1995)].

Sequences within 2 kb of the *AMH* promoter region are sufficient for Sertoli cell- and granulosa cell-specific expression (Peschon *et al.*, 1992). Deletion analysis revealed that regulatory sequences are contained within the most proximal 180 bp of the *AMH* 5'-flanking region (Shen *et al.*, 1994). Besides the element with homology to the consensus binding site for Sry, a second putative element was identified. This 20 bp *AMH*-RE-1 contains a motif that resembles the half site for a number of nuclear hormone receptors (Shen *et al.*, 1994). The orphan nuclear receptor SF-1 shows a similar spatial and sex-specific mRNA expression profile to that of AMH (Figure 1.4A). Indeed, SF-1 was shown to bind the *AMH*-RE-1 and mutation of *AMH*-RE-1 abolished binding of SF-1 to this region. Furthermore, the SF-1 binding site turned out to be essential for sex- and cell-specific *AMH* promoter activity in transgenic mice (Giuili *et al.*, 1997). However, *in vitro*



B. Model for the regulation of AMH mRNA expression. SF-1 regulates the *AMH* promoter activity through binding to the *AMH*-RE-1. WT1 association with SF-1 results in a synergistic activation of the *AMH* promoter. This association is specific for the *AMH* promoter. In the presence of Dax-1, the synergism between WT1 and SF-1 is abrogated [model adapted from (Nachtigal *et al.*, 1998)].

studies revealed that SF-1 by itself is unable to activate the *AMH* gene, but a truncated SF-1 that lacks the putative ligand binding of SF-1 does result in *AMH* induction (Shen *et al.*, 1994). This suggests that an additional factor is required for AMH activation by SF-1. Furthermore, since SF-1 is expressed in both sexes at earlier stages, SF-1 alone cannot account for the testis-specific activation of the *AMH* gene.

Pelletier *et al.* (1991b) have suggested that WT1 might be involved in modulation of AMH mRNA expression. Indeed, *WT1* mutations are often associated with persistent Müllerian duct structures (Little and Wells, 1997). In cotransfection studies, WT1 associates with SF-1, resulting in a synergistic action between WT1 and SF-1 on *AMH* activation (Figure 1.4B) (Nachtigal *et al.*, 1998). Furthermore, it was shown that the synergistic activation of the *AMH* promoter by WT1 only occurs in the presence of the

conserved AMH-RE-1, as WT1 fails to synergize with SF-1 to induce the promoter of the gene encoding the steroidogenic enzyme P450-21-hydroxylase, although this promoter contains a canonical SF-1 response element. In addition, mutated WT1 protein fails to synergize with SF-1 (Nachtigal *et al.*, 1998). The synergism between WT1 and SF-1 on the *AMH* promoter is antagonized by coexpression of DAX-1, the candidate dosage-sensitive sex reversal gene (Figure 1.4B) (Nachtigal *et al.*, 1998). These results suggest that the ratio of expression levels of factors, that are involved in Sertoli cell differentiation, are important in regulation of AMH mRNA expression.

Postnatally, SF-1, WT1 and DAX-1 may also be involved in regulation of AMH mRNA expression, since all these factors are expressed in testicular Sertoli cells and ovarian granulosa cells. During the spermatogenic cycle, the Dax-1 mRNA expression level is low at stages VI-VII, whereas the AMH mRNA expression level is highest at these stages (Baarends *et al.*, 1995a; Tamai *et al.*, 1996). In the ovary, WT-1 mRNA is expressed in small follicles, thereby overlapping with AMH mRNA expression (Baarends *et al.*, 1995b; Hsu *et al.*, 1995). However, the precise regulation of AMH mRNA expression in the adult gonads has not been elucidated thus far.

1.4.3 Functions of AMH

1.4.3.1 Müllerian duct regression

The Müllerian ducts, which form the anlagen of the female reproductive tract, disappear during early embryonic development in the male fetuses. In the above mentioned rabbit experiments, Jost demonstrated that in the absence of the fetal testis but with androgen substitution, the Müllerian ducts remained present in the male (Jost, 1947; Jost, 1953). Purified AMH was shown to induce regression of the Müllerian ducts in cultured female rat urogenital ridges (Picon, 1969).

Regression of the Müllerian ducts is morphologically characterized by a whorl of condensed mesenchymal cells surrounding the Müllerian duct (Dyche, 1979). Breakdown of the basement membrane allows direct contacts between epithelial and mesenchymal cells (Trelstad *et al.*, 1982). Incorporation of [^{3}H]-thymidine in separately cultured epithelial and mesenchymal ductal cells showed that, in the presence of AMH, DNA

synthesis in epithelial cells was not changed; however, the labeling index was significantly reduced in the mesenchymal cells (Tsuji *et al.*, 1992). This suggests that the effect of AMH on the epithelial cells of the Müllerian ducts is via the surrounding mesenchymal cells. The expression of AMHRII mRNA in the mesenchymal cells supports this finding (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994b).

With fluorescent labeling techniques and with the use of chick/quail chimeras, it was demonstrated that the epithelial cells migrate out of the Müllerian ducts during regression and are incorporated in the mesonephric tubules (Hutson *et al.*, 1984; Austin, 1995). However, it has also been argued that programmed cell death (apoptosis) is involved in Müllerian duct regression. The observation of macrophage-infiltration and apoptotic cells in the regressing duct sustained this argument (Price *et al.*, 1977; Catlin *et al.*, 1997).

Regression of the Müllerian ducts is triggered by AMH only during a critical time window of sensitivity (Tsuji *et al.*, 1992). This sensitive time window was defined by exposing female urogenital ridges isolated from rat embryos of different ages to AMH, and was found to be located between E14 and E15, which would compare to E13-E14 in mouse. Exposure of the Müllerian ducts to AMH after this period does not cause Müllerian duct regression (Tsuji *et al.*, 1992).

The role of AMH in Müllerian duct regression has further been demonstrated in gain of function and loss of function experiments. Female transgenic mice with ectopic expression of AMH lack uterus and oviducts (Behringer *et al.*, 1990). AMH-deficient male mice develop, in addition to a normally differentiated Wolffian duct system, Müllerian duct derivatives (Behringer *et al.*, 1994). Absence of Müllerian duct regression in male fetuses was also observed in AMHRII-deficient male mice (Mishina *et al.*, 1996). Loss of AMH function in the human causes a rare form of male pseudohermaphroditism, known as persistent Müllerian duct syndrome (PMDS). PMDS is characterized by the presence of uterus and oviducts in otherwise normally virilized males (Josso *et al.*, 1997). Mutations in the human *AMH* gene have been found, that result in bio-inactive AMH or absent AMH secretion (Knebelmann *et al.*, 1991; Carré-Eusèbe *et al.*, 1992; Imbeaud *et al.*, 1994). Mutations have also been found in the *AMHRII* gene

of PMDS patients (Imbeaud *et al.*, 1995; Imbeaud *et al.*, 1996). However, in a small group of PMDS patients no mutations were detected in either the *AMH* gene or the *AMHRII* gene, reflecting false-negatives in the PCR-SSCP analysis or indicating mutations in downstream genes.

1.4.3.2 Gonadal function

Testis

Although fetal testes and adult gonads express AMH, the role of AMH in gonadal function is not clear. AMH has been suggested to play a role in gonadal cell differentiation during embryonic development. Fetal ovaries exposed to AMH become morphologically masculinized. This may explain why in the bovine freemartin, in which the female fetus is exposed to hormones of the male twin by chorialIantoic anastomoses, Müllerian ducts regress and, in addition, ovaries develop testis cord-like structures (Jost *et al.*, 1972). Exposure of fetal rat ovaries to purified AMH result in a similar sex reversed phenotype (Vigier *et al.*, 1987). Mammalian endocrine sex differentiation is characterized by production of testosterone by the testis and estradiol by the ovary (George and Wilson, 1978; Wilson *et al.*, 1981b). Indeed, AMH exposure affects the pattern of fetal gonadal hormone secretion; AMH inhibits aromatase activity, the enzyme that converts testosterone to estradiol (Figure 1.5), and as a result, AMH-exposed fetal ovaries produce testosterone rather than estradiol (Vigier *et al.*, 1989). Endocrine sex reversal is also observed *in vivo* in female transgenic mice, which ectopically express AMH (Di Clemente *et al.*, 1992). However, AMH does not seem to be essential for normal Sertoli cell differentiation, as PMDS patients showed normal testis development and the infertility in some PMDS patients may be explained by maldescent of the testis caused by the presence of female Müllerian duct derivatives (Hutson and Donahoe, 1986). The abnormal descent of the testis in PMDS boys has led to the suggestion that AMH is involved in the process of testis descent, although this role is still under debate since abnormal testis descent is not observed in other species with PMDS (Tran *et al.*, 1986; Meyers-Wallen *et al.*, 1993; Hutson *et al.*, 1997). Furthermore, AMH has been suggested to cause mitotic arrest of male germ cells (Cate, 1993). A correlation was found between the arrest of germ cell at the prespermatogonia stage and the

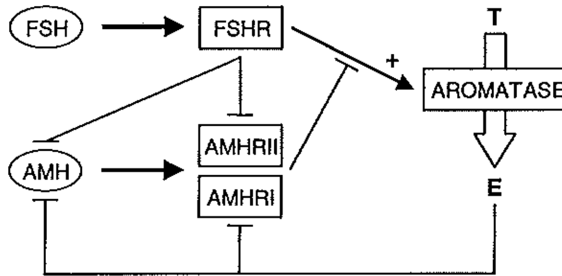


Figure 1.5. Schematic representation of the inhibitory effect of AMH on the FSH-induced aromatase promoter activity.
AMH signals through a type I/type II receptor complex to inhibit aromatase activity. Aromatase converts testosterone (T) to estradiol (E). In the adult ovary, FSH and estradiol may be involved in downregulation of AMH and AMHRII mRNA expression.

initiation of AMH production in ovotestis of $B6^{YDOM}$ mice (Taketo *et al.*, 1991). AMH mRNA is highly expressed at the time of perinatal mitotic arrest, and is switched off after birth in rat and mouse coinciding with the first wave of spermatogenesis (Kuroda *et al.*, 1990; Münsterberg and Lovell-Badge, 1991). In contrast, tissue culture experiments of neonatal mouse testes suggest that AMH is required for the maturation of gonocytes to type A spermatogonia (Zhou *et al.*, 1993). However, such an action of AMH does not seem to be essential for spermatogenesis. Male AMH-deficient mice are found to be infertile, but these mice do produce functional sperm and the infertility appears to be caused by the presence of the female reproductive organs that blocks sperm transfer (Behringer *et al.*, 1994).

The development of loss and gain of function models for AMH revealed a role for AMH in Leydig cell proliferation. Several AMH-deficient male mice incidentally develop Leydig cell hyperplasia (Behringer *et al.*, 1994). In contrast, strong overexpression of AMH causes feminization of external genitalia and impaired Wolffian duct differentiation in transgenic male mice, and it was shown that AMH blocks the differentiation of Leydig cell precursors (Behringer *et al.*, 1990; Racine *et al.*, 1998). In addition, a low

serum concentration of testosterone is measured in transgenic males that overexpress AMH (Lyet *et al.*, 1995).

An inverse relationship between the levels of testosterone and AMH is also observed at puberty in human (Rey *et al.*, 1993). In normal boys, the testosterone level increases at puberty, whereas the AMH level decreases. Also in patients with a high testosterone level, resulting in the onset of puberty at an early age, the AMH level drops to postpubertal values (Rey *et al.*, 1993). However, in patients with androgen-insensitivity syndrome (AIS), due to an inactivating mutation of the androgen receptor, the AMH concentration remains elevated after the onset of puberty (Rey *et al.*, 1994), revealing an inverse relationship between testosterone and AMH, regulated via the androgen receptor protein.

Ovary

The specific expression patterns of AMH and AMHRII mRNAs in preantral and small antral follicles suggest that AMH functions during follicle development (Baarends *et al.*, 1995b). In cultured granulosa cells, AMH decreases aromatase activity and the LH receptor number (Di Clemente *et al.*, 1994a). Furthermore, AMH inhibits the epidermal growth factor (EGF)-induced proliferation and progesterone synthesis of cultured human granulosa/luteal cells (Kim *et al.*, 1992; Seifer *et al.*, 1993). During the process of folliculogenesis, many follicles at the small antral stage become atretic. It is suggested that the follicle-stimulating hormone (FSH) surge at estrus is essential for survival of those small antral follicles that have been selected to ovulate (Hsueh *et al.*, 1994). In this respect, the downregulation of AMH at estrus is of interest (Baarends *et al.*, 1995b). In addition, treatment of prepubertal rats with FSH, which induces aromatase activity, causes downregulation of AMH and AMHRII mRNA expression in some preantral and small antral follicles (Figure 1.5) (Baarends *et al.*, 1995b). Therefore, it was suggested by Baarends *et al.* (1995b) that AMH functions to prevent follicle maturation. However, AMH does not seem to play a dominant role in the ovary, as AMH-deficient mice are fertile, and thus far no gross abnormalities of the ovary have been reported (Behringer *et al.*, 1994); and unpublished observations).

1.5 The role of estrogens in sex differentiation

In reptiles and birds, aromatase activity, which causes the conversion of testosterone to estradiol, plays an important role in sex differentiation. In the turtle, aromatase activity increases in the female fetus during the temperature sensitive sex-determination period (Desvages and Pieau, 1992). In addition, estrogen administration to male fetuses leads to sex reversal (Dorizzi *et al.*, 1991). In birds, estrogen is important for ovarian development. Inhibition of aromatase activity at the stage when gonads are bipotential, causes genetic females to develop a male phenotype with testes capable of spermatogenesis (Elbrecht and Smith, 1992). In mammals, however, the role of estrogens in sex differentiation is less clear.

Estrogens are considered to play a critical role in the development of female secondary sex characteristics, the reproductive cycle, and the maintenance of pregnancy. Furthermore, an essential role in fetal development has been suggested (George and Wilson, 1994). At the age that the fetal testes in rats and mice start to produce testosterone, the fetal ovaries are capable of synthesizing estradiol, even before morphological differentiation has occurred (Greco *et al.*, 1993; Weniger, 1993). It is suggested that estrogens, in part, mediate cellular organization of the ovary. Immunohistochemical analysis revealed the presence of the estrogen receptor α (ERα) in the developing mouse fetus (Greco *et al.*, 1991; Greco *et al.*, 1992). At E15, a similar level of ERα was detected in the reproductive tract of both male and female fetuses. At later developmental stages, an increase in ERα was observed in the mesenchymal cells surrounding the female tract, whereas in the male fetus, a faint immunohistochemical signal remained present in the gonads and ducts (Greco *et al.*, 1991; Greco *et al.*, 1992). Nevertheless, the role of estrogens in sex differentiation is considered controversial. Jost demonstrated that in the absence of gonads, rabbit male and female fetuses developed as phenotypic females, implicating that gonadal steroid hormones are not necessary for female differentiation (Jost, 1947; Jost, 1953). However, a role of maternal estrogens in fetal development was not excluded in this experiment.

To elucidate a possible role of estrogens, an animal model lacking a functional *ERα* was created (Lubahn *et al.*, 1993). Unexpected, both male

and female *ERα* knockout (ERKO) mice are viable, showing normal external phenotypes; however, both sexes are infertile (Lubahn *et al.*, 1993; Eddy *et al.*, 1996). The female knockout mice show hemorrhagic cystic ovaries, due to excessive stimulation by gonadotropins because of the lack of the negative feedback on the pituitary that is normally exerted by estrogens through ERα. Follicle development arrests prior to formation of the ovulatory follicles (Lubahn *et al.*, 1993). Infertility of the ERKO male is related to a lower concentration of epididymidal sperm, due to a defect in the reabsorption of luminal fluid in the head of the epididymides (Hess *et al.*, 1997). This aberrant phenotype most likely reflects a postnatal defect, since disruption of spermatogenesis and degeneration of the seminiferous tubules become evident after 10 weeks of age (Eddy *et al.*, 1996). Morphologically, the extragonadal reproductive tracts of male and female ERKO mice develop normally in the absence of an estrogen response (Lubahn *et al.*, 1993; Eddy *et al.*, 1996). Therefore, estrogens do not seem to play a crucial role during sex differentiation.

The results of the studies on the ERKO mice have been placed in a different perspective, however, with the identification of another estrogen receptor (ERβ) (Kuiper *et al.*, 1996). The detection of about 5% of normal estradiol binding in uteri of ERKO mice (Lubahn *et al.*, 1993), can be explained by the presence of ERβ. In addition, other estrogen responses may be mediated through ERβ. ERβ is more abundantly expressed than ERα during fetal development, especially in the prostate and the fetal ovary and testis (Brandenberger *et al.*, 1997). The generation of *ERβ* knockout mice and *ERα/ERβ* double-knockout mice, will answer questions that still remain about the role of estrogens in fetal sex differentiation.

The presence of estrogen receptors α and β in the reproductive tracts of male and female fetuses indicates that these tracts are potential targets for estrogens (Greco *et al.*, 1991; Greco *et al.*, 1992). Therefore, excess of estrogens during differentiation can cause abnormalities. A case in point is the administration of the synthetic estrogen diethylstilbestrol (DES) to pregnant women, which has led to an increase in genital carcinoma in the daughters (Bornstein *et al.*, 1988). Abnormalities of the reproductive tract, such as the presence of Müllerian duct remnants, were also observed in boys exposed to DES *in utero*, and in male offspring of mice exposed to

DES (McLachlan *et al.*, 1975; Gill *et al.*, 1976; Whitehead and Leiter, 1981). Organ culture of male reproductive tracts of the mouse demonstrated that Müllerian ducts exposed to DES *in vivo* do not regress, even in the presence of control testes, while nonexposed Müllerian ducts regress normally (Newbold *et al.*, 1984). Therefore, it was hypothesized that prenatal DES exposure may change the sensitivity of the Müllerian ducts to AMH. In birds, such an inhibitory action of estrogens on AMH occurs naturally. The right side of a female chicken is masculinized, characterized by the presence of an ovotestis and absence of the Müllerian duct, whereas the left side contains an ovary and a differentiated Müllerian duct. During fetal development of chicken, both male and female gonads produce AMH (Hutson *et al.*, 1981). The failure of the left Müllerian duct to regress in the female, despite the presence of AMH, can be explained by a protective role of estrogens produced by the fetal ovary. This hypothesis is further supported by an increased ERα concentration in the left Müllerian duct (MacLaughlin *et al.*, 1983). Whether a similar protective mechanism can be activated in mammals, which could explain the presence of Müllerian duct remnants in the so-called DES-sons, remains to be studied (see Chapter 5).

1.6 Aim and scope of this thesis

AMH is a member of the TGFβ superfamily of growth and differentiation factors. AMH functions during sex differentiation to induce regression of the Müllerian ducts in the male fetus. Postnatally, the action of AMH is restricted to the gonads. This thesis focuses on the molecular mechanism and developmental aspects of AMH action.

The members of the TGFβ family exert their actions via a type I and a type II receptor complex. The family members, their receptors, and the signaling pathway are described in Chapter 2.

The candidate AMHRII was cloned from rat (Baarends *et al.*, 1994; Grootegoed *et al.*, 1994) and rabbit (Di Clemente *et al.*, 1994b). Chapter 3 describes the development of transgenic mice containing a cDNA construct encoding a dominant negative AMHRII, in order to study the role of this receptor in AMH signaling.

Chapter 4 describes the isolation and characterization of the human gene encoding AMHRII, in order to evaluate possible mutations in the *AMHRII* gene in PMDS patients.

The action of AMH in Müllerian duct regression is inhibited due to prenatal exposure to estrogens, such as the synthetic estrogen diethylstilbestrol (DES). However, the mechanism involved in this inhibitory effect of DES is not known. The influence of prenatal DES exposure on Müllerian duct development, and on the mRNA expression of AMH and AMHRII at several time points during male fetal mouse development, are described in Chapter 5.

Due to the fact that it is difficult to develop a good binding assay for AMH, the known inhibitory effect of AMH on FSH-induced aromatase activity in Sertoli and granulosa cells, was used to develop an *in vitro* model to study the molecular mechanism of AMH action. With this model, ALK2 (activin receptor-like kinase 2) was identified as a candidate AMH type I receptor. The mRNA expression of ALK2 in the target tissues of AMH was studied (Chapter 6).

AMH may induce apoptosis through an AMHRI/AMHRII complex, as a mechanism of Müllerian duct regression. Indeed, apoptotic cells were detected in the regressing Müllerian ducts in male mice, and a constitutively active ALK2 was found to induce apoptosis in cultured cells (Chapter 7).

In the General Discussion (Chapter 8) the possible role of AMH in the origin of Müllerian duct abnormalities in the female is discussed. Furthermore, the implications of the identification of ALK2 in AMH signaling are discussed. Finally, some directions for future research are given.

Chapter Two

Transforming growth factor β family of peptide growth and differentiation factors, and their receptors

Chapter Two

Transforming growth factor β family of peptide growth and differentiation factors, and their receptors

in part submitted

2.1 Introduction

The transforming growth factor β (TGFβ) superfamily consists of a large group of related peptide growth and differentiation factors, which includes the TGFβs, the bone morphogenetic proteins (BMPs), activins and anti-Müllerian hormone (AMH) (Figure 2.1A) (Massagué, 1990). This chapter describes structural and functional aspects of the members of the TGFβ family and their receptors. The identification of factors that function downstream in the TGFβ signaling pathway and the fact that these downstream factors can interact with the signaling cascade of other growth factors may give insight in the diverse biological responses evoked by the TGFβ family members.

2.2 Biological responses

Members of the TGFβ superfamily have a broad range of functions in mesenchymal-epithelial interactions, cell growth, extracellular matrix production and tissue remodeling. The development of several knockout models in mice has given much information about the range of activities *in vivo*. However, many of the activities are masked by functional redundancy among TGFβ family members, which contributes to the complexity of the interpretation of the results. Several mouse knockout models are described below.

Knockout experiments have demonstrated a role for $TGF\beta_1$ in immunogenesis (Shull *et al.*, 1992; Kulkarni *et al.*, 1993), whereas $TGF\beta_3$ is important in craniofacial development and pulmonary maturation (Kaartinen *et al.*, 1995; Proetzel *et al.*, 1995). Analysis of the phenotype of $TGF\beta_2$-deficient mice revealed that $TGF\beta_2$ has a more widespread function: $TGF\beta_2$-deficiency leads to defects in heart, lung, limb, eye, urogenital and craniofacial development (Sanford *et al.*, 1997). Inhibin and possibly also activins are involved in regulation of the secretion of follicle-stimulating hormone (FSH) from the pituitary gland (Ling *et al.*, 1986a; Ling *et al.*, 1986b; Petraglia *et al.*, 1989). Furthermore, activins are involved in craniofacial development and dorsal mesoderm induction (Smith *et al.*, 1990; Van den Eijnden-van Raaij *et al.*, 1990; Matzuk *et al.*, 1995). Inhibin has been suggested to function as a tumor-suppressor gene, since inhibin-

deficient mice developed gonadal somatic cell tumors (Matzuk *et al.*, 1992; Matzuk *et al.*, 1994). Although one might argue, that the tumors arise due to the high activin level that is found in inhibin-deficient mice, and that inhibin functions to suppress activin action rather than acting directly as a tumor-suppressor. BMPs, also known as osteogenic proteins, have a broad expression pattern and knockout experiments revealed functional redundancy. Nevertheless, it was shown that BMPs play a role in bone and cartilage formation, neuronal differentiation, kidney development, spermatogenesis and ventral mesoderm induction (Wozney *et al.*, 1988; Koster *et al.*, 1991; Sampath *et al.*, 1992; Dudley *et al.*, 1995; Zhao *et al.*, 1996). The expression patterns of AMH and growth/differentiation factor-9 (GDF-9) are more restricted than that of other family members. GDF-9 expression is specific for oocytes, and, indeed, in GDF-9-deficient mice oocyte differentiation and folliculogenesis are blocked in the absence of any other phenotypic expression of the loss-of-function mutation (Dong *et al.*, 1996). AMH is expressed by fetal and adult testes, and induces Müllerian duct regression in male fetuses. AMH-deficient male mice show persistence of Müllerian ducts and develop Leydig cell hyperplasia. The latter effect points to a role of AMH in the regulation of Leydig cell proliferation through an autocrine action on Sertoli cells. AMH is also produced by the adult ovary, but thus far no abnormalities of AMH-deficient ovaries have been reported (Behringer *et al.*, 1994).

2.3 General structure

The TGFβ family members are synthesized as large precursor proteins, which form homo- or heterodimers. Cleavage at mono- or dibasic cleavage sites releases the biologically active C-terminal part of the dimeric protein (mature protein) (Figure 2.1B). The dimeric N-terminal fragment plays a role in proper folding and secretion of the dimeric C-terminal fragment. Furthermore, the N-terminal fragment is involved in regulation of the biological activity of the C-terminal fragment. Association of the N-terminal fragment with the C-terminal fragment after cleavage, keeps the protein in an inactive state (Gentry and Nash, 1990). In contrast to other family members, full biological activity of the mature AMH protein is only obtained

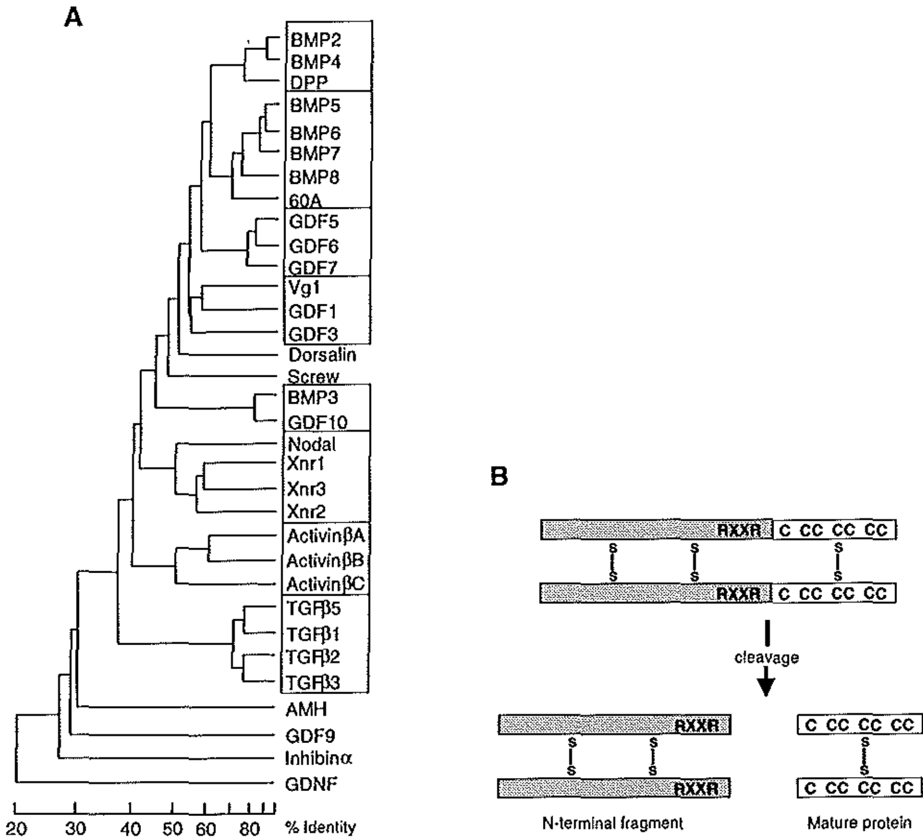


Figure 2.1. **The TGFβ family of growth and differentiation factors.**

A. Similarity between members of the TGFβ family. All factors listed are mammalian, except DPP, 60A and Screw from *Drosophila*; VG1, Xnr and TGFβ5 from *Xenopus*; and Dorsalin from chicken. The percentage identity refers to the amino acid sequences of the bioactive C-terminal domain [modified from Massagué and Weis-Garcia (1996)].

B. Schematic representation of the proteolytic processing of TGFβ related proteins. The proteins are synthesized as precursor molecules with a pro-domain (N-terminal fragment) and a C-terminal mature region. A dibasic cleavage site (RXXR) is found near the junction of the pro and mature domains in most of the family members. AMH contains a monobasic cleavage site (RAGR) at this position. The mature domain contains the seven conserved cysteine residues. Proteolytic cleavage releases the mature protein.

when the N-terminal fragment remains associated with the C-terminal fragment (Wilson *et al.*, 1993). Although the C-terminal fragment of AMH is bioactive, as demonstrated by its ability to induce Müllerian duct regression *in vitro*, its activity is augmented in the presence of the N-terminal fragment (Wilson *et al.*, 1993). Nevertheless, cleavage of AMH is a prerequisite to obtain bioactivity. Mutation of the monobasic site RAGR, which is recognized by members of the proprotein convertase family, into RAGA, abolished cleavage and as a consequence bioactivity (Nachtigal and Ingraham, 1996).

The TGFβ superfamily is characterized by a conserved spacing of seven cysteine residues in the C-terminal fragment (Figure 2.1B). The three-dimensional structure of TGFβ, determined by crystallography, revealed that the dimeric protein consist of two pairs of antiparallel β strands, held together by a disulfide bond formed by one pair of the cysteine residues. The remaining six cysteines form a conserved arrangement of disulfide bridges, known as the "cysteine knot", involved in intrachain folding (Daopin *et al.*, 1992; Schlunegger and Grutter, 1992). The similarity between the different family members ranges from 25-90%, based on the amino acid sequence of the C-terminal domain (Figure 2.1A).

2.4 Serine/threonine kinase receptors

2.4.1 General structure

Members of the TGFβ superfamily exert their actions by binding to transmembrane serine/threonine (S/T) kinase receptors. In binding studies, receptors for TGFβs were identified and termed type I and type II receptors, based upon their respective relative molecular weights of 55 kDa and 80 kDa (Cheifetz *et al.*, 1987; Massagué, 1990). By expression cloning, the genes encoding the TGFβ type II receptor and the activin type II receptor were identified [Lin *et al.*, 1992; Mathews and Vale, 1991). Other S/T kinase receptors, including the type I receptors named activin receptor-like kinases (ALK) 1-7, were identified by PCR using primers based on conserved residues of the kinase domain of the type II receptors (Attisano *et al.*, 1993; Ebner *et al.*, 1993b; Franzén *et al.*, 1993; He *et al.*, 1993; Matsuzaki *et al.*, 1993; Ten Dijke *et al.*, 1993; Ryden *et al.*, 1996). With

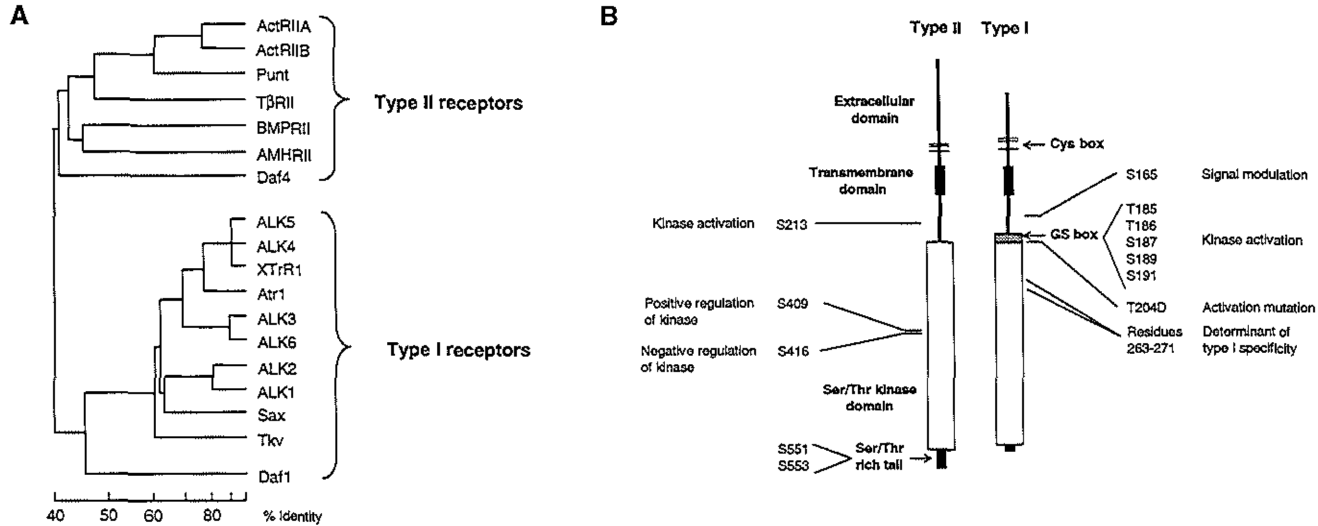


Figure 2.2. **Transmembrane serine/threonine kinase receptors.**

A. Similarity between the two subfamilies of transmembrane S/T kinase receptors. All members listed are human, except Punt, Thick veins, Saxophone and Atr-1 from *Drosophila*; Daf-1 and Daf-4 from *C. elegans*; and XTrR-1 from *Xenopus*. The percentage identity refers to the amino acid sequences of the kinase domain [modified from Massagué and Weis-Garcia (1996)].

B. Schematic structure of the TGFβ type I and type II receptors. The extracellular domain, the conserved Cys box (arrow), the transmembrane domain (filled box), the GS box (grey box and arrow), the S/T kinase domain (large open box), and the type II receptor serine/threonine rich tail (arrow) are shown. Furthermore, known autophosphorylation sites in the TβRII and sites in TβRI phosphorylated by TβRII, and their functional roles are indicated, as well as the activating mutation (T204D), and residues of the TβRI involved in signal specificity and modulation.

the cloning and characterization of the new receptors, it became clear that the S/T kinase receptor family could be subdivided into two subfamilies, based on the structure of the different members (Figure 2.2A and B).

The members of the S/T kinase receptor family share the following overall structure: an N-terminal signal sequence, a short cysteine-rich extracellular region, a single hydrophobic membrane-spanning domain and a cytoplasmic region containing the kinase domain. The extracellular domain of both receptor type I and type II contains a characteristic cluster of cysteine residues, the "cysteine box" (Childs *et al.*, 1993). This cluster is preceded by a conserved pattern of seven cysteine residues in type I receptors, and a variable set of cysteines in type II receptors. The catalytic domain of the S/T kinase domain can be divided into 12 subdomains, which contain stretches of conserved amino acids (Ten Dijke *et al.*, 1994a). The type I receptors contain a highly conserved 30 amino acid sequence in the juxtamembrane region. This conserved region contains a characteristic GSGSG motif, known as the "GS box", and plays a crucial role in signal transduction (see 2.4.2) (Wrana *et al.*, 1994). A unique characteristic of the TGFβ receptor family is the presence of two short inserts in the kinase domain between subdomains VI-A and VI-B and between subdomains X and XI (Ten Dijke *et al.*, 1994a). Furthermore, the type II receptors have a serine/threonine rich C-terminal tail, which is absent in type I receptors (Figure 2.2B).

Sequence comparison of the receptors revealed that the extracellular domain shows little sequence homology. However, different receptors with affinity for the same ligand are more similar (Ten Dijke *et al.*, 1994a). Comparison of the cytoplasmic kinase domain of the different receptors revealed a 30-40% amino acid identity, while comparison within the type I and type II receptor subfamilies results in a sequence identity of 60-80% (Figure 2.2A) (Kingsley, 1994; Wrana *et al.*, 1994).

2.4.2 Mechanism of receptor activation

The mechanism of receptor activation by TGFβ has been well characterized. From studies in TGFβ resistant cell lines, which lack binding of TGFβ to either type I (R mutant) or type II receptors (DR mutant), it became evident that TGFβ has to interact with two receptors, one from

each subfamily (Boyd and Massagué, 1989; Laiho *et al.*, 1990). TGFβ signaling is lost in R and DR mutants, but a somatic cell hybrid of the R mutant and DR mutant cell lines restores full receptor function (Laiho *et al.*, 1991). In addition, expression of either wild type TGFβ type II receptor (TβRII) in the R mutant or wild type TGFβ type I receptor (TβRI) in the DR mutant cell lines restored all TGFβ responsiveness (Wrana *et al.*, 1992; Franzén *et al.*, 1993; Bassing *et al.*, 1994; Ten Dijke *et al.*, 1994b). Evidence that both receptor types are engaged in physical association in a heteromeric receptor complex, was shown by coprecipitation of both receptors using antibodies against either receptor (Wrana *et al.*, 1992; Ebner *et al.*, 1993a; Franzén *et al.*, 1993). From these studies, it was concluded that the type II receptor can bind ligand in the absence of the type I receptor, whereas the type I receptor itself is unable to bind the ligand in the absence of the type II receptor. Upon ligand binding by the type II receptor, the type I receptor is recruited into the complex (Wrana *et al.*, 1994). Formation of the heteromeric receptor complex, besides being triggered upon ligand binding, may also be stabilized by interaction between the cytoplasmic parts of the receptors (Feng and Derynck, 1996).

Several lines of evidence indicate that the type I and II receptors may in fact form a heterotetrameric complex. In cross-linking experiments, it was demonstrated that, in the absence of ligand, the type II receptors are present in a homomeric complex (Chen and Derynck, 1994; Vivien *et al.*, 1995). Since TβRI does not bind TGFβ in the absence of TβRII, it has been difficult to study homodimerization of TβRI in the absence of TβRII. Therefore, chimeric receptors with the extracellular domain of the erythropoietin (Epo) receptor and the cytoplasmic domain of the TβRI (E-RI), or TβRII (E-RII) were constructed. Homodimerization of the extracellular domain of the wild type EpoR is necessary for receptor activation. Epo induced the formation of an active heteromeric complex between chimeric E-RI/E-RII. However, a chimeric E-RI, with a constitutively active cytoplasmic kinase domain of TβRI (see below), cannot signal in the absence of Epo. Epo-induced homodimerization of E-RI is necessary for receptor activity (Luo and Lodish, 1996). In addition, kinase-defective and activation-defective TβRIs can complement each other, suggesting that at least two type I receptors are needed (Weis-Garcia and Massagué, 1996). In the

current model, it is suggested that the signaling complex is formed as a heterotetramer, consisting of two type I and two type II receptors (Figure 2.3).

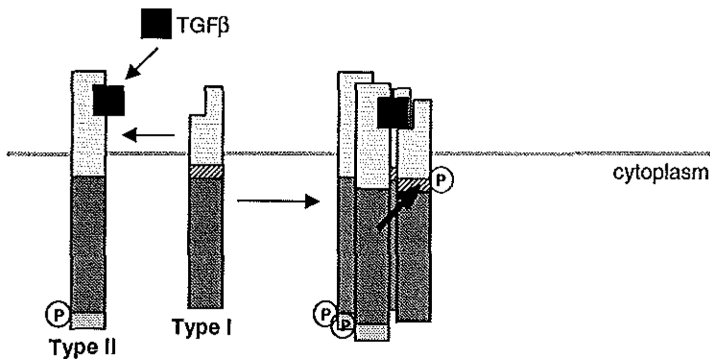


Figure 2.3. **Mechanism of TGFβ receptor activation.**
The type II receptor is the primary TGFβ receptor, which is a constitutively active serine/threonine kinase that recruits the type I receptor upon ligand binding. Subsequent phosphorylation of the GS box by the type II receptor activates the type I receptor to signal to downstream substrates (Wrana *et al.*, 1994).

From studies on the mechanism of receptor activation, it was shown that the TβRII is highly phosphorylated in the absence of ligand and that exposure to TGFβ did not alter the phosphorylation level or the kinase activity. Tryptic phosphopeptide mapping revealed that TβRII is phosphorylated on multiple serine and threonine residues, due to ligand-independent autophosphorylation and possibly also through phosphorylation by other cytoplasmic kinases (Wrana *et al.*, 1994; Chen and Weinberg, 1995). Autophosphorylation of serine residues in TβRII plays a role in the regulation of TβRII signaling. It was shown that phosphorylation of Ser213 and Ser409 are essential for TβRII signaling, while phosphorylation of Ser416 inhibits receptor function, since mutation of Ser416 to an alanine residue yielded a hyperactive receptor with constitutive activity (Figure 2.2B) (Luo and Lodish, 1997). In addition, in a recent paper by Lawler *et al.* (1997), it was shown by anti-phosphotyrosine

Western blotting that TβRII kinase can also autophosphorylate on tyrosine residues. Furthermore, the kinase activity of TβRII can be inhibited by a tyrosine kinase inhibitor or by replacement of three tyrosine phosphorylation sites by phenylalanine residues (Lawler *et al.*, 1997). However, phosphorylation of serine/threonine residues in the C-terminal tail of TβRII is not essential for signaling, since a mutant TβRII, which lacks this tail, showed undiminished signaling activity (Wieser *et al.*, 1993).

An important event in TGFβ receptor activation is transphosphorylation of the type I receptor by the type II receptor after ligand-induced receptor complex formation (Ventura *et al.*, 1994; Wrana *et al.*, 1994). The type I receptor becomes phosphorylated at five clustered serine/threonine residues in the GS box, which are essential for signaling (Figure 2.2B and 2.3). Mutation of any one residue, or two or three residues, results in no loss or partial loss in signaling, respectively. However, mutation of four of the five residues completely inactivates the receptor (Wrana *et al.*, 1994; Franzén *et al.*, 1995; Wieser *et al.*, 1995). The crucial role of transphosphorylation of the TβRI by TβRII in signaling, was further demonstrated by the use of kinase-defective type II receptors. The TβRII-(K277R), which carries a single amino acid substitution in the nucleotide binding fold of the kinase domain, binds ligand and forms a receptor complex to a similar extent as the wild type receptor. However, autophosphorylation of the TβRII-(K277R) is abolished and the receptor is also unable to phosphorylate the type I receptor (Wrana *et al.*, 1994). For the mutant TβRII-(P525L), autophosphoryation was found to be normal. Nevertheless, the catalytically active TβRII-(P525L) is unable to phosphorylate the type I receptor, suggesting that TβR-(P525L) cannot recognize TβRI as a substrate (Carcamo *et al.*, 1995). Transphosphorylation of the type I receptor by the type II receptor does not depend on the kinase activity of the type I receptor, since mutations that disrupt the kinase activity of TβRI do not prevent phosphorylation of TβRI by TβRII. However, disruption of the kinase activity of TβRI prevents signaling (Wrana *et al.*, 1994). This suggests that the type I receptor serves as substrate for the type II receptor, and that the kinase activity of the type I receptor is required to phosphorylate downstream factors (Wrana *et al.*, 1994). The downstream function of TβRI was confirmed by identification of a constitutively active

TβRI (Wieser *et al.*, 1995). In addition to phosphorylation of the residues in the GS box, phosphorylation of two additional threonines located near the start of the kinase domain plays a role in receptor activation. Mutation of these threonines to valine residues inhibits receptor phosphorylation and signaling activity (Wieser *et al.*, 1995). However, replacement of Thr204 with an aspartic acid residue results in a type I receptor with elevated kinase activity that signals in the absence of ligand and TβRII (Wieser *et al.*, 1995). This suggest that the type I receptor determines the onset of downstream signals. Using chimeras between TβRI and one of the other type I receptors (ALK2), it was shown that the juxtamembrane domain, including the GS box, and most regions in the kinase domain could functionally substitute for each other. However, a nine amino acid sequence between subdomains IV and V of the TβRI, which varies between the different type I receptors, is important in determining specific TGFβ signals. Replacement of this sequence in TβRI by the corresponding sequence of ALK2, which is unable to transduce TGFβ responses, inactivated TGFβ signaling, whereas reciprocal introduction of the corresponding TβRI sequence into ALK2 realizes TGFβ signaling (Feng and Derynck, 1997). In addition, serine and threonine phosphorylation sites in the cytoplasmic juxtamembrane region play a role in the determination of TGFβ signals. Mutational analysis revealed that phosphorylation of Ser165 modulates TGFβ signaling. A stronger effect on growth inhibition was observed, whereas the apoptotic signal was lost (Souchelnytskyi *et al.*, 1996). Furthermore, mutation of Ser172 or Thr176 showed that these residues are dispensable for extracellular matrix protein production, but are essential for growth inhibition by TGFβ (Saitoh *et al.*, 1996).

The mechanism of receptor activation by TGFβ may serve as a model for other family members. Signaling of activins and BMPs is also mediated by a type I and type II receptor complex (Attisano *et al.*, 1996). However, variations on this model have been described. BMPs can bind to type I or type II receptors individually with low affinity, but high affinity binding is achieved when both receptor types are present (Liu *et al.*, 1995; Nohno *et al.*, 1995). In addition, receptor complex formation by activins and BMPs show other variable aspects. Both activins and BMPs can bind to two different type II receptors that, in addition, can interact with more than one

type I receptor. Furthermore, some type I receptors, such as ALK2, can serve more than one ligand. An overview of the ligand/receptor combinations and their downstream factors are presented in Figure 2.4.

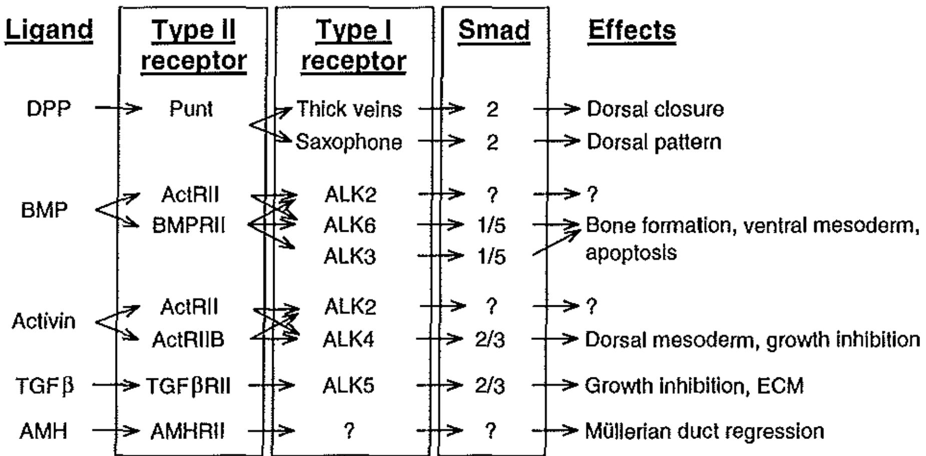


Figure 2.4. **Ligand-receptor combinations and their known effects.**

2.4.3 Downstream signaling molecules

2.4.3.1 Smad proteins

Genetic analysis of *Drosophila* and *C. elegans* mutants has led to identification of a family of proteins that signal downstream of the S/T kinase receptors to the nucleus, where transcription of target genes is initiated.

The *Drosophila* gene *dpp* (decapentaplegic) encodes a member of the TGFβ superfamily. In a screen for genes that enhance weak *dpp* mutants, *Mothers against dpp (Mad)* was identified (Raftery *et al.*, 1995; Sekelsky *et al.*, 1995). Homozygous *Mad* mutants display a similar phenotype as *dpp* mutants. Evidence that Mad functions downstream in the DPP signaling pathway came from the finding that transgenic expression of the *Mad* gene rescued the phenotype in *dpp* mutants (Newfeld *et al.*, 1996). Furthermore,

Mad mutations inhibit signaling of the constitutively active DPP type I receptor encoded by *Thick veins* (Hoodless *et al.*, 1996; Wiersdorff *et al.*, 1996). In *C. elegans*, the genes *sma2*, *sma3*, and *sma4* were identified in a screen for mutants with similar phenotypes as the type II receptor *Daf4* mutants (*Small* mutants) (Savage *et al.*, 1996). These genes are homologous to the *Mad* gene of *Drosophila*. Recently, at least nine genes homologous to *Mad* and *sma* have been identified. To simplify the nomenclature, the homologues of *Mad* and *sma* are designated *Smads*: *Smad1* (*Madr1, Xmad1, bsp1, Dwf-A, JC4-1*), *Smad2* (*Madr2, Xmad2, JV18-1*), *Smad3* (*hMad3, JV15-2*), *Smad4* (*DPC4*), *Smad5* (*Dwf-C, JV5-1*), *Smad6* (*JV15-1*), *Smad7*, *Smad8*, and *Smad9* (*MADH6*) (Raftery *et al.*, 1995; Sekelsky *et al.*, 1995; Baker and Harland, 1996; Chen *et al.*, 1996b; Eppert *et al.*, 1996; Graff *et al.*, 1996; Hahn *et al.*, 1996; Hoodless *et al.*, 1996; Lechleider *et al.*, 1996; Riggins *et al.*, 1996; Savage *et al.*, 1996; Yingling *et al.*, 1996; Imamura *et al.*, 1997; Nakao *et al.*, 1997a; Topper *et al.*, 1997; Watanabe *et al.*, 1997).

The identified Smads can be divided into three subclasses: pathway-specific, common-mediator, and inhibitory Smads. These three subclasses, and the mechanism of Smad activation and translocation to the nucleus will be discussed below.

Pathway-specific Smads

Smad1, Smad2, Smad3, Smad5, Smad8 and Smad9 are designated pathway-specific Smads. Smad1, and probably Smad5 and Smad8, transduce BMP responses (Graff *et al.*, 1996; Thomsen, 1996), whereas Smad2 and Smad3 display TGFβ and activin specificity (Baker and Harland, 1996; Graff *et al.*, 1996). Injection of human or mouse Smad1 mRNA into Xenopus animal cap cells resulted in induction of ventral mesoderm (Liu *et al.*, 1996), similar to a BMP4 response (Koster *et al.*, 1991), suggesting that the function of Smads is conserved across species.

The pathway-specific Smads become activated upon phosphorylation by the type I receptor at their most C-terminal region (Hoodless *et al.*, 1996; Kretzschmar *et al.*, 1997b). This region contains a conserved Ser/Ser/X/Ser (SSXS) motif (Figure 2.5A). By mutational analysis it has been shown that the two most C-terminal serine residues become phosphorylated (Abdollah

et al., 1997; Souchelnytskyi *et al.*, 1997). The pathway-specific Smads interact directly with the type I receptor, as shown by co-immunoprecipitation (Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996; Nakao *et al.*, 1997b). This interaction is transient and can only be detected using a kinase inactive form of the type I receptor (Macias-Silva *et al.*, 1996; Nakao *et al.*, 1997b).

Common-mediator Smads

Upon phosphorylation, the pathway-specific Smad is released from the type I receptor, upon which this Smad protein forms a hetero-oligomer with the common-mediator Smad4 and is then translocated to the nucleus to activate target genes (Figure 2.5B) (Hoodless *et al.*, 1996; Lagna *et al.*, 1996; Liu *et al.*, 1996; Wu *et al.*, 1997). The common-mediator Smad4 forms a complex with Smad2 and Smad3 (Lagna *et al.*, 1996; Nakao *et al.*, 1997b; Wu *et al.*, 1997), but also with Smad1 (Lagna *et al.*, 1996; Kretzschmar *et al.*, 1997b), indicating its common role in signal transduction. Indeed, injection of Smad4 mRNA into *Xenopus* animal caps induced both dorsal and ventral mesoderm (Lagna *et al.*, 1996; Zhang *et al.*, 1997). Smad4 has a similar structure as the pathway-specific Smads. However, Smad4 lacks the SSXS motif and does not bind to, and is also not phosphorylated by the type I receptor, implicating a different function for Smad4 (Figure 2.5A) (Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996; Nakao *et al.*, 1997b). It was suggested that the *Drosophila* gene *medea* encodes a Smad4 homologue (Raftery *et al.*, 1995; Massagué *et al.*, 1997). So far, Smad4 is the only Smad protein identified in different species as a common-mediator.

Inhibitory Smads

Smad6 and Smad7 form the third subclass of Smads (Hayashi *et al.*, 1997; Imamura *et al.*, 1997; Nakao *et al.*, 1997a). Smad6 and Smad7 share the C-terminal domain of the other Smads, but, similar to Smad4, lack the SSXS motif. The most important hallmark of the inhibitory Smads, Smad6 and Smad7, is found in the N-terminal domain, which does not show any homology with the N-terminal domain of the other Smads (Figure 2.5A). Inhibitory Smads have also been identified in *Drosophila* (*Dad*: *daughters against dpp*) (Tsuneizumi *et al.*, 1997), suggesting a conserved function.

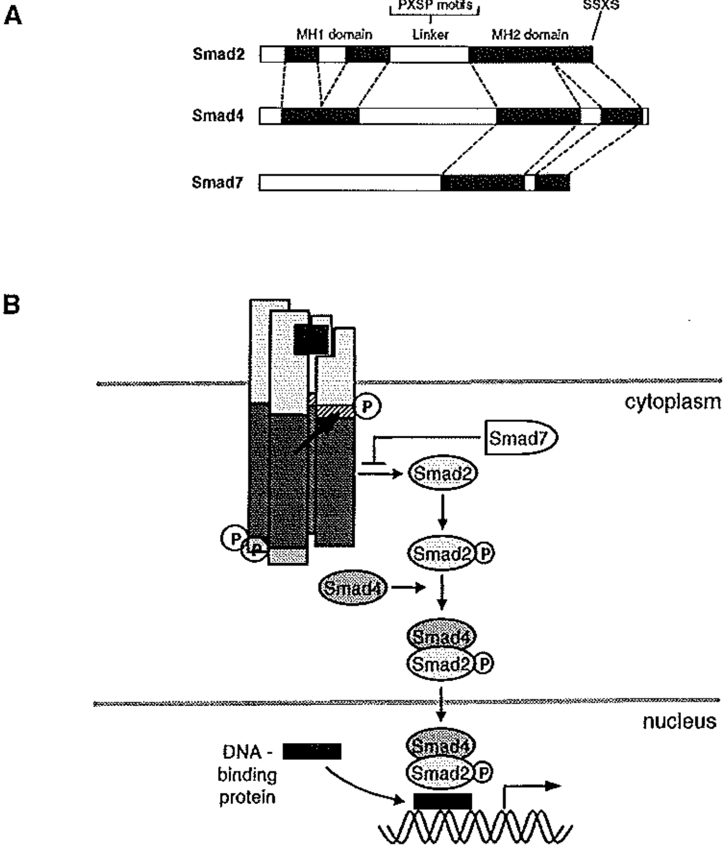


Figure 2.5. **Smads: structure and signaling pathway.**

A. General structure of pathway-specific Smads (Smad2), common-mediator Smad (Smad4) and inhibitory Smads (Smad7). The areas of homology between the different types of Smad proteins are indicated in black. Furthermore, the MH1 domain (repressor domain), linker domain containing the four PXSP motifs (ERK MAP kinase phosphorylation sites), and the MH2 domain (effector domain) with the SSXS phosphorylation site, are indicated.

B. TGFβ downstream signaling model. Upon activation of the type I receptor, pathway-specific Smads (Smad2; present in a homotrimeric form) are phosphorylated, which leads to heterodimerization with the common-mediator Smad (Smad4). The hetero-oligomeric complex translocates to the nucleus where it binds directly, or in a complex with other factors, to DNA. The inhibitory Smads (Smad7) bind to type I receptor and prevent phosphorylation of pathway-specific Smads.

Analogous to the pathway-specific Smads, Smad6 and Smad7 interact directly with the type I receptor. However, since the C-terminal phosphorylation site is absent in the inhibitory Smads, the association with the type I receptor is more stable than observed for the pathway-specific Smads (Hayashi *et al.*, 1997; Imamura *et al.*, 1997; Nakao *et al.*, 1997a), suggesting that Smad6 and Smad7 function by competitive inhibition. By binding to the type I receptor, the inhibitory Smads interfere with phosphorylation of the pathway-specific Smads, thereby preventing the formation of active heteromeric Smad complexes and, subsequently, translocation to the nucleus (Figure 2.5B) (Hayashi *et al.*, 1997; Imamura *et al.*, 1997; Nakao *et al.*, 1997a). Overexpression of Smad7 inhibits phosphorylation of Smad2 and Smad3 by activated TGFβ type I receptor, and also prevents phosphorylation of Smad1 by activated BMP type I receptors. Therefore, it is suggested that Smad7 functions as a general inhibitor (Nakao *et al.*, 1997a). Smad6 may act more specific, by inhibiting phosphorylation of Smad2 but not Smad3. Furthermore, phosphorylation of Smad1 by ALK3, but not by ALK6, is inhibited by Smad6 (Imamura *et al.*, 1997). Recently, it was shown that, in addition to interaction with type I receptors, Smad6 can associate with Smad1, thereby preventing the formation of an active Smad1-Smad4 complex (Hata *et al.*, 1998). In general, the inhibitory Smads may act as an autoregulatory negative feedback system in signal transduction.

In the pathway-specific and common-mediator Smad proteins, two regions of homology are present, the N-terminal and C-terminal domains, termed the Mad-homology domains MH1 and MH2, respectively. These domains are separated by a proline-rich linker sequence. In the inhibitory Smads only the linker region and the MH2 domain can be distinguished (Figure 2.5A). Within the MH1 and MH2 domains, specific residues are highly conserved, and it has been shown that these conserved residues are targets for mutations. A recent paper by Massagué *et al.* (1997) has given insight in the functional role of the Smad domains. The MH2 domain of the pathway-specific Smads functions as the effector domain, since it is fully active in the absence of the MH1 domain (Baker and Harland, 1996). The MH2 domain was shown to contain the ability to induce a transcriptional

response when fused to a GAL4 DNA-binding domain (Liu *et al.*, 1996). Furthermore, the MH2 domain of pathway-specific Smads plays a role in the interaction with Smad4 (Meersseman *et al.*, 1997). Non-stimulated wild type Smad protein is located in the cytoplasm, and upon phosphorylation translocates to the nucleus. In the absence of the MH1 domain, the protein is predominantly located in the nucleus, even in non-stimulated cells (Baker and Harland, 1996). The MH1 domain may function as a negative regulator by interaction with the MH2 domain, thereby preventing hetero-oligomer formation and keeping the Smad protein in an inactive state (Hata *et al.*, 1997). Phosphorylation of the SSXS motif releases the interaction between the MH1 and the MH2 domains, thereby activating the protein.

How Smad proteins propagate the signal from the ligand to target genes, is still under investigation. In a GAL4 fusion experiment, it was shown that Smads mediate transcriptional responses (Liu *et al.*, 1996). However, it is unclear whether Smads interact directly with target genes or whether other factors are involved. The response elements of several target genes have been identified and may serve to identify interacting transcription factors. The *Mix-2* gene contains an activin response element (ARE) (Chen *et al.*, 1996a), and studies on the activation of *Mix-2* have shown that besides Smad2 and Smad4, another factor is involved. This factor, FAST1, is a member of the winged-helix transcription factor family, and forms the major DNA-binding component. A C-terminal domain of FAST1, termed the Smad-interaction domain, is involved in binding of Smad2 and Smad4. This protein complex interacts directly with the ARE of the *Mix-2* gene (Chen *et al.*, 1997a). Whether other target genes of the TGFβ family members are activated by Smad proteins either directly or in complex with another transcription factor remains to be studied.

2.4.3.2 Other downstream factors

In addition to the Smad proteins, other factors have been proposed to function downstream in the TGFβ signaling pathway (Figure 2.6). The involvement of guanine-nucleotide binding proteins (G proteins) and the involvement of mitogen-activated protein kinases (MAPKs) in transducing the mitogenic signal of polypeptide growth factors, have been suggested, and will be discussed next.

G proteins can be divided into several classes, such as the heterotrimeric signal transducing G proteins and the small G proteins (Gilman, 1987; Bourne *et al.*, 1990). The heterotrimeric signal transducing G proteins, G_s and G_i, modulate the activity of adenylyl cyclase (Gilman, 1987), and their function can be altered by bacterial exotoxins. Pertussis toxin (PT) inhibits receptor interaction of G_i, resulting in an attenuated inhibition of adenylyl cyclase, while cholera toxin (CT) inhibits the GTPase activation of G_s (Gilman, 1987). PT and CT also affect some of the biological actions of TGFβ, such as stimulation of protooncogene expression (Howe and Leof, 1989). Stable transfection of $G_{i\alpha 1}$ in NIH 3T3 fibroblasts, a cell line with little responsiveness to TGFβ, restores TGFβ responsiveness to a normal level (Kataoka *et al.*, 1993). Furthermore, the stimulation of GTPase activity and GTPγS binding is induced by TGFβ, suggesting an involvement of G protein action in TGFβ signaling (Howe *et al.*, 1989; Kataoka *et al.*, 1993). In this respect, the presence of a consensus G protein activating sequence in the TGFβ type II receptor is of particular interest (Kataoka *et al.*, 1993). To determine which G protein or whether α or βγ subunits mediate the effects of TGFβ requires more studies.

Many studies have shown a central role of small G proteins (Ras, Rac, Rho and Cdc42) in the downstream signaling of tyrosine kinase receptors. The association between Ras and tyrosine kinase receptors is made by protein intermediates, such as Grb2 (McCormick, 1993). Grb2 binds to phosphotyrosine and a guanine nucleotide exchange factor, Sos. Next, Sos interacts with and activates membrane-bound Ras, leading to the activation of the MAPK family (Figure 2.6) (Egan and Weinberg, 1993; McCormick, 1993; Montminy, 1993; Hallberg *et al.*, 1994).

Three related MAPK cascades have been identified, which include the extracellular signal-regulated kinases (ERKs) (Avruch *et al.*, 1994), p38-MAPK (Han *et al.*, 1994), and the c-Jun N-terminal kinases (JNKs), also known as the stress-activated protein kinases (SAPKs) (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994). The ERKs are central elements in mitogenic signal transduction downstream of Ras (Avruch *et al.*, 1994). P38-MAPKs and JNKs/SAPKs participate in the pathway of pro-inflammatory cytokines and environmental stress, and, in addition, JNKs/SAPKs respond to apoptotic signals (Derijard *et al.*, 1994; Han *et al.*, 1994; Kyriakis *et al.*, 1994; Chen *et*

al., 1996c; Kyriakis and Avruch, 1996; Meyer *et al.*, 1996). The ERK, JNK/SAPK, and p38-MAKP cascades include a role for the MAPKKKs, MAPKKs, and MAPKs (Figure 2.6) (Davis, 1993; Cowley *et al.*, 1994; Minden *et al.*, 1994; Derijard *et al.*, 1995; Raingeaud *et al.*, 1995).

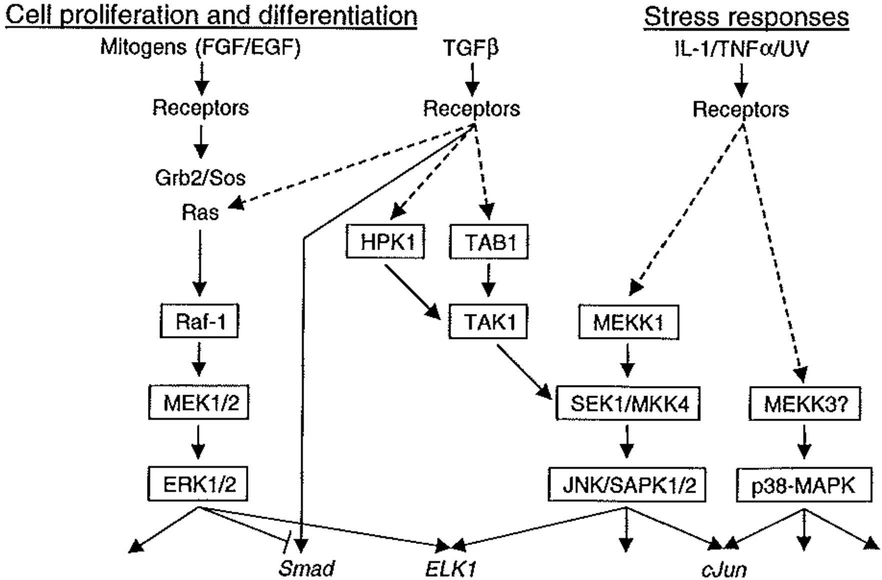


Figure 2.6. The mammalian MAPK-dependent signal transduction pathways. The components of the ERKs, JNK/SAPKs and p38-MAPK pathways are indicated. Homologous kinases are indicated within boxes at equivalent sites in the pathways. The interaction of TGFβ with the MAPK pathways through Ras or TAK1 is shown. The stippled arrows indicate that the mechanism of activation, and the identity of the possible upstream components, are unknown.

Recent studies suggest that the small G proteins and the MAPK cascade are also included in the signaling pathway of S/T kinase receptors. Using a TGFβ sensitive cell line, Mulder and Morris (1992) demonstrated activation of small G proteins by TGFβ. TGFβ stimulation resulted in a rapid increase in GTPγS bound to Ras (Mulder and Morris, 1992). Furthermore, constitutively active small G proteins stimulate

transcription of the TGFβ reporter construct p3TPlux, while in the presence of dominant-negative G proteins this TGFβ response was inhibited (Atfi *et al.*, 1997b). The involvement of G proteins, in particular Ras, in downstream signaling of S/T kinase receptors, was also demonstrated in the *Xenopus* animal cap assay. When animal caps dissected from blastula stage embryos are maintained *in vitro*, addition of activins or BMPs leads to mesoderm formation (Green and Smith, 1990). Expression of dominant-negative Ras mutants can prevent this mesoderm-inducing effect of activin, suggesting that activin utilizes the Ras signal transduction pathway (Whitman and Melton, 1992). Similar experiments have been performed in the presence of BMP4. BMP4 was shown to increase the activity of AP1, a heterodimer of cFos and cJun (Xu *et al.*, 1996). Phosphorylation of cJun is critical for AP1 activity (Whitmarsh and Davis, 1996). Since cJun is one of the targets of the JNK/SAPK and p38-MAPK cascades, the involvement of Ras, Raf, and cJun in the BMP4 signaling pathway has been studied. Indeed, dominant-negative mutants of Ras, Raf, and cJun inhibit the BMP4-induced increase in AP1 activity (Xu *et al.*, 1996). Collectively, these results suggest that signal transduction induced by members of the TGFβ family can make use of the small G proteins and the MAPK cascade. Indeed, TGFβ has been shown to activate all three MAPK cascades (Frey and Mulder, 1997a). In an intestinal epithelial cell line and a breast cancer cell line, TGFβ treatment of the cells resulted in growth inhibition and, in addition, rapid activation of ERK1 or ERK2 was observed (Hartsough and Mulder, 1995; Frey and Mulder, 1997a; Frey and Mulder, 1997b). Furthermore, transfection of dominant negative ERK1 into TGFβ-responsive NIH 3T3 fibroblasts, abolished stimulation of plasminogen activator inhibitor-1 (*PAI-1*) promoter activity by TGFβ (Mucsi *et al.*, 1996).

The activation of the JNK/SAPK cascade by TGFβ has become more evident with the cloning of *Tak1* (TGFβ-activated kinase 1) (Figure 2.6) (Yamaguchi *et al.*, 1995). *Tak1* was identified in a functional expression screen for MAPKKK cDNAs that counteract defects of the MAPK pathway, and was shown to induce the TGFβ-responsive *PAI-1* promoter *in vitro* (Yamaguchi *et al.*, 1995). In addition, a kinase-inactive TAK1 mutant inhibited the TGFβ response on the *PAI-1* promoter. Furthermore, dose-dependent activation of TAK1 was specific for TGFβ and could not be

induced by EGF (Yamaguchi *et al.*, 1995). The upstream TAK1 binding protein (TAB1), that activates TAK1, was shown to be important in TGFβ signaling in a mammalian cell line (Shibuya *et al.*, 1996). TAK1 can activate p38-MAPK through SEK1 (Yamaguchi *et al.*, 1995; Moriguchi *et al.*, 1996). Since SEK1 is also an activator of JNK/SAPK, the involvement of TAK1 in the JNK/SAPK pathway was studied. Indeed, a dominant-negative TAK1 mutant blocked JNK/SAPK activation, indicating a role for TAK1 in JNK/SAPK mediated pathways (Wang *et al.*, 1997). In addition, transfection of dominant-negative JNK/SAPK blocked the TGFβ-induced p3TPlux activation, thereby further demonstrating a role for the JNK/SAPK cascade in TGFβ signaling (Figure 2.6) (Atfi *et al.*, 1997b).

The factors, which directly mediate the transduction of the signal form the TGFβ receptors, to activate the MAPK cascade have not been identified yet. In addition to the Smad proteins, *schnurri (shn)* has been identified in *Drosophila* (Arora *et al.*, 1995; Staehling-Hampton *et al.*, 1995). *Shn* encodes a zinc finger transcription factor, homologous to the human major histocompatibility complex-binding proteins. The strikingly similar phenotypes of mutations in SHN and in the DPP receptors suggests, that SHN plays a role downstream of DPP signaling (Arora *et al.*, 1995; Staehling-Hampton *et al.*, 1995), although it is unclear in which signaling cascade and at what position.

In a recent study by Atfi *et al.* (1997b) it was demonstrated that the MAPK cascade and the Smad signaling pathway might interact. In the presence of dominant-negative cJun, the Smad3/Smad4-induced 3TPlux activity was inhibited, suggesting a requirement of the JNK/SAPK cascade for functioning of the Smad proteins (Atfi *et al.*, 1997a). Whether integration of the actions of Smad proteins and the MAPK cascades is a general feature in TGFβ signaling, remains to be studied. Nevertheless, the involvement of several downstream signaling pathways in response to TGFβ family members suggests that the specificity of the response to the ligand is determined, not by specific actions of a given type I/type II receptor complex, but rather by the combination of downstream factors which is present in the cell. This might explain the apparent contradictory responses of TGFβ in different cell types, such as growth inhibition versus growth stimulation.

2.5 Crosstalk between signaling pathways

Inductive interaction is a widely observed mechanism during embryogenesis. The induction of mesoderm and mesenchymal-epithelial interactions are important processes in various aspects of organogenesis. Many of these processes are regulated by members of the TGFβ family. However, also factors that propagate their signal by tyrosine kinase receptors, such as fibroblast growth factor (FGF) and epidermal growth factor (EGF), are involved in proper organogenesis. Several studies have indicated that the signaling pathways of S/T kinase receptors and tyrosine kinase receptors may cooperate.

TGFβ and bFGF together exert a myogenic promoting activity on cultured chick paraxial mesoderm, while this effect was not seen when the factors were added alone (Stern *et al.*, 1997). Synergistic interaction was also observed between FGF4 and the TGFβ family member BMP2, for induction of cardiogenesis in chick mesoderm explants (Lough *et al.*, 1996). In addition, experiments with dominant-negative receptors have demonstrated that both bFGF and activin are required for proper mesoderm induction in *Xenopus* embryos (Amaya *et al.*, 1991; Hemmati-Brivanlou and Melton, 1992).

Besides synergistic effects, antagonistic effects between members of the TGFβ family and FGF or EGF have also been described. FGF4 and BMPs are both involved in regulation of mouse limb outgrowth. FGF4 opposes the antiproliferative effect of BMP2 during limb bud outgrowth, and prevents the ability of BMP4 to induce interdigital membrane apoptosis (Niswander and Martin, 1993a; Niswander and Martin, 1993b; Ganan *et al.*, 1996). Furthermore, in mandibular arches of mouse embryos, BMP2 and BMP4 prevent the induction by FGF8 of Pax9, a marker for prospective tooth mesenchyme (Neübuser *et al.*, 1997).

The mechanism by which the synergistic or antagonistic interactions are regulated, is unknown. However, in a recent study using cultured mink lung epithelial cells, it was shown that the signaling pathways of EGF and BMP interact functionally (Kretzschmar *et al.* 1997a). BMP stimulates the phosphorylation of Smad1 at the C-terminal domain and induces nuclear accumulation of Smad1, whereas EGF signaling is mediated by the ERK family of MAPKs. In the presence of EGF, Smad1 became phosphorylated

at specific serine residues within the linker region, thereby preventing nuclear accumulation of Smad1 (Figure 2.6) (Kretzschmar *et al.*, 1997a). Mutational analysis of the linker region of Smad1 demonstrated that four repeated PXSP motifs become phosphorylated in the presence of EGF (Kretzschmar *et al.*, 1997a). These PXSP motifs are consensus substrate sites for MAP kinases (Hill and Treisman, 1995), and are also found in other pathway-specific Smads. Thus, regulation of Smad proteins by MAP kinases may be a general mechanism for crosstalk between the signaling activity of TGFβ family members and other growth factors. The inhibition of autophosphorylation of the EGF receptor in a human vulvar tumor cell line, and the inhibition of EGF-induced proliferation and progesterone production of human granulosa-luteal cells, by recombinant human AMH (Kim *et al.*, 1992; Maggard *et al.*, 1996), suggests that the antagonistic interactions between TGFβ family members and mitogenic growth factors may function in a reciprocal manner.

Members of the TGFβ superfamily acting through S/T kinase receptors, and mitogenic growth factors acting through tyrosine kinase receptors, are both involved in control of many aspects of growth and differentiation. Crosstalk between the different signaling pathways, which are activated by these receptor systems, may serve as a tool to balance the specific signals, to achieve coordinated control of developmental processes.

Chapter Three

Generation of transgenic mice expressing a dominant-negative AMH type II receptor

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Generation of transgenic mice expressing a dominant-negative AMH type II receptor

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Abstract

Anti-Müllerian hormone (AMH) induces regression of the Müllerian ducts in male fetuses. AMH is produced sex-specifically only by the fetal testis, but postnatally AMH mRNA is expressed both in testis and ovary. Furthermore, the AMH type II receptor (AMHRII) mRNA is expressed in the mesenchymal cells of the fetal Müllerian ducts, and also in fetal and postnatal testis and ovary. This expression poses the question whether AMH might have some role in development and maintenance of gonadal functions. To study the role of AMH during fetal and postnatal development, transgenic mice, expressing a dominant-negative AMHRII (ΔAMHRII) under the control of the inducible mouse metallothionein-1 (MT-1) promoter, were generated. Five founder mice were obtained and were found to express ΔAMHRII mRNA in several tissues including the Müllerian ducts and the gonads. However, both without and with the induction of ΔAMHRII mRNA expression, no aberrant phenotype was observed. It is concluded that a dominant-negative effect of the transgene may require a high expression level of the transgene.

Introduction

In mammals, differentiation of the male reproductive tract is regulated by two testicular hormones: testosterone and anti-Müllerian hormone (AMH) (Jost, 1953). The fetal Sertoli cells produce AMH, also known as Müllerian-inhibiting substance (MIS). AMH induces the regression of the fetal male Müllerian ducts, which form the anlagen of the oviducts, uterus and upper part of the vagina (Josso *et al.*, 1993). The fetal Leydig cells start to produce testosterone, which causes the differentiation of the Wolffian ducts into vasa deferentia, epididymides and seminal vesicles. The differentiation of the female reproductive tract is considered to follow a default pathway: in the absence of gonadal hormones the Müllerian ducts differentiate, whereas the Wolffian ducts degenerate. Failure of AMH action during male fetal development, as a result of a gene mutation leading to inactive AMH, causes persistent Müllerian duct syndrome (PMDS), a rare form of male pseudohermaphroditism (Carré-Eusèbe *et al.*, 1992; Imbeaud *et al.*, 1994; Imbeaud *et al.*, 1995). PMDS is characterized by the presence of oviducts

and uterus in otherwise normally virilized males. Also in male mice that lack AMH, Müllerian ducts do not regress (Behringer *et al.*, 1994). These mice have morphologically normal testes and can produce functional sperm. However, they often become infertile due to obstruction of the genital tract caused by the presence of Müllerian duct derivatives. In addition, several mice develop Leydig cell hyperplasia at adulthood, suggesting that AMH might be involved in maintenance of normal Leydig cell function (Behringer *et al.*, 1994). An inhibitory effect of AMH on Leydig cell function is observed in testes of transgenic mice that chronically express a high level of AMH; these mice develop Leydig cell hypoplasia (Behringer *et al.*, 1990). In female rats, AMH mRNA is expressed after birth in the granulosa cells of preantral and small antral follicles, suggesting that AMH might play a role during follicle development (Baarends *et al.*, 1995b). In transgenic female mice that overexpress AMH also during fetal life, ovarian aromatase activity is decreased and the ovaries develop cord structures resembling testicular cords (Behringer *et al.*, 1990). Inhibition of aromatase activity was also observed in cultured fetal ovaries exposed to AMH (Vigier *et al.*, 1989; Di Clemente *et al.*, 1992).

During folliculogenesis in adult rats and mice, many small antral follicles become atretic. The follicle-stimulating hormone (FSH) surge at estrus is suggested to be essential for the survival of those small follicles that are selected to ovulate (Hsueh *et al.*, 1994). At estrus, the ovarian expression of AMH mRNA is downregulated, and in addition, treatment of prepubertal rats with FSH causes a downregulation of AMH expression in some preantral and small antral follicles (Baarends *et al.*, 1995b). Therefore, it was suggested that AMH may exert an inhibitory effect on follicle maturation (Baarends *et al.*, 1995b). However, AMH-deficient female mice appeared to be fertile, and no gross abnormalities have been observed thus far in the ovaries of these mice (Behringer *et al.*, 1994).

AMH is a member of the transforming growth factor β (TGFβ) family of growth and differentiation factors (Massagué, 1990). These factors signal through a serine/threonine kinase receptor complex, consisting of a type I and a type II receptor. Upon ligand binding by the type II receptor, the type I receptor is recruited into the complex. Phosphorylation of the type I receptor by the type II receptor is essential for signaling (Wrana *et al.*,

1994), and kinase-deficient type II receptors were shown to function as a dominant-negative receptor in TGFβ-, activin-, and BMP-signaling, both *in vitro* and *in vivo* (Hemmati-Brivanlou and Melton, 1992; Ishikawa *et al.*, 1995; Tsuchida *et al.*, 1995; Attisano *et al.*, 1996).

Previously, an AMH receptor was identified that has the general structure of a type II receptor (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994b; Grootegoed *et al.*, 1994). Expression of mRNA for this novel type II receptor was found in defined and putative target cells of AMH, i.e. the mesenchymal cells surrounding the Müllerian ducts, the Sertoli cells of fetal and adult testes, and the granulosa cells of fetal and adult ovaries (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994b; Baarends *et al.*, 1995a; Baarends *et al.*, 1995b). Therefore, it was suggested that the novel type II receptor represents an AMH type II (AMHRII) receptor. Conclusive evidence for this hypothesis was obtained by analysis of *AMHRII* knockout mice (Mishina *et al.*, 1996), and the finding of mutations in the human *AMHRII* gene in PMDS patients (Imbeaud *et al.*, 1995; Imbeaud *et al.*, 1996).

To study developmental aspects of the role of AMHRII in AMH signal transduction, transgenic mice were generated that express a dominant-negative AMHRII (ΔAMHRII) under the control of the inducible metallothionein-1 promoter (MT-1). Upon zinc induction, a high expression level of the ΔAMHRII transgene is expected, and induction of the transgene should evoke abrogation of AMH signaling at specific time points. Furthermore, since in AMH-deficient mice testicular function in adult mice is disturbed due to the presence of Müllerian duct remnants, an inducible ΔAMHRII may be required to permit normal male fetal development, in order to study AMH function in the adult testis.

Materials and Methods

Construction of the MT-ΔAMHRII transgene

The endogenous CMV promoter of the pcDNA1 vector was removed by MluI/BamHI digestion and replaced by a 260 bp BglI-BglII fragment of the mouse MT-1 (mMT-1) promoter from the pMK' plasmid (Stuart *et al.*, 1984). A 740 bp EcoRI-StuI fragment of the rat AMHRII cDNA, encoding the

extracellular and transmembrane domains, was subcloned into EcoRI/XhoI sites of pcDNA-MT-1 to generate the construct pMT-ΔAMHRII (Figure 3.1).

Figure 3.1. Schematic representation of the MT-ΔAMHRII construct.
A 260 bp mouse metallothionein-1 (mMT-1) promoter was fused to the truncated rat ΔAMHRII cDNA, encoding the extracellular and transmembrane domains. A 700 bp fragment containing the Simian virus 40 (SV40) 3' untranslated region and polyadenylation signal was placed downstream of ΔAMHRII. A 1.8 kb NruI/ScaI linear DNA fragment containing the MT-ΔAMHRII transgene was used for microinjection of fertilized mouse eggs.

A 1.8 kb NruI-ScaI pMT-ΔAMHRII linear fragment was isolated and used for microinjection into the pronucleus of fertilized eggs generated from inbred FVB mice. Microinjected eggs were transferred to the oviducts of pseudopregnant foster mothers. Tail genomic DNA was isolated and tested either by Southern blotting (founder mice) or by PCR analysis (subsequent homozygous generations). All transgenic founder and F1 mice were intercrossed with wild type or transgenic littermates at 6 weeks of age. The activity of the MT-1 promoter was induced by maintaining animals on drinking water containing 25 mM $ZnSO_4$. Animals were kept under standard animal housing conditions in accordance with the NIH Guidelines for the Care and Use of Experimental Animals.

RNA analysis

RNA was isolated from mouse tissues using the LiCl/urea method (Auffray and Rougeon, 1980). A 310 bp EcoRI-KpnI rat AMHRII cDNA fragment encoding the extracellular domain, was subcloned in pBluescript KS and used to generate [^{32}P]-UTP-labeled anti-sense probe. The control glyceraldehyde-3-phosphate dehydrogenase (GAPD) RNA probe was synthesized using a construct containing 163 bp AccI-Sau3AI fragment of

the rat GAPD cDNA. RNase protection assay of 10 μg total RNA with these probes was performed as described by Baarends *et al.* (1994).

Morphology and histological analysis

Transgenic and zinc-exposed transgenic mice of lines 14 and 79 were analyzed at 1 month of age for general functional and morphological abnormalities. For histological analysis, fetuses were isolated from pregnant control and transgenic mice at E13, E15, E17 and E19. Vaginal plug detection was considered day 0 (E0) and pregnant mice were given drinking water with or without 25 mM $ZnSO_4$ from E0 onwards. Fetuses were fixed overnight in Bouin's fixative, embedded in paraffin, sectioned transversally at 7 μm and stained with hematoxylin/eosin.

Results

Generation of MT-ΔAMHRII transgenic mice and analysis of expression

The MT-ΔAMHRII transgene, encoding the extracellular and transmembrane domains of the rat AMHRII, was expressed under the control of a mouse MT-1 promoter (Figure 3.1). This promoter has been shown to yield a high expression level of the transgene upon zinc induction in a variety of fetal and adult tissues (Palmiter *et al.*, 1983). Based on the high sequence conservation of transmembrane serine/threonine kinase receptors between species (Ten Dijke *et al.*, 1994a), it is anticipated that the rat ΔAMHRII transgene encodes a functional protein that can compete with the endogenous AMHRII for AMH binding.

Five founder transgenic mice were generated by pronuclear injection of fertilized mouse eggs (Figure 3.2). All founder mice were males, of which line 82 was infertile. Upon closer examination of founder 82, no anatomical abnormalities were observed, and sperm was found in the epididymides. However, no vaginal plugs were found in females that were mated with founder 82.

PCR and Southern blot analysis of genomic DNA from offspring from the other four founder mice demonstrated that all four lines transmitted the transgene. However, with breeding of lines 81 and 92 the transgene was transmitted only to male offspring, suggesting that the transgene had

integrated on the Y chromosome. As expected, the integrated transgene copy number was different between the various transgenic mouse lines: line 79 had the highest copy number and line 82 the lowest, although the exact copy numbers were not determined (Figure 3.2).

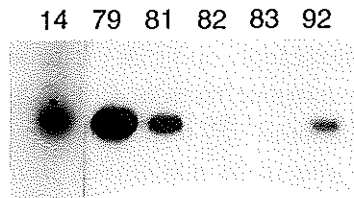

Figure 3.2. Southern blot analysis of transgenic DNA.
Transgenic mice were identified by Southern blot analysis using the 1.8 kb transgene as probe. The approximate order of mouse lines with respect of the number of transgene copies, is as follows: line 79 ≥ line 14 ≥ line 81 ≥ line 92 ≥ line 82. Number 83 represents a non-transgenic littermate.

RNase protection assay was performed to analyze the expression of the MT-ΔAMHRII transgene in both reproductive and nonreproductive tissues of lines 14 and 79. In both mouse lines, the transgene is expressed in several tissues. In line 14, zinc-induced mRNA expression was observed in ovary, uterus, prostate, colon and muscle, whereas testis expressed a substantial level of the MT-ΔAMHRII transgene mRNA without zinc induction (Figure 3.3). Also in the testis of line 79, a high expression level of the transgene, which could not be increased upon zinc induction, was found. Zinc-induced expression of the transgene was found in the ovary and kidney of line 79 (Figure 3.3).

Morphological and histological analysis of MT-ΔAMHRII transgenic mice
In *AMH* or *AMHRII* knockout mice, disruption of the AMH signal leads to persistence of Müllerian duct derivatives in males (Behringer *et al.*, 1994; Mishina *et al.*, 1996). However, upon macroscopic inspection, adult transgenic mice of lines 14 and 79 did not display anatomically abnormal urogenital and gonadal development. Furthermore, no derivatives of

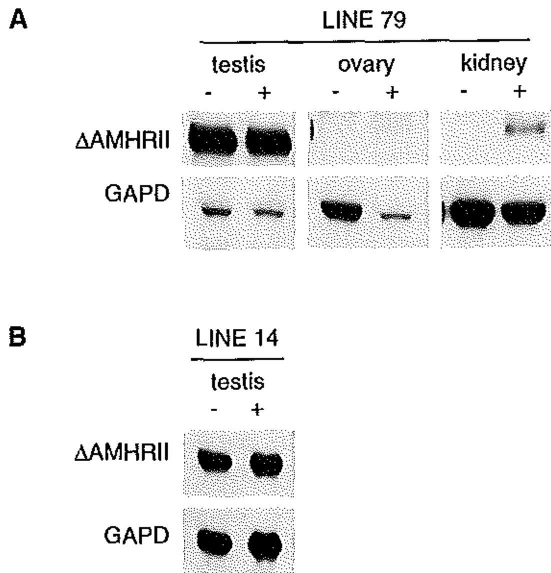

Figure 3.3. **Expression of the MT-ΔAMHRII transgene.**

A. Tissue distribution of MT-ΔAMHRII transgene expression (line 79) in the absence (-) or presence (+) of $ZnSO_4$ in the drinking water.

B. Testicular expression of MT-ΔAMHRII (line 14) was not increased upon zinc induction.

Müllerian origin were observed in any of the male transgenic mice. In addition, the transgenic male mice that were exposed to zinc, starting prenatally at E0, developed normally, and no Müllerian duct remnants were observed after birth.

Male fetuses were examined in more detail during the critical period of Müllerian duct regression at E13 and E15. However, histological examination of sections did not reveal any difference in Müllerian duct regression between control and zinc-exposed transgenic animals (Figure 3.4A-D), and no Müllerian duct remnants were found at E19 (Figure 3.4E/F). The testes and ovaries of zinc-exposed transgenic mice, and the gonad-associated structures, did not show any anatomical abnormalities (results not shown).

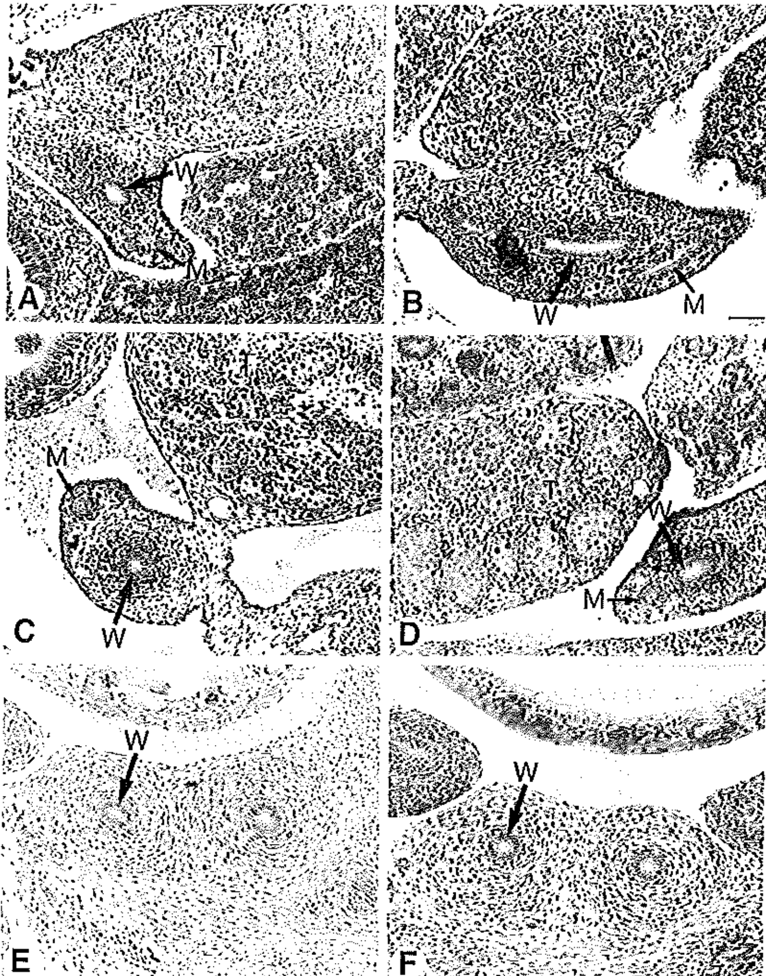

Figure 3.4. Histology of Müllerian duct regression in MT-ΔAMHRII transgenic male fetuses

Müllerian duct regression was studied in the absence (left panel) or presence (right panel) of $ZnSO_4$ in the drinking water of the mothers during pregnancy. No difference in Müllerian duct regression was observed between control and zinc-exposed transgenic fetuses at E13 (A and B) and at E15 (C and D). The expression of MT-ΔAMHRII did not result in the presence of Müllerian duct remnants at E19 (E and F).

Discussion

Members of the TGFβ family are believed to exert their actions through a serine/threonine receptor complex consisting of a type I and a type II receptor. The type I receptor induces the downstream signaling, which was demonstrated with the use of a constitutively active type I receptor that can signal in the absence of ligand and type II receptor (Wieser *et al.*, 1995). However, normally the kinase activities of both receptors are important for signal transduction. A kinase-deficient type I receptor can be phosphorylated by the type II receptor but is unable to activate downstream factors. The kinase activity of the type II receptor is not required for ligand binding or receptor complex formation, but it is essential for activation of the type I receptor (Wrana *et al.*, 1994), indicating a primary role for the type II receptor in signal transduction. Overexpression of kinase-deficient activin type II (ActRII) receptors or TGFβ type II (TβRII) receptors inhibits ligand-induced 3TPlux activity (Tsuchida *et al.*, 1995; Attisano *et al.*, 1996). Furthermore, a truncated TβRII that lacks the serine/threonine kinase domain acts as a dominant-negative receptor, since it blocks TGFβ signaling in neonatal cardiac myocytes (Brand *et al.*, 1993; Filvaroff *et al.*, 1994). Truncated type II receptors also function as a dominant-negative receptor *in vivo*. Expression of truncated bone morphogenetic protein type II (BMPRII) receptors or ActRII in *Xenopus* embryos inhibits mesoderm formation (Hemmati-Brivanlou and Melton, 1992; Ishikawa *et al.*, 1995). In addition, introduction of a dominant-negative TβRII under the control of the MT-1 promoter in mice inhibits TGFβ signaling (Bottinger *et al.*, 1997a; Bottinger *et al.*, 1997b). Primary hepatocytes and pancreatic acinar cell cultures generated from these transgenic mice have lost growth inhibition in response to TGFβ (Bottinger *et al.*, 1997b).

Although truncated type II receptors, with deficient kinase activity, have been shown to function as dominant-negative receptors *in vitro* as well as *in vivo*, no inhibition of AMH signaling was observed in the MT-ΔAMHRII transgenic mice generated in the present study. The absence of an AMH-deficient phenotype, such as partial PMDS, in MT-ΔAMHRII transgenic mice, may be due to a low expression level of the transgene. Exposure to zinc resulted in an increased mRNA expression of the transgene in several

tissues, but the level of transgenic mRNA expression was lower than the mRNA expression of the endogenous *AMHRII* gene in adult gonads (results not shown). The expression of the transgene may be limited by several factors. The chromosomal integration site and the copy number of the transgene may vary, and as a result, different lines of the same transgene can display different levels of expression (Simoni, 1994). Furthermore, it has been suggested that expression of a transgene is higher, when the construct contains the genomic exon and intron structure (Simoni, 1994), although in transgenic experiments that used cDNA sequences, the expression of the transgene was not hampered (Murakami *et al.*, 1993; Davis *et al.*, 1994; Zsigmond *et al.*, 1994). In the present study, cDNA encoding the truncated rat ΔAMHRII under the control of the mMT-1 promoter was used to generate the transgenic construct. The mMT-1 promoter has been used in several transgenic studies, although most studies made use of a 1.7 kb mMT-1 promoter fragment, whereas we used a 260 bp fragment (Wang *et al.*, 1994; Zsigmond *et al.*, 1994; Bottinger *et al.*, 1997b; Guo *et al.*, 1998). This small fragment contains the five potential zinc responsive regulatory elements. However, it cannot be excluded that additional 5' regulatory elements might be necessary to obtain a high expression level of the transgene (Stuart *et al.*, 1984). Bottinger *et al.* (1997b) generated ΔTGFβRII transgenic mice using the larger 1.7 kb mMT-I promoter fragment and 5' and 3' MT locus control regions. These mice demonstrated a high mRNA expression level of the transgene also without zinc induction (Bottinger *et al.*, 1997b).

In the testis of the MT-ΔAMHRII mice, but not in most other tissues, a substantial level of mRNA expression of the transgene was measured, and this expression was not increased further upon zinc induction. This lack of zinc induction of the transgene could be due to a high endogenous zinc level in the testis, which may result in maximal stimulation of the transgene. Zinc plays an important role in testicular function, since zinc deficiency leads to degeneration of the seminiferous epithelium and spermatogenic arrest (Apgar, 1985). Furthermore, it has been reported that the endogenous *MT-1* gene is constitutively transcribed in male germ cells (De *et al.*, 1991), probably as a result of the high endogenous zinc level. It may well be possible that also the MT-1-ΔAMHRII transgene is constitutively

transcribed in the testis. Therefore, the use of the MT-1 promoter to generate an inducible transgene to study testicular function, has to be reconsidered.

An additional reason for the overall low extragonadal expression and high testicular expression of the transgene, may be the methylation status of the transgene. One of the mechanisms to silence gene expression, is methylation of the promoter region of the respective gene (Chomet, 1991). In a study by Salehi-Ashtiani *et al.* (1993), it was shown that expression of a transgene driven by the mouse MT-1 promoter was repressed in all somatic tissues, even when heavy metals were administered, whereas the transgene was transcribed in the testis. Examination of the transgene methylation status revealed that expression was inversely correlated with hypermethylation of the locus. The promoter region was fully methylated in somatic tissues, but was undermethylated in the testis (Salehi-Ashtiani *et al.*, 1993). However, whether the methylation status of the MT-ΔAMHRII transgene plays a role in the regulation of its expression in the present experiments, remains to be investigated.

While the present study was ongoing, *AMHRII* knockout mice were generated (Mishina *et al.*, 1996). The phenotype of the AMHRII-deficient mice appears to be an exact phenocopy of the AMH-deficient mice, suggesting that AMHRII is the only type II receptor for AMH (Mishina *et al.*, 1996). AMH- and AMHRII-deficient male mice become infertile, but this is probably due to obstruction of the genital tract caused by the presence of Müllerian duct derivates. Furthermore, the testes of several of the AMH- and AMHRII-deficient mice developed Leydig cell hyperplasia (Behringer *et al.*, 1994; Mishina *et al.*, 1996), suggesting a role for AMH in Leydig cell function, probably through Sertoli cell-Leydig cell interaction. Similar to the AMH-deficient mice, thus far no histological abnormalities of the ovaries have been found in AMHRII-deficient mice (Mishina *et al.*, 1996).

The intrinsically high testicular expression of transgenic MT-1 constructs interferes with studies on the effect of transgene-encoded proteins, using the *MT-1* promoter in testis development. Hence, the recently developed technique of tissue- and developmental stage-specific inactivation of genes (conditional knockout mice), using the Cre-LoxP system, may prove to be more suitable (Gu *et al.*, 1994; Zou *et al.*, 1994;

Porter, 1998). To generate gonadal AMHRII-deficient mice, the promoter of the follicle-stimulating hormone receptor could be used to control Cre activity.

Chapter Four

Structure and chromosomal localization of the human anti-Müllerian hormone type II receptor gene

Chapter Four

Structure and chromosomal localization of the human anti-Müllerian hormone type II receptor gene

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Abstract

Using the rat anti-Müllerian hormone type II receptor (AMHRII) cDNA as a probe, two overlapping lambda phage clones containing the AMHRII gene were isolated from a human genomic library. Sequence analysis of the exons was performed and the exon/intron boundaries were determined. The coding region was found to consist of 11 exons, divided over 8 kb. The genomic structure resembles that of the related activin type II receptor gene. The AMHRII gene was mapped to human chromosome 12q12-q13. The results reported are essential for identification of AMHRII gene alterations in patients with persistent Müllerian duct syndrome.

Introduction

Anti-Müllerian hormone (AMH) is a member of the TGFβ family of growth and differentiation factors (Cate *et al.*, 1986). AMH plays a critical role during male fetal sex differentiation, by inducing regression of the Müllerian ducts, the anlagen of the female urogenital tract (Jost, 1947; Josso *et al.*, 1993; Lee and Donahoe, 1993). During fetal life, AMH is produced only by the testicular Sertoli cells, but postnatally this factor is also found in ovarian granulosa cells (Hirobe *et al.*, 1992).

Previous studies have shown that members of the TGFβ family exert their action via a heteromeric signaling complex that includes a type I and a type II receptor (Wrana *et al.*, 1994). Both receptors are transmembrane serine/threonine kinases. The type I receptors constitute a smaller subfamily, that is characterized by a specific cysteine spacing in the extracellular domain and a GS-rich juxtamembrane motif (Attisano *et al.*, 1994).

Recently, the rat (Baarends *et al.*, 1994) and the rabbit (Di Clemente *et al.*, 1994b) AMH type II receptor (AMHRII) cDNAs were cloned, as new members of the serine/threonine kinase receptor family. AMHRII mRNA is expressed in the mesenchymal cells surrounding the Müllerian ducts, during the period of fetal development that the Müllerian ducts respond to AMH (Baarends *et al.*, 1994). Furthermore, COS cells transfected with AMHRII cDNA are able to bind AMH (Di Clemente *et al.*, 1994b). AMHRII mRNA was also found in fetal gonads, and in Sertoli cells and granulosa cells of adult rats, although the

function of AMH in the gonads remains to be established (Baarends *et al.*, 1995a; Baarends *et al.*, 1995b).

Failure of AMH action leads to the Persistent Müllerian Duct Syndrome (PMDS), a rare form of male pseudohermaphroditism characterized by the presence of uterus and fallopian tubes in otherwise normally virilized males (Josso *et al.*, 1991). In some cases, this phenotype is caused by a mutation in the AMH gene (Knebelmann *et al.*, 1991). However, the presence of bioactive AMH in other PMDS patients (Guerrier *et al.*, 1989) indicates resistance of the Müllerian ducts to AMH, possibly as a result of alterations in the AMHRII gene. A mutation in the AMHRII gene that may result in alternative splicing has recently been reported (Imbeaud *et al.*, 1995).

Herein, we present the genomic structure of the human AMHRII gene and its chromosomal localization.

Materials and Methods

Library screening

Approximately 1.7 x 10^6 plaques of a phage lambda EMBL3 human CMLO (chronic myeloid leukemia cell line) genomic DNA library were transferred in duplicate to Hybond-N^+ nylon filters (Amersham International plc, Little Chalfont, UK), and screened with the full length 1.9 kb rat AMHRII cDNA as a probe (Baarends *et al.*, 1994). The probe was labeled with [^{32}P]-dATP (Amersham) by random-priming. Hybridization was done o/n at 42 C in 50% formamide, 5xSCC, 5x Denhardt's, 1% SDS, 50 mM Na_2HPO_4/NaH_2PO_4 (pH6.8) and 100 µg/ml denatured herring sperm DNA. The filters were washed in 2x SCC and 0.1% SDS at 42 C for 10 minutes prior to autoradiography.

Positive phage lambda clones

Four positive clones were identified on initial screening of the genomic library. These clones were purified to homogeneity by secondary and tertiary screening using the rat AMHRII cDNA probe. Phage DNA of 2 positive clones was isolated, digested with several restriction enzymes and analyzed by gel electrophoresis to orient the genomic DNA with respect to the lambda arms. The DNA was transferred to Hybond-N^+ filters (Amersham) and probed with fragments of the rat AMHRII cDNA to determine the positions of the 5' and 3'

ends of the gene, the overlap, and the coding regions. Fragments of the coding regions were subcloned into the appropriate sites of the vector pBluescript KS(+) (Stratagene, La Jolla, CA, U.S.A.) for sequence analysis.

DNA sequence analysis

DNA sequencing was performed on plasmid DNA by the dideoxy chain termination method, using M13 and human AMHRII primers. Sequence alignments were performed using the sequence analysis program DNAMAN (Lynnon Biosoft, Vaudrieul, Quebec, Canada) and Clustal V.

In situ hybridization to metaphase chromosomes

Phage DNA was labeled with biotin-14-dATP using the bio-NICK system (Life Technologies, Gaithersburg, MD, U.S.A.). The labeled DNA was precipitated with ethanol in the presence of herring sperm DNA. Precipitated DNA was dissolved and denatured at 80 C for 10 min followed by incubation for 30 min at 37 C, and added to heat-denatured chromosome spreads where hybridization was carried out o/n in a moist chamber at 37 C. After posthybridization washing (50% formamide, 2 x SSC, at 42 C) and blocking with nonfat dry milk powder, the hybridized probe was detected using avidin-FITC (Vector Laboratories, Burlingame, CA, U.S.A.) with two amplification steps using rabbit-anti-FITC (Dako, Glostrup, Denmark) and mouse-anti-rabbit FITC (Jackson Immunoresearch, West Grove, U.S.A.). Chromosome spreads were mounted in antifade solution with blue dye DAPI.

Results and Discussion

Isolation and characterization of the human AMHRII gene

A human CMLO genomic library was screened with the rat AMHRII cDNA as a probe. Four positive clones were identified and two of these clones were used to construct a restriction enzyme map of the human AMHRII gene. Restriction enzyme mapping, Southern blot analysis using specific regions of the rat AMHRII cDNA as a probe, and DNA sequencing revealed that the AMHRII gene consists of 11 exons and 10 introns, and spans approximately 8 kb (Figure 4.1).

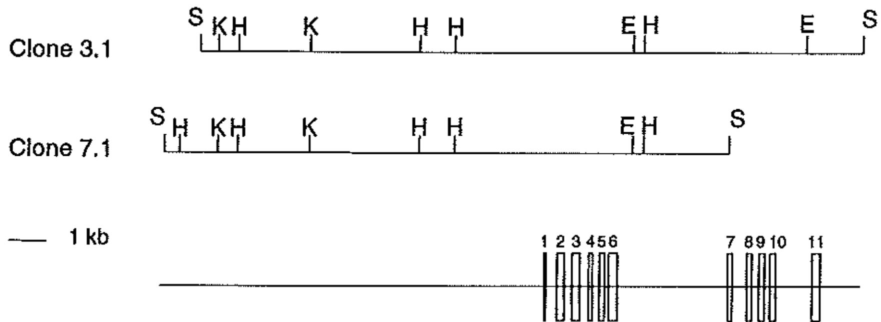

Figure 4.1. Structure of the human AMHRII gene.
The overlapping phage lambda clones 3.1 and 7.1 are shown with the unique restriction enzymes that were used to construct a linear map. The relative length and localization of the exons (boxes) and introns are shown underneath (drawn to scale). E: EcoRI; H: HindIII; K: KpnI; S: SalI. The SalI sites are contained in the lambda EMBL3 arms and do not occur in the human AMHRII gene.

All eleven exons of the human AMHRII gene, and the intron/exon boundaries were sequenced. The DNA and protein sequences of the deduced open reading frame and the DNA sequences of the intron-exon boundaries are shown in Figure 4.2. Exons 2-10 vary in length from 78 bp to 234 bp, and the coding regions of the first (exon 1) and last (exon 11) exons are 49 bp and 297 bp, respectively. These lengths are consistent with the observation that the length of coding exons usually does not exceed 300 bp (Robberson *et al.*, 1990). Introns 4 and 10 are relatively long, 3 kb and 1 kb, respectively, and contribute to approximately half of the gene. The other introns are much shorter, varying from 120 bp to 425 bp. All of the introns start with GT and end with AG, consistent with the donor/acceptor splice rule (Balvay *et al.*, 1993).

Comparison of the deduced human AMHRII amino acid sequence to the sequences of the rat and rabbit receptors, revealed well-conserved sequences with few amino acid changes (Figure 4.3). The amino acid identity is approximately 80 %. In the AMH receptor the Ser^{335} in the sequence HRDLS is conserved in the three mammalian species investigated (Figure 4.3; see also Grootegoed *et al.*, 1994). This feature distinguishes the AMH type II

A

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                                                                                                   exon 1 <> exon 2
GGCTTATGCTCTTCTCCTTCTGCTGCTGCCATCCTCCAGCAAGATGCTAGGGTCTTTGGGGCTTTGGGCATTACTTCCCACAGCTGTGGAAGCACCCCCAAACAGGCGAACCTGTGTGTT 120
                                             M  L  G  S  L  G  L  W  A  L  L  P  T  A  V  E  A  P  P  N  R  R  T  C  V  F 26

CTTTGAGGCCCCTGGAGTGCGGGGAAGCACAAAGACACTGGGAGAGCTGCTAGATACAGGCACAGAGCTCCCCAGAGCTATCCGCTGCCTCTACAGCCGCTGCTGCTTTGGGATCTGGAA 240
 F  E  A  P  G  V  R  G  S  T  K  T  L  G  E  L  L  D  T  G  T  E  L  P  R  A  I  R  C  L  Y  S  R  C  C  F  G  I  W  N 66
                         exon 2 <> exon 3
CCTGACCCAAGACCGGGCACAGGTGGAAATGCAAGGATGCCGAGACAGTGATGAGCCAGGCTGTGAGTCCCTCCACTGTGACCCAAGTCCCCGAGCCCACCCCAGCCCTGGCTCCACTCT 360
 L  T  Q  D  R  A  Q  V  E  M  Q  G  C  R  D  S  D  E  P  G  C  E  S  L  H  C  D  P  S  P  R  A  H  P  S  P  G  S  T  L 106
                                                                                                            exon3 <> exon4
CTTCACCTGCTCCTGTGGCACTGACTTCTGCAATGCCAATTACAGCCATCTGCCTCCTCCAGGGAGCCCTGGGACTCCTGGCTCCCAGGGTCCCCAGGCTGCCCCAGGTGAGTCCATCTG 480
 F  T  C  S  C  G  T  D  F  C  N  A  N  Y  S  H  L  P  P  P  G  S  P  G  T  P  G  S  Q  G  P  Q  A  A  P  G  E  S  I  W 146
                                                   exon 4 <> exon 5
GATGGCACTGGTGCTGCTGGGGCTGTTCCTCCTCCTCCTGCTCGTGCTGGGCAGCATCATCTTGGCCCTGCTACAGCGAAAGAACTACAGAGTGCGAGGTGAGCCAGTGCCAGAGCCAAG 600
 M  A  L  V  L  L  G  L  F  L  L  L  L  L  V  L  G  S  I  I  L  A  L  L  Q  R  K  N  Y  R  V  R  G  E  P  V  P  E  P  R 186
                                                  exon 5 <> exon 6
GCCAGACTCAGGCAGGGACTGGAGTGTGGAGCTGCAGGAGCTGCCTGAGCTGTGTTTCTCCCAGGTAATCCGGGAAGGAGGTCATGCAGTGGTTTGGGCCGGGCAGCTGCAAGGAAAACT 720
 P  D  S  G  R  D  W  S  V  E  L  Q  E  L  P  E  L  C  F  S  Q  V  I  R  E  G  G  H  A  V  V  W  A  G  Q  L  Q  G  K  L 226

GGTTGCCATCAAGGCCTTCCCACCGAGGTCTGTGGCTCAGTTCCAAGCTGAGAGAGCATTGTACGAACTTCCAGGCCTACAGCACGACCACATTGTCCGATTTATCACTGCCAGCCGGGG 840
 V  A  I  K  A  F  P  P  R  S  V  A  Q  F  Q  A  E  R  A  L  Y  E  L  P  G  L  Q  H  D  H  I  V  R  F  I  T  A  S  R  G 266
                                              exon 6 <> exon 7
GGGTCCTGGCCGCCTGCTCTCTGGGCCCCTGCTGGTACTGGAACTGCATCCCAAGGGCTCCCTGTGCCACTACTTGACCCAGTACACCAGTGACTGGGGAAGTTCCCTGCGGATGGCACT 960
 G  P  G  R  L  L  S  G  P  L  L  V  L  E  L  H  P  K  G  S  L  C  H  Y  L  T  Q  Y  T  S  D  W  G  S  S  L  R  M  A  L 306
                                          exon 7 <> exon 8
GTCCCTCGCCCAGGGCCTGGCATTTCTCCATGAGGAGCGCTGGCAGAATGGCCAATATAAACCAGGTATTGCCCACCGAGATCTGAGCAGCCAGAATGTGCTCATTCGGGAAGATGGATC 1080
 S  L  A  Q  G  L  A  F  L  H  E  E  R  W  Q  N  G  Q  Y  K  P  G  I  A  H  R  D  L  S  S  Q  N  V  L  I  R  E  D  G  S 346
                                                                                                  exon 8 <> exon 9
GTGTGCCATTGGAGACCTGGGCCTTGCCTTGGTGCTCCCTGGCCTCACTCAGCCCCCTGCCTGGACCCCTACTCAACCTCAAGGCCCAGCTGCCATCATGGAAGCTGGCACCCAGAGGTA 1200
 C  A  I  G  D  L  G  L  A  L  V  L  P  G  L  T  Q  P  P  A  W  T  P  T  Q  P  Q  G  P  A  A  I  M  E  A  G  T  Q  R  Y 386

CATGGCACCAGAGCTCTTGGACAAGACTCTGGACCTACAGGATTGGGGCATGGCCCTCCGACGAGCTGATATTTACTCTTTGGCTCTGCTCCTGTGGGAGATACTGAGCCGCTGCCCAGA 1320
 M  A  P  E  L  L  D  K  T  L  D  L  Q  D  W  G  M  A  L  R  R  A  D  I  Y  S  L  A  L  L  L  W  E  I  L  S  R  C  P  D 426
  exon 9 <> exon 10
TTTGAGGCCTGACAGCAGTCCACCACCCTTCCAACTGGCCTATGAGGCAGAACTGGGCAATACCCCTACCTCTGATGAGCTATGGGCCTTGGCAGTGCAGGAGAGGAGGCGTCCCTACAT 1440
 L  R  P  D  S  S  P  P  P  F  Q  L  A  Y  E  A  E  L  G  N  T  P  T  S  D  E  L  W  A  L  A  V  Q  E  R  R  R  P  Y  I 466
              exon 10 <> exon 11
CCCATCCACCTGGCGCTGCTTTGCCACAGACCCTGATGGGCTGAGGGAGCTCCTAGAAGACTGTTGGGATGCAGACCCAGAAGCACGGCTGACAGCTGAGTGTGTACAGCAGCGCCTGGC 1560
 P  S  T  W  R  C  F  A  T  D  P  D  G  L  R  E  L  L  E  D  C  W  D  A  D  P  E  A  R  L  T  A  E  C  V  Q  Q  R  L  A 506

TGCCTTGGCCCATCCTCAAGAGAGCCACCCCTTTCCAGAGAGCTGTCCACGTGGCTGCCCACCTCTCTGCCCAGAAGACTGTACTTCAATTCCTGCCCCTACCATTCTCCCCTGTAGGCC 1680
 A  L  A  H  P  Q  E  S  H  P  F  P  E  S  C  P  R  G  C  P  P  L  C  P  E  D  C  T  S  I  P  A  P  T  I  L  P  C  R  P 546

TCAGCGGAGTGCCTGCCACTTCAGCGTTCAGCAAGGCCCTTGTTCCAGGAATCCTCAGCCTGCCTGTACCCTTTCTCCTGTGTAAATATGCAGTTTATATCAGTTCTGACCAGTGACTT  1799
 Q  R  S  A  C  H  F  S  V  Q  Q  G  P  C  S  R  N  P  Q  P  A  C  T  L  S  P  V  *                                     573
```

human AMHRII gene

B

EXON	(SIZE)	EXON 3'	INTRON	(SIZE)	EXON 5'	EXON
exon 1	(>49 bp)	GTG GAA G V E	gtaagt...intron 1	(303 bp)...tgggcctcag	CA CCC CCA A P P	exon 2
exon 2	(183 bp)	ATG CAA G M Q	gtgaat...intron 2	(215 bp)...catccatcag	GA GCC CGA G C R	exon 3
exon 3	(193 bp)	GCC CCA G A P	gtagcc...intron 3	(264 bp)...tgatgtccag	GT GAG TCC G E S	exon 4
exon 4	(78 bp)	ATC TTG G I L	gtacta...intron 4	(212 bp)...tctgttccag	CC CTG CTA A L L	exon 5
exon 5	(119 bp)	TTC TCC CAG F S Q	gtgccc...intron 5	(120 bp)...tccccagcag	GTA ATC CGG V I R	exon 6
exon 6	(234 bp)	CAT CCC AAG H P K	gtgagc...intron 6	(~ 3 kb)...gtttccccag	GGC TCC CTG G S L	exon 7
exon 7	(115 bp)	CAG AAT G Q N	gtgggt...intron 7	(425 bp)...tcccccacag	GC CAA TAT G Q Y	exon 8
exon 8	(178 bp)	ATC ATG GAA I M E	gtgagt...intron 8	(204 bp)...tgctctccag	GCT GGC ACC A G T	exon 9
exon 9	(148 bp)	AGG CCT G R P	gtaagg...intron 9	(165 bp)...cttcctccag	AC AGC AGT D S S	exon 10
exon 10	(137 bp)	TTT GCC ACA F A T	gtaaga...intron 10	(~ 1 kb)...ttccccccag	GAC CCT GAT D P D	exon 11
exon 11	(>297 bp)					

Figure 4.2. **Human AMHRII gene**

A. Nucleotide and amino acid sequence of the deduced open reading frame. The arrowheads indicate the beginning (>) and end (<) of the respective exon.

B. Intron/exon boundaries in the human AMHRII gene. The nucleotide sequence for each intron/exon boundary and the size of each exon and intron are shown. The consensus acceptor/donor sequences are shown in bold.

receptor from the other members of the TGFβ receptor family, which contain a lysine residue at that position. Another conspicuous characteristic of the AMHRII is the cysteine spacing in the extracellular ligand binding domain, which may reflect the evolutionary distance of the AMH-AMHRII pair to the other ligand-serine/threonine kinase receptor pairs.

Similar to the activin type II receptor (ActRII) (Matzuk and Bradley, 1992) and the bone morphogenetic protein 2/4 type I receptor (BMPRI) (Mishina *et al.*, 1995) gene, the human AMHRII gene contains 11 exons. However, the AMHRII gene is remarkably small: 8 kb compared to >60 kb for the ActRII gene and 38.2 kb for the BMPRI gene. The different exons encode separate functional domains in the AMHRII protein molecule. The signal sequence is encoded by exon 1, the extracellular ligand binding domain by exons 2 and 3, and the transmembrane domain is encoded by exon 4. The remaining exons 5-11 contain the sequence information for the kinase domain. When the gene structure of the receptors of the TGFβ family is compared, the spacing of the exons and even the division of the functional protein domains into different numbers of exons indicate that the receptors show distant evolutionary relationship. Thus, this comparison provides no information on similarities in mechanism of action of the different serine/threonine kinase receptors.

Chromosomal localization of the AMHRII gene

Fluorescent *in situ* hybridization to metaphase chromosomes using the two isolated lambda phages, showed fluorescent signals at 12q12-13 (Figure 4.4). One of the clones (clone 3.1) also showed a signal at 9q, but this was not observed with clone 7.1 (not shown). Other genes in the 12q12/13 region include the Wingless-type MMTV integration site 1 gene (Wnt1), the Keratin 3 gene (Krt3), the retinoic acid receptor-γ gene (Rarg) and the Homeobox C cluster genes (Hoxc) (Online Mendalian Inheritance in Man). In the mouse, we mapped the mouse AMHRII gene to chromosome 15F (result not shown). This region (15F(50-58)) is syntenic with the human chromosome 12, and contains mouse homologues of the genes mentioned above (Mouse Genome, 1994).

In conclusion, we have cloned and sequenced the human gene encoding the AMHRII. The AMHRII is well conserved between mammalian species. This information is essential to identify possible gene alterations in diseases involving the Müllerian ducts.

```
hAMHRII     1   MLGSLGLWALLPTAVEAPPNRRTCVFFEAPGCRGSTKTLGELLDTGTELPRAIRCLYSRC
ocAMHRII    1   MLGTLGLWALLPAAVQAPPNRRTCVFFEAPGVRGSTKTLGELLDAGPGPPRVIRCLYSRC
rAMHRII     1   MLGTLGLWTLLPAAAQVSPNRRTCVFFEAPGVRGSTKTLGEVVDAGPGPPKGIRCLYSHC

hAMHRII    61   CFGIWNLTQDRAQVEMQGCRDSDEPGCESLHCDPSPRAHPSPGSTLFTCSCGTDFCNANY
ocAMHRII   61   CFGIWNLTRDQAQVEMQGCRDSDEPGCESLSCDPSPRARASSGSTLFTCSCGADFCNANY
rAMHRII    61   CFGIWNLTHGRAQVEMQGCLDSDEPGCESLHCDPVPRAHPSPSSTLFTCSCGTDFCNANY

hAMHRII   121   SHLPPPGSPGTPGSQGPQAAPGESIWMALVLLGLFLLLLLVLGSIILALLQRKNYRVRGE
ocAMHRII  121   SHLPPLGGPGTPGPQGPQAAPGESPWMALALLGLVLLLLLLLGGIVVALLQRKAYRVQSG
rAMHRII   121   SHLPPSGNRGAPGPQEPQATPGGPIWMAQLLLGVFLVLLLSI--IILALLQRKACRVQGG

hAMHRII   181   PVPEP PDSGRDWSVELQELPELCFSQVIREGGHAVVWAGQLQGKLVAIKAFPPRSVAQF
ocAMHRII  181   --PEPEPDSGRDCSEELPELPQLCFSQVIREGGHAAVWAGQLQGELVAIKVFPRRAVAQF
rAMHRII   179   SDPEPEPGSGGDCSEELPELAELRFSQVIQEGGHAVVWAGRLQGEMVAIKAFPPRAVAQF

hAMHRII   241   QAERALYELPGLQHDHIVRFITASRGGPGRLLSGPLLVLELHPKGSLCHYLTQYTSDWGS
ocAMHRII  239   RAERALYELPGLQHNHVVRFIAAGQGGPGPLPSGPLLVLELHPKGSLCQYLSQHTSDWGS
rAMHRII   239   RAERAVYQLLGLQHNHIVRFITAGQGGPGPLPSGPLLVLELYPKGSLCHYLTQYTSDWGS

hAMHRII   301   SLRMALSLAQGLAFLHEERWQNGQYKPGIAHRDLSSQNVLIREDGSCAIGDLGLALVLPG
ocAMHRII  299   SLRMALSLAQGLAFLHEERWQDGQYKPGIAHRDLSSQNVLIREDGSCAIGDLGLALVLPG
rAMHRII   299   SLRMALSLAEGLAFLHGERWQDGQYKPGIAHRDLSSQNVLIREDRSCAIGDLGLALVLPG

hAMHRII   361   LTQPPAWTPTQPQGPAAIMEAGTQRYMAPELLDKTLDLQDWGMALRRADIYSLALLLWEI
ocAMHRII  359   FAQPRAWAPPQPRGPAAIMEAGTQRYMAPELLDKSLDLQDWGTALRRADVYSLALLLWEI
rAMHRII   359   LAQPPALAPTQPRGPAAILEAGTQRYMAPELLDKTLDLQDWGTALQRADVYSLALLLWEI

hAMHRII   421   LSRCPDLRPDSSPPPFQLAYEAELGNTPTSDELWALAVQERRRPYIPSTWRCFATDPDGL
ocAMHRII  419   LSRCPDLRPDGRPPPFQLAYEAELGSAPTTCELWALAVEERRRPDIPSSWCCFATDPGGL
rAMHRII   419   LSRCSDLRPDHRPPPFQLAYEAELGSNPSACELWALAVAERKRPNIPSSWSCSATDPRGL

hAMHRII   481   RELLEDCWDADPEARLTAECVQQRLAALAHPQESHPPPESCPRGCPPLCPED--CTSIPA
ocAMHRII  479   RELLEDCWDADPEARLTAECVQQRLVALVHPQEAQP----CPEGRPHSHPEDWPPAPAPA
rAMHRII   479   RELLEDCWDADPEARLTAECVQQRLAALAYPQVASSPPESCPQGCPENYP------AAPA

hAMHRII   539   PTILPCRPQRSACHFSVQQGPCSRNPQPACTLSPV
ocAMHRII  535   PALLPGSPQPGACHFGVQQGLCSRNPGAACASSDV
rAMHRII   533   SAAFPCRPQQSSCLLSVQQGSGSKS----------
```

Figure 4.3. **Comparison of the human (hAMHRII), the rabbit (ocAMHRII) and the rat (rAMHRII) AMHRII amino acid sequences.** Reversed script is used to highlight amino acid residues that are found at the same position in at least two of the three receptors.

Figure 4.4. Chromosomal localization of the human AMHRII gene.
The photomicrograph shows localization of the AMHRII probe on 12q12-q13 using fluorescence *in situ* hybridization (left panel). The identification of individual chromosomes was deduced from converted DAPI banding patterns (right panel). The hybridization sites are marked by arrows.

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Chapter Five

Effect of prenatal exposure to diethylstilbestrol on Müllerian duct development in fetal male mice

Chapter Five

Effect of prenatal exposure to diethylstilbestrol on Müllerian duct development in fetal male mice

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Abstract

The clinical use of diethylstilbestrol (DES) by pregnant women has resulted in an increased incidence of genital carcinoma in the daughters born from these pregnancies. Also in the so-called DES-sons abnormalities were found, mainly the presence of Müllerian duct remnants, which indicates that fetal exposure to DES may have an effect on male sex differentiation. Fetal regression of the Müllerian ducts is under testicular control through anti-Müllerian hormone (AMH). In male mice, treated *in utero* with DES, the Müllerian ducts do not regress completely, although DES-exposed testes do produce AMH. We hypothesized that incomplete regression in DES-exposed males is caused by a diminished sensitivity of the Müllerian ducts to AMH. Therefore, the effect of DES on temporal aspects of Müllerian duct regression and AMH type II receptor (AMHRII) mRNA expression in male mouse fetuses was studied.

It was observed that Müllerian duct regression was incomplete at E19 (19 days *post coitum*), upon DES administration during pregnancy from E9 through E16. Furthermore, analysis of earlier time points of fetal development revealed that the DES treatment had clearly delayed the onset of Müllerian duct formation by approximately two days; in untreated fetuses Müllerian duct formation was complete by E13, whereas fully formed Müllerian ducts were not observed in DES-treated male fetuses until E15.

Using *in situ* hybridization, no change in the localization of AMH and AMHRII mRNAs expression was observed in DES-exposed male fetuses. The mRNA expression was quantified using RNase protection assay, showing an increased expression level of AMH and AMHRII mRNAs at E13 in DES-exposed male fetuses. Furthermore, the mRNA expression levels of Hoxa 11 and Steroidogenic Factor 1 (SF-1) were determined as a marker for fetal development. Prenatal DES exposure had no effect on Hoxa 11 mRNA expression, indicating that DES did not exert an overall effect on the rate of fetal development. In DES-exposed male fetuses, SF-1 showed a similar increase in mRNA expression as AMH, in agreement with the observations that the *AMH* gene promoter requires an intact SF-1 DNA binding site for time- and cell-specific expression, although an effect of DES on SF-1 expression in other tissues such as the adrenal and pituitary

gland cannot be excluded. However, the increased expression levels of AMH and AMHRII mRNAs do not directly explain the decreased sensitivity of the Müllerian ducts to AMH. Therefore, it is concluded that prenatal DES exposure of male mice delays the onset of Müllerian duct development, which may result in an asynchrony in the timing of Müllerian duct formation with respect to the critical period of Müllerian duct regression, leading to persistence of Müllerian duct remnants in male mice.

Introduction

Anti-Müllerian hormone (AMH), a member of the transforming growth factor β (TGFβ)-superfamily of peptide growth and differentiation factors, is the earliest protein known to be secreted by the fetal Sertoli cells (Cate *et al.*, 1986; Massagué, 1990). In contrast to other family members, which have a broad range of functions, AMH has a very specific role during sex differentiation. AMH, which is only produced by the fetal testes and not by the ovaries during fetal development, might play a role in gonadal differentiation, as indicated by the formation of "ovotestes" in female mice overexpressing AMH (Behringer *et al.*, 1990). Most importantly, in the male AMH induces the regression of the Müllerian ducts, which form the anlagen of the uterus, oviducts, and upper part of the vagina. It has been shown that the timing of AMH action on the Müllerian ducts is very critical. In the rat, exposure of female fetuses to AMH after E16 (16 days *post coitum*) does not result in Müllerian duct regression (Münsterberg and Lovell-Badge, 1991; Tsuji *et al.*, 1992).

The cellular and molecular mechanisms by which AMH induces Müllerian duct regression are poorly understood. However, the identification and cloning of the AMH type II receptor (AMHRII) has contributed to the elucidation of this question (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994b). AMHRII mRNA is expressed in the fetal gonads and in the mesenchymal cells located adjacent to the Müllerian duct epithelium, which corresponds to the sites of action of AMH (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994b). Recent results have shown that AMH elicits its effect on the Müllerian duct epithelium via the surrounding

mesenchymal cells, a process which may also involve induction of programmed cell death (Tsuji *et al.*, 1992; Catlin *et al.*, 1997).

AMHRII is a member of the transmembrane serine/threonine kinase receptor family, to which also the TGFβ and activin receptors belong (Ten Dijke *et al.*, 1994a). Members of the TGFβ-superfamily exert their action through a heteromeric signaling complex, consisting of a type I and a type II receptor (Wrana *et al.*, 1994). Failure in AMH action, as a result of a gene mutation leading to either inactive AMH or AMHRII, causes inhibition of Müllerian duct regression, resulting in a rare form of pseudohermaphroditism in man known as persistent Müllerian duct syndrome (PMDS) (Imbeaud *et al.*, 1994; Imbeaud *et al.*, 1995). Gene knockout experiments in mice have confirmed that in the absence of AMH or AMHRII, Müllerian ducts do not regress (Behringer *et al.*, 1994; Mishina *et al.*, 1996).

In chickens, unilateral regression of Müllerian ducts occurs in the female. The left Müllerian duct is retained whereas the right Müllerian duct regresses, due to the fact that, in contrast to mammalian species, AMH is also expressed by the fetal ovary (Hutson *et al.*, 1981). It has been suggested that estrogens protect the left duct from regression. This is supported by the observation that the concentration of estrogen receptor in the left duct is higher than that in the right duct (MacLaughlin *et al.*, 1983). Furthermore, inhibition of estrogen production in female chick fetuses, by treatment with an aromatase inhibitor during egg incubation, resulted in regression of both ducts (Elbrecht and Smith, 1992). Exposure to estrogen during egg incubation prevents Müllerian duct regression in both male and female chick fetuses (Hutson *et al.*, 1982; Doi and Hutson, 1988).

Although it is a large step from chicken to human, it is of interest to compare the data from the experiments with chickens with clinical data. In human, intrauterine exposure to diethylstilbestrol (DES), a potent synthetic estrogen that has been administrated during pregnancy to prevent miscarriages, has led to an increased incidence of reproductive tract abnormalities. The effects of prenatal DES exposure in so-called DES-daughters, such as an increased risk of genital carcinoma, have been well documented (Bornstein *et al.*, 1988). However, also the sons born from DES-controlled pregnancies have an increased incidence of genital tract

abnormalities, including epididymal cysts, cryptorchidism, and the presence of Müllerian duct remnants (Gill *et al.*, 1976; Whitehead and Leiter, 1981). This indicates that DES has an effect on male sex differentiation. To study the prenatal effects of DES on the developing genital tract in an animal model, McLachlan *et al.* (1975) injected DES daily into pregnant mice during the phase of growth and differentiation of the fetal reproductive tract. Observations on the male offspring of these DES-treated mice indicated that the developing reproductive tract of the fetus is sensitive to DES exposure. Hypoplastic testes and Müllerian duct remnants were found (McLachlan *et al.*, 1975; Newbold *et al.*, 1987). It is, however, not clear how DES mediates its inhibitory effect on reproductive tract differentiation.

In a mouse organ culture system, after *in vivo* DES treatment, Newbold *et al.* (1984) studied whether the inhibitory effect of DES on Müllerian duct regression results from suppression of fetal testicular AMH production or a change in responsiveness of the Müllerian ducts to AMH. Control Müllerian ducts regressed normally when cultured in the presence of control testes, whereas DES-exposed Müllerian ducts in the presence of DES-exposed testes did not regress. Combination of control Müllerian ducts and DES-exposed testes resulted in normal regression. However, in the reciprocal combination, DES-exposed ducts and control testes, only partial regression of the Müllerian ducts was observed. These results indicate that DES-exposed testes still produce bioactive AMH, and that the effect of DES is mainly due to a decrease in AMH responsiveness of the Müllerian ducts.

We hypothesized that the change in sensitivity of the Müllerian ducts to AMH may result from an effect of DES on the expression of AMHRII. In this paper we describe the effects of DES exposure of mouse male fetuses on the Müllerian ducts, in particular AMH and AMHRII mRNA expression, during the period of reproductive tract differentiation. As a control for possible effects of DES exposure on general fetal development (Krumlauf, 1994; Hsieh-Li *et al.*, 1995), the expression of Hoxa 11 mRNA was measured. The mRNA expression level for Steroidogenic Factor 1 (SF-1) mRNA, an orphan nuclear receptor essential for the development of steroidogenic tissues (Luo *et al.*, 1994), was measured as a control for possible effects of DES exposure on urogenital ridge development. The results of this study

may contribute to our knowledge about the possible involvement of exposure to exogenous estrogenic compounds in the postulated increased incidence of reproductive tract disorders in wild-life, and perhaps also in humans (Sharpe and Skakkebaek, 1993; Guillette and Guillette, 1996).

Materials and Methods

Animals and treatment

FVB mice were kept under standard animal housing conditions in accordance with NIH Guidelines for the Care and Use of Experimental Animals. Vaginal plug detection was considered day 0 (E0) of pregnancy. Pregnant mice were given daily subcutaneous injections with DES (100 μg/kg body weight) (Janssen Chimica, Beerse, Belgium) dissolved in olive oil, or olive oil alone, on days E9 through E16 of gestation. Pregnant mice were sacrificed by cervical dislocation at E13, E14, E15, E17 or E19 of gestation. Fetuses were isolated and snap frozen in liquid nitrogen and stored at -80 C. Total RNA was isolated using the LiCl/urea method (Auffray and Rougeon, 1980). In addition, fetuses from the same litter were also fixed overnight in 4% paraformaldehyde, embedded in paraffin, and sectioned transversally at 7 μm. PCR reactions using placental genomic DNA (Hogan *et al.*, 1986) were performed as described by Mitchell *et al.* (1991), using primers for the mouse genes *Sbx* and *Sby* (Kay *et al.*, 1991; Mitchell *et al.*, 1991), to determine the sex of the fetuses.

In situ hybridization

A PstI fragment containing bp 1243-1640 of the rat AMHRII cDNA and an NheI fragment containing bp 38-400 of the rat AMH cDNA were subcloned in pBluescript KS (Stratagene, Westburg, Leusden, The Netherlands) and used to generate sense and antisense [^{35}S]-UTP-labeled (Amersham, 's Hertogenbosch, The Netherlands) transcripts *in vitro*. *In situ* hybridization was performed as described by Zeller and Rogers (Zeller and Rogers, 1991) with some modifications (Baarends *et al.*, 1994). Sections were mounted on slides that were coated with 3-aminopropyl-ethoxysaline. After deparaffinization, sections were treated with 0.2 N HCl (20 min), treated with proteinase K (1 μg/ml in 0.2 M Tris pH 7.5, 2 mM $CaCl_2$; incubation for

15 min at 37 C) and postfixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline. After treatment with dithiothreitol and blocking of non-specific binding with 0.1 M triethanolamine, followed by 0.1 M triethanolamine and acetic anhydride, sections were incubated with [^{35}S]-UTP-labeled antisense and sense AMH and AMHRII RNA probes at a final concentration of 5x10^5 cpm/µl. Hybridization was carried out as described previously (Baarends *et al.*, 1994). Sections were exposed at 4 C for 1 week, developed, and counterstained with haematoxylin and mounted.

RNase protection assay

A mouse AMHRII DNA template for *in vitro* transcription was generated by RT-PCR. The RT-PCR reaction was carried out on 100-200 ng total RNA, extracted from 25-day-old mouse testis, using random hexamers. A sample of the reverse transcription reaction product was used in the PCR reaction using the primers 5'GCTCCGGAGCTCTTGGACAAG3' (forward primer) and 5'CAGGCGCTGCTGCACACACTC3' (reverse primer) corresponding to kinase subdomains VIII, IX, and X of the *AMHRII* gene transcript. A 350 bp PCR product was subcloned in pBluescript KS and used to generate [^{32}P]-UTP-labeled anti-sense probe. The AMH RNA probe was obtained using a 430 bp PstI fragment, containing exon 1, of mouse genomic DNA. The SF-1 RNA probe was obtained using a 252 bp HindIII-EcoRI fragment of mouse SF-1 cDNA (Lala *et al.*, 1992). The Hoxa 11 RNA probe was obtained using a 300 bp BamHI-BglII fragment of the mouse Hoxa 11 cDNA (Hsieh-Li *et al.*, 1995). The control glyceraldehyde 3-phosphate dehydrogenase (GAPD) RNA probe was synthesized using a construct containing 163 bp AccI-Sau3AI fragment of the rat GAPD cDNA. RNase protection assays of 50 µg total fetal RNA with these probes were performed as described by Baarends *et al.* (1994). GAPD was used as a control for RNA loading. The relative amount of protected mRNA band was quantified through exposure of the gels to a phosphor screen (Molecular Dynamics, B & L Systems, Zoetermeer, The Netherlands), followed by a calculation of the relative density of the obtained bands using a phospho-imager and Image Quant (Molecular Dynamics) as computer analysis software. The arbitrary units are expressed as the rations after division by the corresponding GAPD values.

Results

Effect of DES exposure on Müllerian duct formation

The development of the Müllerian duct was studied in male fetuses at E13, E15, and E19, at three positions along the axis of the Müllerian ducts (Figures 5.1, 5.2, and 5.3). Position I indicates the most cranial part of the ducts, at the level of the fetal testes. Position II is at the level where the Müllerian and Wolffian ducts cross each other. Position III indicates the caudal part of the Müllerian ducts, near the urogenital sinus.

In control fetuses at E13, no morphological signs of regression could be detected along the axis of the Müllerian ducts (Figure 5.1A/D). In DES-exposed E13 fetuses, on the other hand, Müllerian ducts were only found at position I (Figure 5.1F). Caudally, at positions II and III, the Müllerian ducts were not formed, indicating a delay in their formation caused by the DES treatment (Figure 5.1I, position III).

At E15, differences in Müllerian duct regression between control and DES-exposed fetuses were observed. In E15 control fetuses, regression of the Müllerian ducts had started but was not complete (Figure 5.2A/D). The regression of the Müllerian ducts was initiated cranially at position I, and concomitantly we observed the characteristic presence of a whorl of mesenchymal cells surrounding the Müllerian ducts (Figure 5.2A). No signs of Müllerian duct regression could be detected at positions II and III at E15, indicating that degeneration of the Müllerian ducts is initiated cranially and then progresses caudally (Figure 5.2D). In contrast, regression of the Müllerian ducts in the DES-exposed E15 fetuses was not initiated at all three positions, as indicated by the absence of the typical whorl of mesenchymal cells (Figure 5.2F/I). The appearance of the Müllerian ducts in DES-exposed fetuses at E15 corresponds to that of the Müllerian ducts in control fetuses at E13, implicating that the onset of Müllerian duct regression is delayed by approximately two days.

It was observed that regression of the Müllerian ducts in control male fetuses resulted in their complete absence at E19 (Figure 5.3A). In the DES-exposed male fetuses at E19, regression of the cranial part of the Müllerian ducts was complete at positions I and II, as no Müllerian structures could be detected (results not shown). However, more caudally, at position III, the Müllerian ducts were still present (Figure 5.3B). The epithelial and

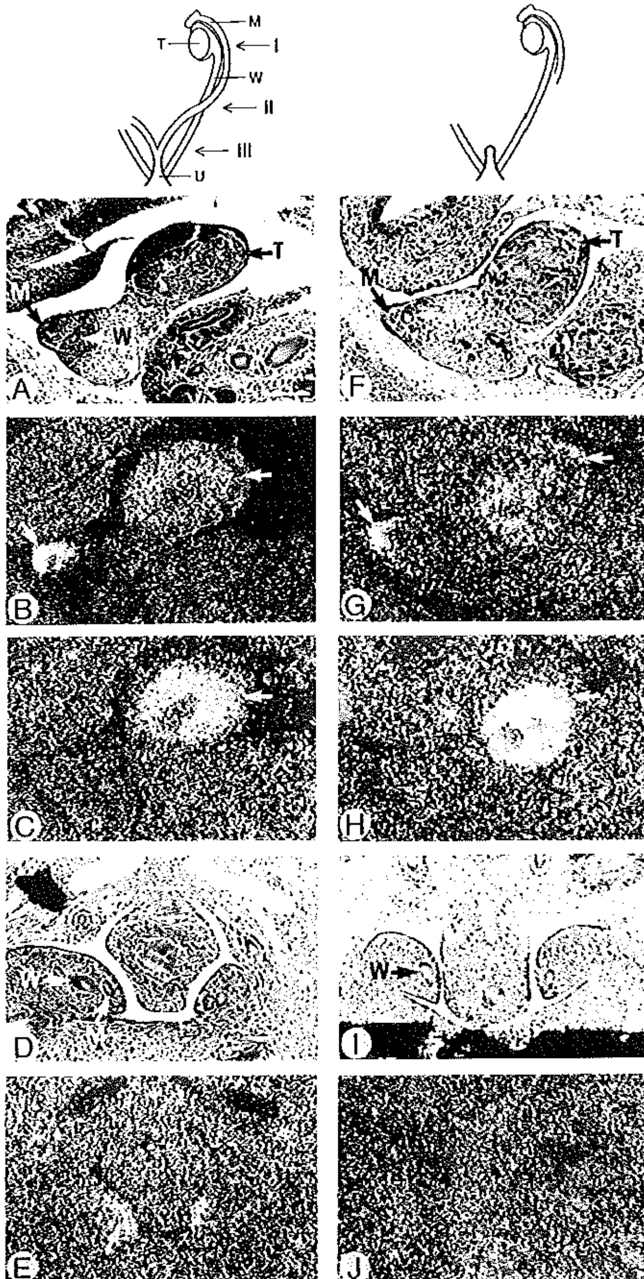
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Figure 5.1. **Histology of Müllerian ducts and expression of AMH and AMHRII mRNAs in control and DES-exposed male mouse fetuses at E13.**
The formation of the Müllerian ducts is represented schematically in the top panel. The roman numerals indicate positions I, II, and III, at which sections were taken for morphology study and *in situ* hybridization. The left figures (position I: A, B, C; position II: D, E) and the right figures (position I: F, G, H; position II: I, J) are sections from control and DES-exposed fetuses, respectively. At position I, the Müllerian ducts are present in control fetuses (A) and in DES-exposed (F) fetuses, although less differentiated. At position II, the Müllerian ducts are found in control (D) but not in DES-exposed fetuses (I). Expression of AMH and AMHRII mRNAs was determined using *in situ* hybridization. Darkfield views of AMHRII (control: B, E; DES-exposed: G, J) and AMH (control: C; DES-exposed: G) mRNAs are shown in adjacent sections. No difference between control and DES-exposed fetuses is found in the localization of AMH and AMHRII mRNAs expression; AMH mRNA expression is found in the testes, and AMHRII mRNA expression in the testes and the mesenchymal cells surrounding the Müllerian ducts. Arrows indicate expression sites. T, testis; W, Wolffian duct; M, Müllerian duct. Scale bar represents 100 µm.

mesenchymal cells of the Müllerian duct remnants, in DES-exposed male fetuses at E19 (Figure 5.3B), were differentiated and had an appearance comparable to that found in control female fetuses of the same developmental stage (results not shown).

These results are schematically summarized in the top panels of Figures 5.1, 5.2, and 5.3.

Expression of AMH and AMHRII mRNAs

The expression of AMH and AMHRII mRNAs was studied by *in situ* hybridization. AMH mRNA expression was localized in the gonads of DES-exposed male fetuses, similar to control fetuses, although differences in the quantitative level of expression were detected. The testes of DES-exposed fetuses at E13 showed a marked increase in AMH mRNA expression compared to control testes (Figure 5.1C/H). This increase in AMH mRNA expression was also present on E15 (Figure 5.2C/H), while testicular expression of AMH mRNA could hardly be detected in both control and DES-exposed E19 fetuses (results not shown).

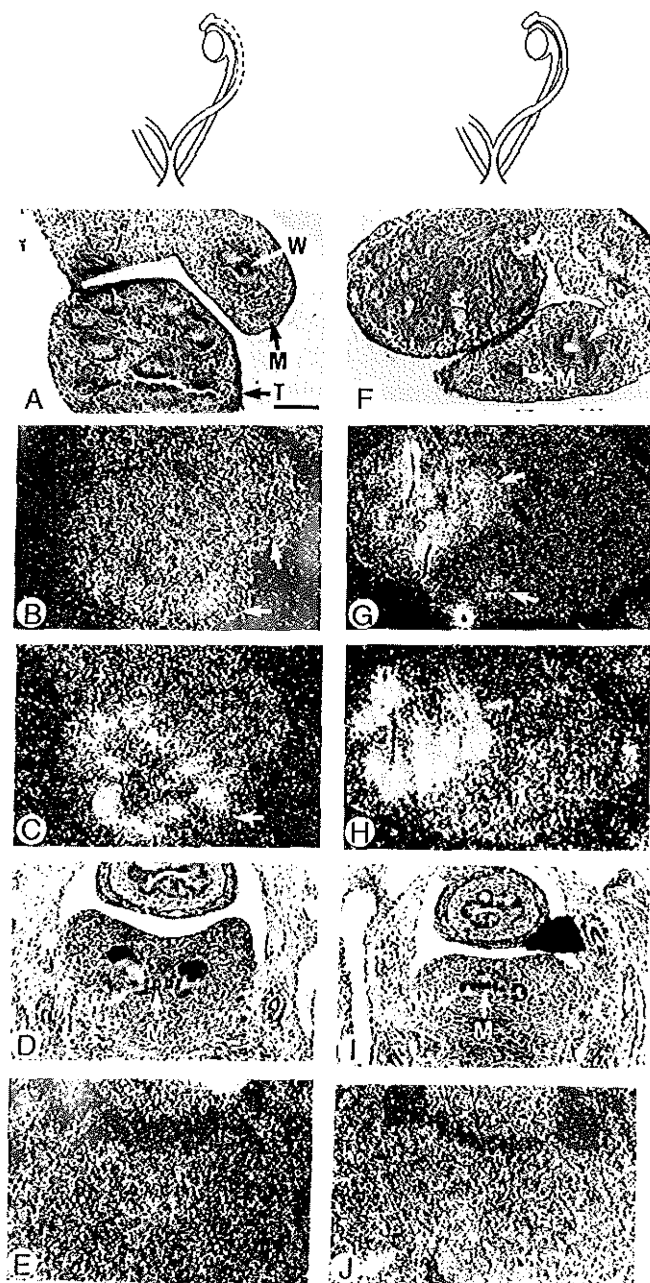
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Figure 5.2. **Histology of Müllerian ducts and expression of AMH and AMHRII mRNAs in control and DES-exposed male mouse fetuses at E15.**
In the top panel, the regression of the Müllerian ducts is represented schematically. The stippled lines indicate the regressed Müllerian ducts. The left figures (position I: A, B, C; position III: D, E) and the right figures (position I: F, G, H; position III: I, J) are sections from control and DES-exposed fetuses, respectively. Regression has initiated in control fetuses at position I (A), but not at position II and III (D). In DES-exposed fetuses the Müllerian ducts are completely present (F, I), although no signs of regression are found at position I (F). Note the presence of a whorl of mesenchymal cells surrounding the Müllerian duct in control fetuses (A). Expression of AMH and AMHRII mRNAs was determined using *in situ* hybridization. Darkfield views of AMHRII (control: B, E; DES-exposed: G, J) and AMH (control: C; DES-exposed: H) mRNAs are shown in adjacent sections. AMH and AMHRII mRNA expression are increased in fetal testes of DES fetuses. At position I, mesenchymal cells of control fetuses do not express AMHRII mRNA, whereas in DES-exposed fetuses a low expression is found. At position III no expression of AMHRII was found. Arrows indicate expression sites. T, testis; W, Wolffian duct; M, Müllerian duct. Scale bar represents 100 µm.

AMHRII mRNA expression was also studied at the three positions indicated in Figure 5.1. Expression of AMHRII mRNA in DES-exposed fetuses was found in the same tissues as in control fetuses; the fetal gonads and the mesenchymal cells surrounding the Müllerian ducts (Figure 5.1B/G). It is important to note that, although the formation of the Müllerian ducts was not complete by E13 in DES-exposed fetuses, AMHRII mRNA was already expressed. More caudally, at positions II and III, the Müllerian ducts were absent in DES-exposed fetuses; hence, expression of AMHRII mRNA could not be detected at these sites (Figure 5.1J). In control fetuses, AMHRII mRNA was expressed along the whole axis of the Müllerian ducts, although expression decreased caudally (Figure 5.1B/E).

In control fetuses at E15, expression of AMHRII mRNA could no longer be detected in the mesenchymal cells surrounding the Müllerian ducts, at all three positions studied (Figure 5.2B/E). The mesenchymal cells of the cranial Müllerian ducts in DES-exposed E15 fetuses did still express AMHRII mRNA (Figure 5.2G), although the expression was lower compared to that in E13 DES-exposed fetuses. Caudally, at position III, expression

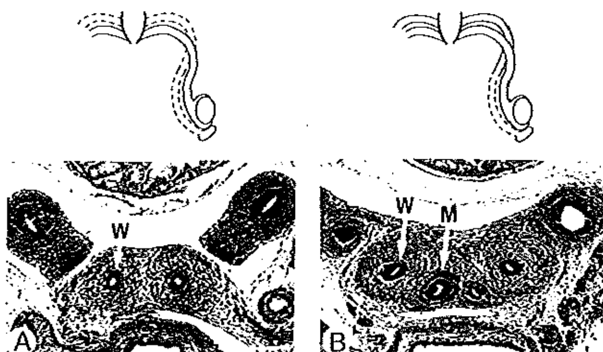

Figure 5.3. Histology of Müllerian ducts in control and DES-exposed male mouse fetuses at E19.
In the top panel the regression of the Müllerian ducts is represented schematically. At position III, the Müllerian ducts have completely regressed in control fetuses (A), whereas in DES-exposed fetuses remnants of Müllerian ducts are clearly visible (B). The Müllerian duct remnants show differentiation of epithelial and mesenchymal cells. W, Wolffian duct; M, Müllerian duct. Scale bar represents 100 µm.

could not be detected (Figure 5.2J). In the testes of control fetuses, AMHRII mRNA was only weakly expressed, whereas the testes of DES-exposed fetuses at E15 still showed a clear AMHRII mRNA expression (Figure 5.2B/G). An increase in testicular AMH mRNA expression in DES-exposed fetuses compared to control fetuses was still observed at E15 (Figure 5.2C/H).

At E19, testicular AMHRII mRNA expression was equally low, in both control and DES-exposed fetuses. AMHRII mRNA expression in the mesenchymal cells of the Müllerian ducts could not be detected in control and DES-exposed fetuses, although the Müllerian ducts were still present in DES-exposed male fetuses near the urogenital sinus (results not shown).

Expression levels of AMH and AMHRII mRNAs were quantified more precisely using an RNase protection assay (Figure 5.4A). Furthermore, the expression of SF-1 mRNA was included as a marker for urogenital ridge development. The results of the RNase protection showed that the

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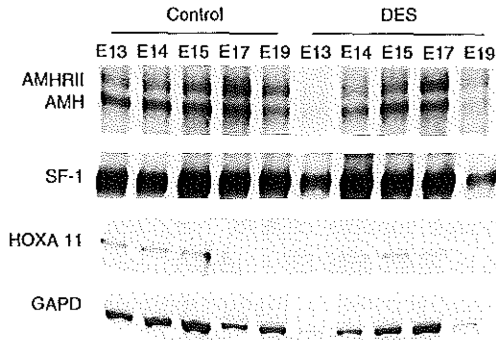

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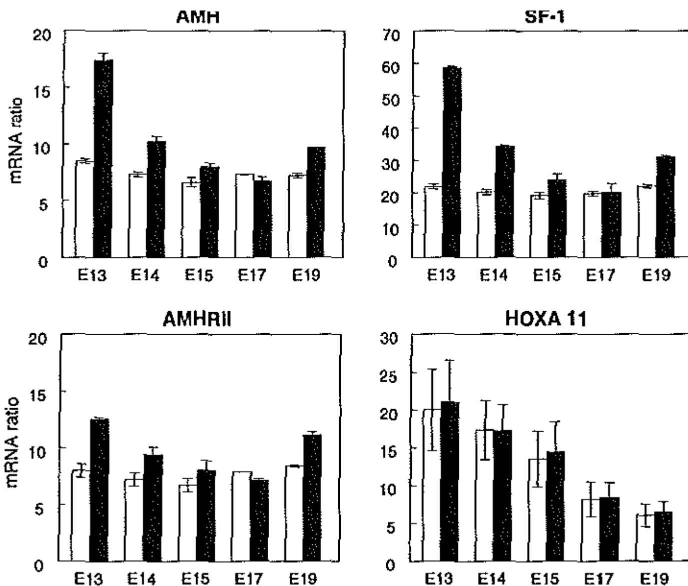

Figure 5.4 **Quantitative analysis of the expression of AMH, AMHRII, SF-1, and Hoxa 11 mRNAs in control and DES-exposed fetuses, studied by RNase protection assay.**

Expression in whole fetuses was determined at E13, E14, E15, E17, and E19. **A.** The results of the RNase protection assay. **B.** Quantitative analysis of the expression levels. The mRNA expression level is expressed as the ratio AMH/GAPD, AMHRII/GAPD, SF1/GAPD, and Hoxa11/GAPD. The open bars represent control fetuses and filled bars the DES-exposed fetuses. The bars and error bars represent the mean and standard deviation of two fetuses, isolated from different treatments.

expression patterns of AMH and AMHRII mRNAs mimic the expression pattern of SF-1 mRNA (Figure 5.4B). In DES-exposed male fetuses at E13 the expression of SF-1 mRNA is strongly increased compared to control fetuses. A similar increase in AMH mRNA expression was measured in DES-exposed male fetuses, as was observed with *in situ* hybridization. An increase of AMHRII mRNA expression was found using RNase protection assay in DES-exposed fetuses at E13, although this increase was less evident compared to AMH and SF-1 mRNAs expression. At E14 and E15, DES-exposed fetuses showed a higher expression of SF-1, AMH, and AMHRII mRNAs than control fetuses, although less pronounced than at E13. From E15 onwards, changes in mRNA expression of SF-1, AMH and AMHRII were limited to a slight increase at E19.

In addition to the expression of SF-1 mRNA, the Hoxa 11 mRNA expression level was determined, as a control for a possible effect of DES treatment on the general rate of fetal development. It was observed that DES-treated fetuses were born one day later compared to control fetuses. This might indicate that the DES-induced delay in Müllerian duct formation would reflect a general delay in fetal development of DES fetuses. However, DES-treated and control fetuses did not show a difference in fetal Hoxa 11 mRNA expression at all time points studied (Figure 5.4), indicating similar rates of general fetal development in the two treatment groups. Furthermore, no differences in length, width, or digit differentiation were observed between control and DES-treated fetuses during fetal development (results not shown).

Discussion

This paper describes the effect of prenatal DES exposure on regression of the Müllerian ducts of fetal male mice. In agreement with previous studies (McLachlan *et al.*, 1975; Newbold *et al.*, 1987), we found incomplete Müllerian duct regression upon DES exposure. In male fetuses from DES-treated mice, regression had initiated in the cranial part of the Müllerian ducts, but did not progress completely caudally, leaving remnants of Müllerian ducts at the position of the urogenital sinus. The nonregressed parts of the Müllerian ducts showed female-like differentiation, indicating

that the Müllerian duct remnants might be responsive to estrogens. In female mice, prenatal exposure to DES also causes uterine epithelial cell hypertrophy (Wordinger *et al.*, 1991). These findings indicate that the Müllerian ducts are a target for DES action in both male and female fetuses.

In addition to the appearance of Müllerian duct remnants, we observed that DES exposure resulted in a delay in Müllerian duct formation of approximately two days. In control fetuses, the complete Müllerian ducts were present at E13, whereas in DES-exposed fetuses fully formed Müllerian ducts were not found before E15. In addition, DES-exposed fetuses were born one day later compared to control fetuses. These observations suggest that DES causes a delay in general embryonic development. Also in rats, exposure to estrogens during pregnancy leads to a prolonged gestation (Zimmerman *et al.*, 1991), but this is explained by an inhibiting effect of DES on the onset of uterine contraction. Caesarian sections, performed to rescue the litter, revealed no difference in size of fetuses from control and DES-treated mothers (Zimmerman *et al.*, 1991). Transgenic mice, overexpressing the estrogen receptor α (ERα), have similar problems with birth, with gestation lengths prolonged up to four days (Davis *et al.*, 1994). Exposure to DES in neonatal mice results in an increase of ERα mRNA expression in uterine cells (Sato *et al.*, 1992), suggesting that the longer gestation time in DES-exposed mice may be a phenocopy of the change in pregnancy in ERα transgenic mice, and reflects a maternal effect rather than a delay in fetal development. No differences in body size or digit differentiation were observed between control and DES-treated fetuses during fetal development (results not shown). Furthermore, the expression of Hoxa 11 mRNA was studied as a marker for general fetal development (Krumlauf, 1994; Hsieh-Li *et al.*, 1995). Hoxa 11 mRNA is expressed in the limbs, the kidneys, and in the stromal cells surrounding the Müllerian and Wolffian ducts, and this expression is detected at E10, several days before reproductive tract differentiation (Hsieh-Li *et al.*, 1995). In the present study, no difference in Hoxa 11 mRNA expression between control and DES-exposed fetuses was observed, at all embryonic stages studied. This indicates that the rate of general fetal development is not affected, but that DES elicits a specific effect on

reproductive tract development. The variation in the results with the Hoxa 11 probe is due to the large differences in specific activity of the probe in different experiments.

The anlagen of the reproductive tract, the Wolffian and Müllerian ducts, are formed separately. The Wolffian duct is formed as an excretory duct of the mesonephros and is recognizable before the gonads are formed. At the time of gonad formation, the Müllerian ducts develop in a cranial to caudal direction along the Wolffian ducts, which function as a guiding structure for early growth of the Müllerian ducts (Byskov and Hoyer, 1994, and references therein). The genes involved in Müllerian duct formation have not been identified yet. It has been suggested that the Wolffian ducts release epithelial cells which contribute to the developing Müllerian ducts (Dohr and Tarmann, 1984). It has also been suggested that the growth of Müllerian ducts is autonomous (Grünwald, 1941). In our studies, DES treatment affects the formation of the Müllerian ducts rather than formation of the Wolffian ducts, because DES was administered after completion of Wolffian duct formation. However, an effect of estrogens on Wolffian duct formation cannot be ruled out. It has been observed that the Wolffian ducts are affected by exogenous estrogen exposure, resulting in several abnormalities, such as seminal vesicle tumors and prostate inflammation (Newbold, 1995). Also in female fetuses, the Wolffian ducts are a target for DES action. Retention of Wolffian ducts, postnatally, was observed in females, both in human and mouse (Newbold *et al.*, 1983; Haney *et al.*, 1986). These effects of DES on Wolffian and Müllerian duct differentiation may point to a common mechanism in the development of these duct systems. Both Wolffian and Müllerian ducts can respond to estrogens, since the estrogen receptor ERα is present in both structures during development (Greco *et al.*, 1993). The identification of a novel estrogen receptor, ERβ (Kuiper *et al.*, 1996), may contribute to our understanding of the mechanism of DES action. Recently, it was reported that ERα and ERβ, when activated by estradiol, signal in opposite ways via an AP1 site (Paech *et al.*, 1997). DES, therefore, may cause different effects, depending on the tissue studied. ERβ is highly expressed in prostate and ovary, whereas ERα shows a higher expression in the uterus (Kuiper *et al.*, 1997). Studying the effects of prenatal DES exposure in *ERα*, *ERβ*, or

double-knockout mice, will reveal which ER type is mainly involved in DES action.

In previous studies it has been proposed that incomplete regression of the Müllerian ducts in fetuses exposed to exogenous estrogens, is a result of a change in sensitivity of the ducts to AMH (Newbold *et al.*, 1984). Therefore, we have studied the effect of DES on AMHRII mRNA expression. The expression of AMH and AMHRII mRNAs was studied by *in situ* hybridization, and the expression levels in total fetuses were quantified by RNase protection. With *in situ* hybridization, a strong increase in AMH mRNA expression in the fetal testes of DES-exposed fetuses was evident. Quantification of the expression revealed a two-fold increase of AMH mRNA expression in DES-exposed fetuses compared to controls. This increase was most significant at E13. Nevertheless, this higher AMH mRNA expression did not result in complete Müllerian duct regression. This is in agreement with the observations in *in vitro* studies, that addition of a relatively high dose of AMH did not result in full regression of Müllerian ducts from DES-exposed fetuses (Newbold, personal communication). The DES-induced increase in AMH mRNA expression implies a direct effect of estrogens on the regulation of AMH mRNA expression. Indeed, a 13 basepair palindromic sequence, nearly identical to the estrogen response element (ERE), has been identified in the *AMH* gene promoter (Guerrier *et al.*, 1990). In footprinting experiments, this site was shown to bind ERα. Furthermore, 39 ERE half-sites were identified in the 5' flanking sequences of the *AMH* gene (Dresser *et al.*, 1995). Clusters of half-sites or degenerate palindromic sites can be effective, as was shown *in vitro*, where several ERE half-sites can act synergistically to control expression of the ovalbumin gene (Kato *et al.*, 1992). However, the functionality of the ERE half-sites in the *AMH* gene has not been proven. Recent papers have shown that AMH expression is dependent on SF-1 (Shen *et al.*, 1994; Giuili *et al.*, 1997). SF-1, an orphan nuclear receptor expressed in adrenals, gonads and the gonadotrophes of the pituitary gland, was characterized as a transcription factor which regulates several genes, such as genes encoding steroidogenic enzymes (Lala *et al.*, 1992). *SF-1* knockout mice lack gonads and adrenals, revealing an essential role for SF-1 in sexual differentiation

and formation of primary steroidogenic tissues (Luo *et al.*, 1994). In *in vivo* experiments, it was demonstrated that the proximal *AMH* gene promoter requires an intact binding site for SF-1 for time- and cell-specific expression (Giuili *et al.*, 1997). We observed a strong increase in SF-1 mRNA expression in DES-exposed fetuses, which was most significant at E13 and decreased towards E17. The increased expression of AMH mRNA in DES-exposed mice was found to have a similar temporal pattern as the SF-1 mRNA expression, corresponding with the role of SF-1 in regulation of AMH gene expression. These data suggest that DES has an effect on fetal gonadal gene expression. An effect of prenatal exposure to estrogenic compounds on SF-1 mRNA expression has been reported previously, although the described effect was a downregulation of SF-1 mRNA expression (Majdic *et al.*, 1997). In that study by Majdic *et al.* (1997), DES or the estrogenic compound 4-octylphenol were injected twice during pregnancy (E11 and E15) and expression of SF-1 mRNA was measured in the fetal testis at E17 (Majdic *et al.*, 1997). The disagreement between their and our results may be explained by the animal model, the experimental procedure, and the time points at which expression was determined.

In the present study, expression of AMHRII mRNA was also found to be increased at E13 in DES fetuses, although this increase was less obvious and could not be detected by *in situ* hybridization. In *in vitro* studies, no direct regulation of the *AMHRII* promoter by estrogens was found (Visser *et al.*, unpublished results). Therefore, it is likely that DES influences AMHRII mRNA expression indirectly. In the DNA sequence of both the human and mouse *AMHRII* gene promoter, an SF-1 response element was identified (Imbeaud *et al.*, 1995, and results not shown). Although regulation of AMHRII mRNA expression by SF-1 has not been reported, the increased AMHRII mRNA expression in DES-exposed fetuses might be a consequence of an increased SF-1 level. In accordance with developmental changes in SF-1 and AMH mRNAs expression, the most pronounced increase in AMHRII mRNA expression was found at E13, and this increase becomes less evident in older fetuses.

The increased mRNA expression levels of AMH and AMHRII do not directly explain the decreased sensitivity of the Müllerian ducts to AMH. However, a DES-induced effect on factors downstream of AMHRII, such as

a type I receptor or Smad proteins, can not be excluded. One can hypothesize that a DES-induced inhibition of downstream signaling factors influences a negative feedback loop, resulting in an increased expression of AMH and AMHRII mRNA, although the existence of such a feedback system for AMH has not been reported yet. Furthermore, whether the increase mRNA expression levels result in higher protein levels remains to be studied.

The *in situ* hybridization demonstrated that AMHRII mRNA expression can be detected along the entire axis of the Müllerian ducts in control fetuses at E13, but it decreases in caudal direction towards the urogenital sinus. At E15, expression of AMHRII mRNA could not be detected in the regressed cranial part of the Müllerian ducts. However, also in the caudal part of the Müllerian ducts, AMHRII mRNA expression could hardly be detected. These observations suggest that the onset of the critical period for AMH sensitivity of the Müllerian ducts (E13), is at the time point when Müllerian ducts are completed and express the AMH type II receptor, whereas the end of this critical period (E15) is demarcated by disappearance of the receptor. In DES-exposed fetuses at E13, AMHRII mRNA expression was found in the cranial part of the Müllerian ducts. The caudal parts have not been formed, and expression could not be detected at this site, suggesting that AMHRII mRNA expression is dependent on the presence of a formed Müllerian duct. At E15, a time point at which the Müllerian ducts have completely formed in the DES-exposed fetuses, AMHRII mRNA expression was detectable in the cranial ducts although expression was much lower compared to E13. Caudally, expression could hardly be detected, comparable to expression in control E15 fetuses. In DES-exposed mice just prior to birth (E19), the Müllerian duct remnants had lost expression of AMHRII mRNA and, therefore, are unable to respond to AMH at this late developmental time point. Although the formation of the Müllerian ducts is delayed in DES-treated fetuses, the timing of AMHRII mRNA expression is not delayed. This probably leads to a temporal asynchrony between the presence of the Müllerian ducts and the onset of the critical period of Müllerian duct regression.

The present observation of a DES-induced delay in Müllerian duct formation, contributes to our understanding of the diversity of

developmental defects in affected DES-sons. In humans, exposure of mothers to DES during early pregnancy results in a two-fold increase in the prevalence of malformations in their sons (Wilcox *et al.*, 1995). The formation of the Müllerian ducts is completed before the 11th week of gestation, and Müllerian duct regression is initiated at the 11th week. Exposure to DES after this period results in less abnormalities, while exposure before the 11th week results in a higher incidence of Müllerian duct remnants in the DES-sons (Wilcox *et al.*, 1995). This is in concordance with the present observations in mice, and we suggest that, also in humans administration of DES during early pregnancy causes an asynchrony between Müllerian duct formation and the critical period of Müllerian duct regression.

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Chapter Six

Identification of ALK2 as a candidate anti-Müllerian hormone type I receptor

Chapter Six

Identification of ALK2 as a candidate anti-Müllerian hormone type I receptor

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Submitted

Abstract

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor β (TGFβ) family of growth and differentiation factors. In mammalian male fetuses, AMH produced by the testes, causes the regression of the Müllerian ducts, which otherwise would differentiate into fallopian tubes, uterus and upper part of the vagina. In mouse and human, absence of AMH action caused by gene defects, results in persistence of the Müllerian ducts and development of duct derivatives in the male. In transgenic mice that overexpress AMH in many tissues including the ovary, Müllerian duct regression is also found in female fetuses. In addition, the ovaries of these mice develop testis cord-like structures and have suppressed aromatase activity. Inhibition of aromatase activity by AMH is also shown in cultured fetal ovaries and postnatal granulosa cells.

Members of the TGFβ family are believed to exert their actions through a receptor complex consisting of a type I and a type II receptor. Although the signaling mechanism of many TGFβ family members has been studied in much detail, little is know about AMH signaling. The AMH type II receptor cDNA was isolated recently, but an AMH type I receptor is unknown. To study AMH signaling, we developed an *in vitro* model based on the inhibitory effect of AMH on follicle-stimulating hormone (FSH)-induced aromatase activity. HEK-293 cells were stably transfected with cDNAs encoding the follicle-stimulating hormone (FSH) receptor and the AMH type II receptor, resulting in HEK-293FAN cells. Because promiscuity in ligand binding by type I receptors has been shown, we hypothesized that one of the known type I receptors might function as an AMHRI. Therefore, we investigated whether one or more of the six different type I receptors, known as activin receptor-like kinases (ALK1-6), could inhibit the FSH induction of the aromatase promoter. The HEK-293FAN cells were transiently cotransfected with an aromatase promoter-luciferase reporter construct, together with constitutively active mutated forms of the different ALKs. Only the constitutively active ALK2 and ALK6 were able to repress FSH-induced aromatase promoter activity. In addition, both wild type ALK2 and wild type ALK6 were found to be able to signal in response to AMH, although the inhibition found with ALK6 was less than that observed with ALK2.

Expression of an AMH type I receptor is expected at the potential target sites of AMH that also express AMHRII receptor, which are the mesenchymal cells surrounding the Müllerian ducts and the fetal and adult gonads. ALK2 and ALK6 mRNAs are expressed in the urogenital ridge of both male and female fetuses. *In situ* hybridization revealed that ALK2 mRNA is expressed by the mesenchymal cells of the Müllerian ducts. In addition, the fetal gonads express ALK2 mRNA, whereas ALK6 mRNA could not be detected.

Based on the temporal and spatial mRNA expression pattern of ALK2, and its ability to inhibit FSH-induced aromatase promoter activity in response to AMH, we suggest that ALK2, but not ALK6, can function as an AMH type I receptor.

Introduction

Anti-Müllerian hormone (AMH), also known as Müllerian-inhibiting substance (MIS), is a member of the transforming growth factor β (TGFβ) family of growth and differentiation factors. This family includes the TGFβs, activins, and the bone morphogenetic proteins (BMPs; Massagué, 1990). In contrast to these other family members, which fulfill a broad range of functions, AMH has a very specific role during sex differentiation. In male fetal mice, AMH is the earliest known protein secreted by the testicular Sertoli cells (Münsterberg and Lovell-Badge, 1991). The secreted protein acts as a hormonal factor to induce regression of the Müllerian ducts, which form the anlagen of the fallopian tubes, the uterus and the upper part of the vagina. Failure of AMH action, such as caused by a genetic defect, results in absence of Müllerian duct regression and thereby in a rare form of male pseudohermaphroditism in human, known as persistent Müllerian duct syndrome (PMDS; Carré-Eusèbe *et al.*, 1992; Imbeaud *et al.*, 1994). Indeed, gene knockout experiments in mice have demonstrated that in the absence of AMH, Müllerian ducts do not regress (Behringer *et al.*, 1994).

In addition to its specific role in sex differentiation, AMH also may play a role in the control of germ cell and gonadal development (Taketo *et al.*, 1991). In AMH-deficient mice, no abnormalities in spermatogenesis were

observed, but closer examination of AMH-deficient testes revealed the occasional presence of Leydig cell hyperplasia, suggesting a role for AMH in the control of Leydig cell development and/or function (Behringer *et al.*, 1994). This is supported by the finding that transgenic mice, which overexpress AMH showed Leydig cell hypoplasia (Behringer *et al.*, 1990). In the ovary, AMH is only expressed in the granulosa cells of pre-antral and small antral follicles, and it has been suggested that AMH is involved in a negative control of follicle maturation (Baarends *et al.*, 1995). However, in the ovaries of *AMH* knockout mice no obvious abnormalities in follicle development have been reported (Behringer *et al.*, 1994). In contrast, ovarian differentiation is disturbed in transgenic mice that overexpress AMH. At birth, ovaries of these mice had less germ cells than normal and showed masculinization, i.e. their somatic cells had differentiated into testis cord-like structures. The change of ovarian differentiation into a more testis-like phenotype was correlated with a decreased aromatase activity in these transgenic ovaries, leading to a higher testosterone level (Behringer *et al.*, 1990), since aromatase is the only enzyme that can convert testosterone to estradiol. Inhibition of aromatase activity was also shown when *in vitro* cultured fetal ovaries were exposed to AMH, resulting in release of testosterone rather than estradiol (Vigier *et al.*, 1989; Di Clemente *et al.*, 1992). In addition, suppression of aromatase activity by AMH has been shown in an *in vitro* assay of cultured granulosa cells isolated from postnatal ovaries (Di Clemente *et al.*, 1994a).

The members of the TGFβ family exert their action via a family of related serine/threonine kinase receptors, which can be subdivided into the type I or activin receptor-like kinases (ALKs) and the type II receptors. The six known type I receptors contain a Gly/Ser-rich motif known as the GS box, that is located N-terminal of the kinase domain, while the type II receptors are characterized by a short Ser/Thr-rich C-terminus, which is absent in type I receptors (Ten Dijke *et al.*, 1994a; Massagué and Weis-Garcia, 1996). Upon ligand binding to a type II receptor, a type I receptor is recruited into the hetero-dimeric receptor complex. Finally, a tetrameric receptor complex is formed, consisting of two type II receptors and two type I receptors (Wrana *et al.*, 1994; Luo and Lodish, 1996; Weis-Garcia and Massagué, 1996). A prerequisite for signaling is the phosphorylation of the

type I receptor on serine residues in the GS box by the type II receptor, which has constitutive kinase activity (Wrana *et al.*, 1994; Massagué and Weis-Garcia, 1996). The final signal is generated by the type I receptor through its kinase activity. The signaling capacity of type I receptors was demonstrated by a mutated TGFβ type I receptor (TβRI), in which a threonine residue located near the kinase domain was replaced by an aspartic acid, resulting in a constitutively active receptor that signals in the absence of ligand and type II receptor (Wieser *et al.*, 1995). Recently, a family of downstream phosphorylation targets of type I receptors was identified. These so-called Smad proteins become phosphorylated by type I receptors and interact with a common signaling component named Smad4. The complex subsequently translocates to the nucleus where it regulates gene transcription, either directly after DNA binding or as a cofactor in association with DNA-binding proteins (Massagué, 1996; Heldin *et al.*, 1997).

Although the signaling mechanism of many members of the TGFβ family has been studied in much detail, little is known about AMH signaling, also due to the fact that the type I receptor of AMH has not been identified yet. The type II receptor for AMH (AMHRII) was cloned and identified (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994b; Grootegoed *et al.*, 1994), and AMHRII mRNA is expressed in the mesenchymal cells surrounding the Müllerian ducts, coinciding with the site of action of AMH (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994). Furthermore, AMHRII mRNA is expressed in the granulosa and Sertoli cells of fetal and adult gonads (Baarends *et al.*, 1995a; Baarends *et al.*, 1995b). Similar to AMH-deficient mice, male *AMHRII* knockout mice possess a female genital tract in addition to a complete male reproductive tract (Mishina *et al.*, 1996). Furthermore, Leydig cell hyperplasia was also observed in testes of AMHRII-deficient mice (Mishina *et al.*, 1996). Since AMHRII-deficient mice are an exact phenocopy of the AMH-deficient mice, these *in vivo* experiments proved that AMH is the only and necessary ligand for a single type II receptor, the AMHRII. This conclusion is further supported by the identification of mutations in the human *AMHRII* gene in patients with PMDS (Imbeaud *et al.*, 1995; Imbeaud *et al.*, 1996).

To study the signaling mechanism of AMH, the identification of the AMH type I receptor is a prerequisite. Binding studies with AMH were not conclusive, since radiolabeling of AMH appears to abolish its ability to bind to the receptor and decreases its biological activity (Donahoe *et al.*, 1977). Iodinated AMH bound in a dose-dependent manner to cells transfected with AMHRII cDNA, but the level of non-specific binding was very high (Di Clemente *et al.*, 1994b). So far, AMH action can only be studied in an *in vitro* Müllerian duct regression assay, or in a fetal ovary assay in which inhibition of aromatase activity is measured.

Herein, we describe a novel *in vitro* assay, which is based on the ability of AMH to inhibit FSH-induced aromatase activity. Furthermore, we describe the functional identification of ALK2 as a candidate AMH type I receptor, using this assay.

Materials and Methods

DNA constructs

The expression vector for AMHRII was constructed by cloning an EcoRI-XhoI fragment encoding the full-length rat AMHRII cDNA (Baarends *et al.*, 1994) into EcoRI-BamHI sites of pSG5 (Green *et al.*, 1988). The expression vectors for rat follicle-stimulating hormone receptor (FSHR), *Neo*, and the wild type and constitutively active ALK1-Q201D, ALK3-Q233D, ALK4-T206D, ALK5-T204D, and ALK6-Q203D, activin type II receptors (ActRIIA and ActRIIB), and bone morphogenetic type II receptor (BMPRII) have been described previously (Van Doren *et al.*, 1984; Ten Dijke *et al.*, 1994c; Wieser *et al.*, 1995; Hoodless *et al.*, 1996; Martens *et al.*, 1997; Kraaij *et al.*, 1998). The wild type ALK2 cDNA was subcloned into the EcoRI site, and the constitutively active ALK2-Q204D (J. de Winter, unpublished) was subcloned into the BamHI site, of pSG5. The aromatase promoter-luciferase reporter construct was generated by cloning the HindIII-XbaI fragment encoding the rat aromatase promoter (Fitzpatrick and Richards, 1993) into the SmaI site of pGL2-basic (Promega, Leiden, The Netherlands).

Transfection and luciferase assay

HEK-293 (human embryonic kidney) cells were maintained in DMEM-F12 (Life Technologies, Breda, The Netherlands) and 10% FCS (SEBAK, Aidenbach, Germany) at 37 C in a 5% CO_2 atmosphere. Transfection was conducted using the calcium phosphate method (Chen and Okayama, 1987) at 35 C and 3% CO_2. Stable cell lines, denoted HEK-293FAN cells, expressing FSHR and AMHRII, were generated and selected for neomycin resistance, after cotransfection with 5 µg FSHR, 5 µg AMHRII, and 1 µg pK0-Neo. The HEK-293FAN cells were transiently transfected with 10 µg aromatase promoter-luciferase reporter construct or 1 µg pADneo2-C6-BGL, a vector that contains six cAMP response elements (Himmler *et al.*, 1993). In the case of cotransfection with the constitutively active type I receptors, 1 µg of expression plasmid was used. Cotransfection with the wild type ALK2 or wild type ALK6 was carried out with 5 µg of expression plasmid. To correct for transfection efficiencies, the β-galactosidase expression plasmid pCH110 (1 µg/75 mm dish; NUNC, Life Technologies) was added (Hall *et al.*, 1983). Following transfection, cells were split into 12 well plates (NUNC) and allowed to attach overnight. Subsequently, the cells were incubated with vehicle, 1 IU/ml recFSH (Organon, Oss, The Netherlands), 1 IU/ml recFSH together with 1.8 µg/ml AMH, or 1.8 µg/ml AMH alone, in DMEM-F12 containing 0.1% (w/v) BSA. AMH was obtained by concentrating medium of HEK-293 cells coexpressing AMH and prohormone convertase 5 (PC5). PC5 is able to process AMH, and PC5-cleaved AMH was proven to have full bioactivity in the Müllerian duct regression assay (Nachtigal and Ingraham, 1996).

An additional transfection experiment was conducted in which HEK-293 cells were cotransfected with the aromatase promoter-luciferase reporter construct and 1 µg of the ActRIIA, ActRIIB, or BmpRII expression plasmids together with 5 µg of ALK2, or ALK6 expression plasmids. Subsequently, cells were incubated with vehicle, 1 IU/ml recFSH, 1 IU/ml recFSH together with 20 ng/ml activin, or 100 ng/ml BMP2, or 100 ng/ml BMP7, or activin, BMP2, or BMP7 alone.

After 48 hours, cells were lysed, and luciferase and β-galactosidase activities were measured (Miller, 1972; Blok *et al.*, 1992).

RNA isolation
FVB mice were kept under standard animal housing conditions in accordance with the NIH Guidelines for the Care and Use of Experimental Animals. Vaginal plug detection was considered day 0 (E0) of pregnancy. Pregnant mice were sacrificed by cervical dislocation on E13, 14, 15 or 18. Fetal tissues and adult gonads (40 days) were isolated and snap frozen in liquid nitrogen and stored at -80 C. Total RNA from fetal tissues was isolated using a microscale version of the method described by Chomczynski and Sacchi (1987), and total RNA from adult tissues was isolated using the LiCl/urea method (Auffray and Rougeon, 1980) In addition, fetuses were fixed overnight in Bouin's fixative, embedded in paraffin, and sectioned transversally at 8 μm. PCR reactions using placental genomic DNA (Hogan *et al.*, 1986) were performed as described by Mitchell *et al.* (1991), using primers for the mouse genes *Sbx* and *Sby* (Kay *et al.*, 1991; Mitchell *et al.*, 1991), to determine the sex of the fetuses.

RNAse protection assay and in situ hybridization
A mouse AMHRII DNA template for *in vitro* transcription was generated by RT-PCR. The RT-PCR reaction was carried out on 100-200 ng total RNA, extracted from 25-day-old mouse testis, using random hexamers. A sample of the reverse transcription reaction product was used in the PCR reaction using the primers 5'GCTCCGGAGCTCTTGGACAAG3' (forward primer) and 5'CAGGCGCTGCTGCACACACTC3' (reverse primer), corresponding to the kinase subdomains VIII, IX, and X of the *AMHRII* gene. A 350 bp PCR product was subcloned in pBluescript KS and used to generate [^{32}P]-UTP-labeled anti-sense RNA probe. The AMH RNA probe was obtained using a 430 bp mouse genomic DNA PstI fragment of the *AMH* gene, containing exon 1. An EcoRI-HindIII fragment containing bp 1-472 of the mouse ALK2 cDNA, and a HpaI-ApaI fragment containing bp 79-551 of the mouse ALK6, both encoding the extracellular domain, were subcloned in pBluescript KS and used to generate [^{32}P]-UTP-labeled anti-sense RNA probes. The control glyceraldehyde 3-phosphate dehydrogenase (GAPD) RNA probe was synthesized using a construct containing 163 bp AccI-Sau3AI fragment of the rat GAPD cDNA. RNase protection assays of 10 μg of total RNA with these probes were performed as described by Baarends *et al.* (1994).

The EcoRI-HindIII fragment containing bp 1-472 of the mouse ALK2 cDNA was also used to generate sense and anti-sense [^{35}S]-UTP-labeled transcripts for *in situ* hybridization. The *in situ* hybridization procedure was performed as described by Zeller and Rogers (1991) with some modifications (Baarends *et al.*, 1994).

Results

Development of an AMH in vitro assay

To study several aspects of the molecular mechanism of AMH action, we developed an *in vitro* assay based on the inhibitory effect of AMH on FSH-induced aromatase promoter activity (Vigier *et al.*, 1989; Di Clemente *et al.*, 1992; Di Clemente *et al.*, 1994). Stable HEK-293 cell lines expressing the cDNAs encoding the FSHR and AMHRII were generated, and the cell line with the highest FSHR and AMHRII mRNA expression level (HEK-293FAN cells) was used in further experiments.

The aromatase promoter-luciferase reporter construct was transfected transiently into the HEK-293FAN cells. Upon FSH stimulation, a three- to four-fold increase in aromatase promoter activity was measured compared to nonstimulated cells (Figure 6.1). The main signal transduction pathway by FSH is activation of adenylyl cyclase through G_s. The FSH-induced rise in intracellular cAMP regulates expression of several genes through interaction of CREBP (cAMP response element binding protein) with CREs (Birnbaumer, 1992; Lalli and Sassone-Corsi, 1994). Using a luciferase reporter construct with six cAMP response elements (6xCRE), a four- to five-fold increase in luciferase was obtained, while basal luciferase activity using this reporter construct was much higher (Figure 6.1). These results indicate the presence of a functional FSHR and cAMP transduction system in the HEK-293FAN cells.

Identification of type I receptors with inhibitory signaling ability

To identify type I receptors that are able to signal the inhibition of FSH-induced aromatase activity, mutant receptors ALK1-ALK6 with constitutive kinase activity were cotransfected with the aromatase promoter-luciferase

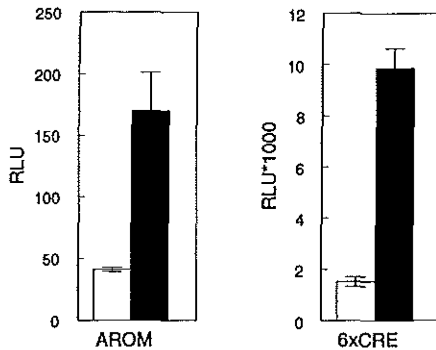

Figure 6.1. FSH-response in HEK-293FAN cells.
HEK-293 cells were stably transfected with cDNAs encoding FSHR and AMHRII and selected for neomycin resistance, resulting in HEK-293FAN cells. The HEK-293FAN cells were transiently transfected with either the aromatase promoter-luciferase reporter construct (AROM) or with a luciferase reporter containing six cAMP response elements (6xCRE). Cells were incubated without (open bars) or with (black bars) human recFSH (1 IU/ml) for 4 hours. Luciferase activity was measured in cell lysates and given as mean ± SEM (3 independent experiments).

reporter construct into HEK-293FAN cells. These mutant receptors have a mutation C-terminal of the GS box, leading to constitutive activity in theabsence of ligand and type II receptor (ALK1-Q201D, ALK2-Q204D, ALK3-Q233D, ALK4-T206D, ALK5-T204D, and ALK6-Q203D). The presence of the control vector pSG5 (results not shown) or wild type ALKs did not affect the FSH-induced aromatase promoter activity (Figure 6.2). This was also found with the constitutive active mutants of ALK1 and ALK4, while ALK3-Q233D and ALK5-T204D caused a slight increase of basal reporter activity (Figure 6.2). In contrast, transfection of the constitutive active ALK2-Q204D and ALK6-Q203D caused inhibition of FSH-induced aromatase promoter activity (Figure 6.2). In addition, the basal activity of the aromatase promoter-luciferase reporter construct was suppressed in the presence of constitutive active ALK2-Q204D (Figure 6.2).

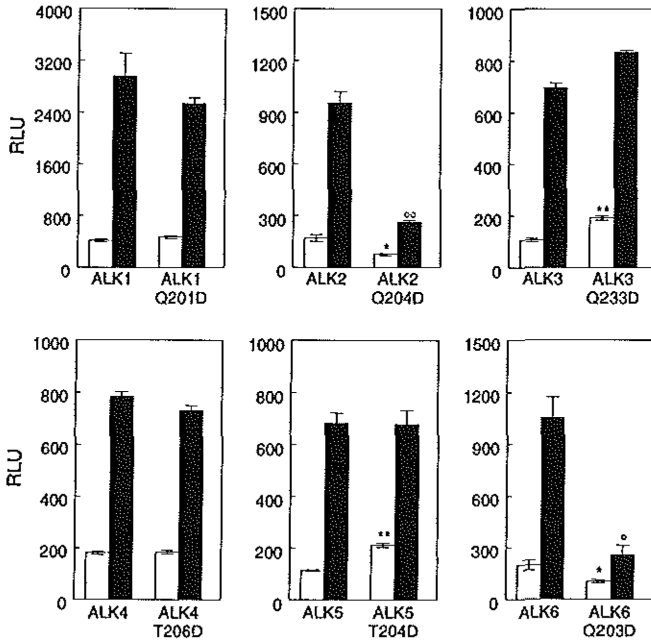

Figure 6.2. **Inhibition of FSH-induced aromatase promoter activity by constitutively active ALKs.**

HEK-293FAN cells were transiently cotransfected with the aromatase promoter-luciferase reporter construct, and with cDNAs encoding the indicated wild type or constitutively active receptors. The expressed receptors are: wild type ALK1-6 and mutant receptors ALK1-Q201D, ALK2-Q204D, ALK3-Q233D, ALK4-T206D, ALK5-T204, and ALK6-Q203D. Wild type and mutant receptor are expressed by identical vectors. The transfected cells were incubated for 48 hours without (open bars) or with (black bars) recFSH (1 IU/ml). Luciferase activity was measured in cell lysates and given as mean ± SEM (3 independent experiments). Data were subjected to Student's t-test: *, significantly different from non-treated wild type receptor ($P < 0.05$); **, significantly different from non-treated wild type receptor ($P < 0.005$); °, significantly different from FSH-treated wild type receptor ($P < 0.005$); °°, significantly different from FSH-treated wild type receptor ($P < 0.0005$).

AMH signaling through ALK2 and ALK6

To study the ability of ALK2 and ALK6 to relay the signal of AMH, HEK-293FAN cells were cotransfected with the aromatase promoter-luciferase reporter construct and wild type ALK2 or wild type ALK6. In the presence of ALK2 or ALK6, stimulation with FSH alone resulted in an increase of aromatase promoter activity (Figure 6.3A). However, incubation of HEK-293FAN cells expressing ALK2 with both FSH and AMH resulted in approximately 50% inhibition of the aromatase promoter activity compared to stimulation with FSH alone (Figure 6.3A). Incubation with a higher concentration of AMH did not result in a further decrease of aromatase promoter activity (results not shown). Incubation of HEK-293FAN cells expressing ALK2 with AMH alone decreased the basal activity of the aromatase promoter (Figure 6.3A).

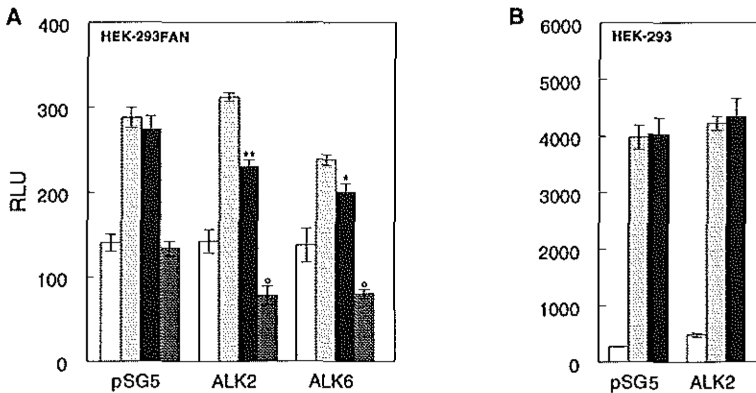

Figure 6.3. Inhibition of FSH-induced aromatase promoter activity by AMH.
A. HEK-293FAN cells were transiently cotransfected with the aromatase promoter-luciferase reporter, together with cDNA encoding the indicated wild type receptor or with the pSG5 vector. **B.** To obtain cells not expressing AMHRII, HEK293 cells were transiently cotransfected with cDNAs encoding FSHR, the aromatase promoter-luciferase reporter and ALK2. The transfected cells were incubated for 48 hours with recFSH (1 IU/ml; light grey bars), recFSH together with AMH (1 IU/ml and 1.8 μg/ml, respectively; black bars), AMH alone (1.8 μg/ml; dark grey bars), or vehicle (open bars). Luciferase activity is given as mean ± SEM (3 independent experiments). Data were subjected to Student's t-test; °, significantly different from non-treated cells ($P < 0.05$); *, significantly different from FSH-treated cells ($P < 0.05$); **, significantly different from FSH-treated cells ($P < 0.001$).

Similar results were obtained with HEK-293FAN cells transfected with ALK6 (Figure 6.3A), but the inhibition of FSH-induced aromatase promoter activity was statistically less strong than the inhibition measured in the presence of ALK2. In the absence of AMHRII, incubation with AMH did not affect the FSH-induced aromatase promoter activity in HEK-293 cells cotransfected with FSHR, the aromatase promoter-luciferase reporter construct and ALK2 (Figure 6.3B), demonstrating that also in HEK-293 cells AMHRII is essential for AMH signaling.

ALK2 and ALK6 may also function as activin and/or BMP type I receptors. Therefore, HEK-293 cells were cotransfected with the aromatase promoter-luciferase reporter construct and the cDNAs encoding the activin type II receptors (ActRIIA or ActRIIB), or BMP type II receptor (BMPRII), together with ALK2 or ALK6. Transfected cells were stimulated with recFSH alone or with recFSH together with activin, BMP2, or BMP7. However, no inhibition of FSH-induced aromatase activity was found with either of these combinations (Figure 6.4). These results suggest that the inhibition of FSH-induced aromatase activity is specific for AMH, signaling through an AMHRII/ALK2 receptor complex.

Expression of ALK2 and ALK6 mRNAs.

The results with the aromatase promoter assay suggest that ALK2 and ALK6 can function as an AMH type I receptor (AMHRI) *in vitro*. However, to fulfill the role of AMHRI *in vivo*, the type I receptor has to be expressed in the target tissues of AMH, which are the mesenchymal cells surrounding the Müllerian ducts, the fetal testis and possibly also the adult testis and ovary. Using RNase protection assay, the expression of ALK2 and ALK6 mRNAs was studied in isolated urogenital ridges and gonads at E13, E14, E15, and E18 (Figure 6.5A). Both ALK2 and ALK6 mRNAs are expressed in the urogenital ridges of male and female fetuses at all ages, although the mRNA expression level of ALK2 is higher compared to the level of ALK6. Expression of AMHRII mRNA is lost in the urogenital ridges during male fetal development, whereas it remained expressed in the female urogenital ridge (Figure 6.5A). The loss of AMHRII mRNA expression is not correlated with a comparable loss of ALK2 mRNA expression. Expression of both

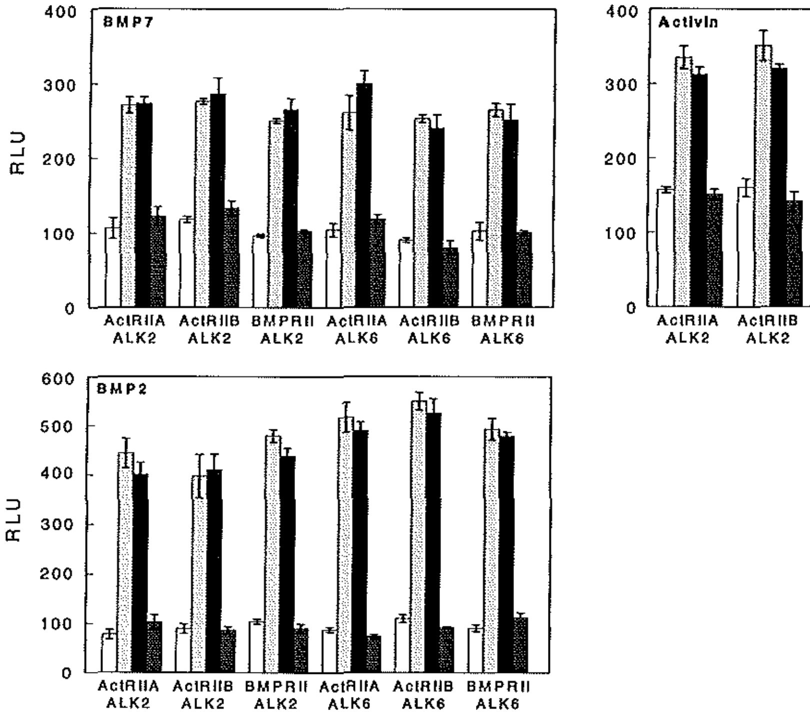

Figure 6.4. **Activin, BMP2, and BMP7 do not inhibit FSH-induced aromatase promoter activity.**
HEK-293 cells were transiently cotransfected with the aromatase promoter-luciferase reporter, together with cDNAs encoding the FSHR, the ActRII receptors, or BMPRII, and the indicated wild type type I receptor. The transfected cells were incubated for 48 hours with recFSH (1 IU/ml; light grey bars), recFSH (1 IU/ml) together with activin (20 ng/ml), BMP2 or BMP7 (100 ng/ml) (black bars), activin, BMP2, or BMP7 alone (dark grey bars), or vehicle (open bars). Luciferase activity is given as mean ± SEM (2 independent experiments). The ligand used in each experiment is indicated at the top.

AMHRII and ALK2 mRNAs is also found in the fetal gonads of both sexes, but mRNA expression of ALK2 was lower in the fetal ovary than in the fetal testis (Figure 6.5A). In contrast, ALK6 mRNA expression was not detected in the fetal gonads of both sexes, at all time points studied (Figure 6.5A).

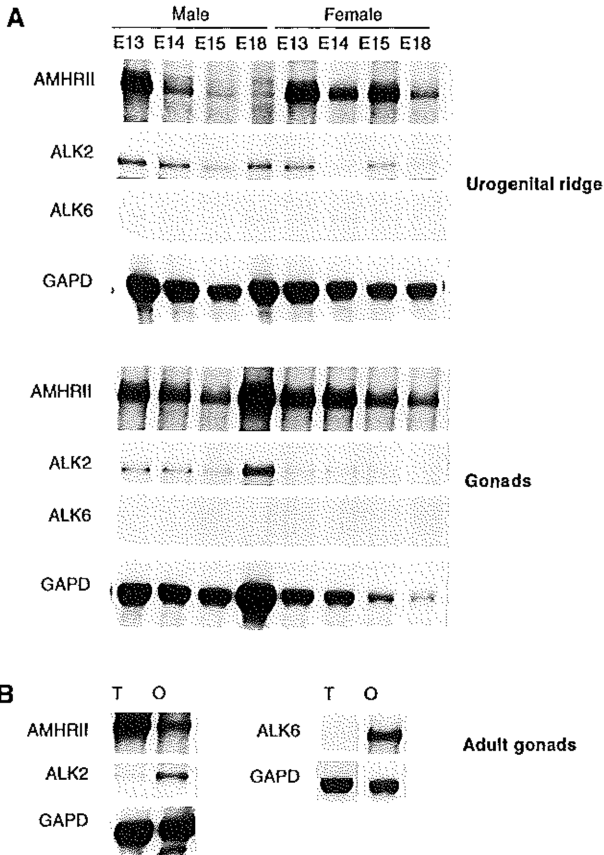

Figure 6.5. **Expression of AMHRII, ALK2, and ALK6 mRNAs in AMH target tissues.**

A. Expression in fetal urogenital ridge and fetal gonads. The fetal ages at which the tissues were obtained and the sex of the fetuses are indicated at the top of the lanes.

B. Expression in the adult gonads. ALK6 mRNA expression in testis and ovary was measured in separate experiments. T, testis; O, ovary. AMHRII, ALK2, ALK6, and GAPD indicate the position of the respective protected fragments. The GAPD mRNA level was included as a control for RNA loading.

Postnatally, ALK6 mRNA is only expressed in the adult ovary but not in the adult testis, whereas ALK2 mRNA is expressed in the ovary and also, albeit at lower level, in the testis (Figure 6.5B).

In situ hybridization was used to study the localization of ALK2 mRNA expression in mouse embryos at the AMH sensitive period of Müllerian duct regression (E13). ALK2 mRNA is highly expressed in fetal liver and adrenal, tissues in which AMHRII mRNA is not present (Figure 6.6). In addition, ALK2 mRNA expression was detected in the mesenchymal cells surrounding the Müllerian ducts, but not in association with Wolffian ducts (Figure 6.6B). No mRNA expression above background could be detected in the fetal testes (Figure 6.6B). We were not successful in detecting ALK6 mRNA expression using *in situ* hybridization, possibly caused by the very low mRNA expression level of ALK6 (Figure 6.5A). The expression data indicate that in all tissues that express AMHRII, ALK2 is also expressed, allowing a possible role of ALK2 as AMHRI.

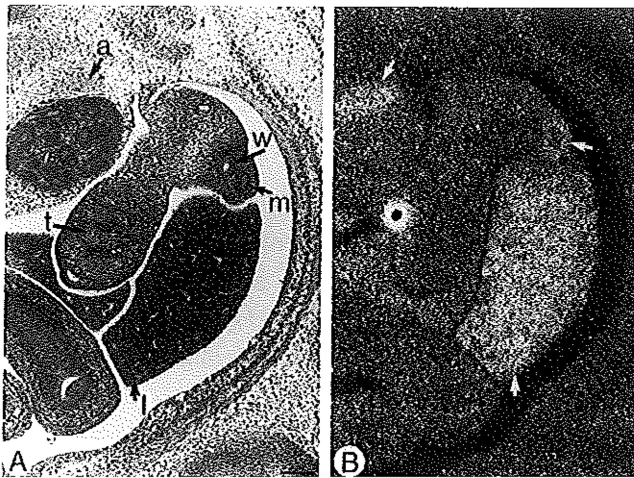

Figure 6.6. Expression of ALK2 in male fetuses at E13, determined using *in situ* hybridization.
A. Haematoxylin-eosin stained section. B. Darkfield view of ALK2 mRNA. a, adrenal; l, liver; t, testis; m, Müllerian duct; w, Wolffian duct. White arrows indicate the sites of expression (the ring structure at the middle-left is an artifact). Scale bar represents 100 µm.

Discussion

The molecular signaling mechanism of several members of the TGFβ family of peptide growth and differentiation factors, such as TGFβ and BMPs, has been studied in detail. However, little is known about the AMH signaling cascade. The AMHRII receptor has been identified recently (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994; Grootegoed *et al.*, 1994), but the identity of the type I receptor of AMH has remained unknown. So far, binding studies with AMH have appeared to be difficult, in contrast to observations on binding of TGFβs, activins and BMPs. All members of the TGFβ family are synthesized as large precursor proteins that contain a proteolytic cleavage site. Cleavage at this site generates the C-terminal mature growth factor and the N-terminal pro-domain, and the dissociation of the two domains is required for bioactivity (Gentry and Nash, 1990). However, to obtain full bioactivity of AMH, continuous association of the mature growth factor with the pro-domain after proteolytic cleavage is necessary (Wilson *et al.*, 1993). The instability of the hormone upon labeling (Donahoe *et al.*, 1977) and its size (140 kDa) have precluded binding experiments with sufficient specific binding to AMHRII (Di Clemente *et al.*, 1994b0. Therefore, the use of binding experiments for identification of the AMH type I receptor may not be feasible.

AMH has first been identified by its induction of Müllerian duct regression in male fetuses (Picon, 1969). However, AMH was also shown to be capable of inhibiting FSH-induced aromatase activity in gonadal cells. The aromatase activity of cultured fetal ovaries and postnatal granulosa cells is suppressed in the presence of AMH (Vigier *et al.*, 1989; Di Clemente *et al.*, 1992; Di Clemente *et al.*, 1994a). This feature of AMH, the inhibition of FSH-induced aromatase activity, was used to study the mechanism of AMH action. The stable cell line HEK-293FAN, expressing FSHR and AMHRII, was generated. In these cells, the aromatase promoter-luciferase reporter construct was transfected transiently. The 641 bp aromatase promoter region contains sequences required for cAMP induction (Fitzpatrick and Richards, 1993). Indeed, upon FSH or dbcAMP stimulation, a three- to four-fold increase in aromatase promoter activity was obtained in this assay.

To study the downstream signaling pathway of AMH, our aim was to identify type I receptors that are able to signal the inhibition of FSH-

induced aromatase activity. Ligand binding to type II receptors is very specific, as illustrated by the fact that the TGFβ type II receptor only binds TGFβ and the BMP type II receptor (BMPRII) only binds BMPs (Lin *et al.*, 1992; Estevez *et al.*, 1993). Also, AMH is the exclusive ligand for the AMHRII, since *AMHRII* knockout mice are an exact phenocopy of *AMH* knockout mice (Behringer *et al.*, 1994; Mishina *et al.*, 1996). However, ligand binding to type I receptors shows a high degree of promiscuity. ALK5 was shown to be a functional TGFβ type I receptor, and ALK3 and ALK6 both function as BMP type I receptors (Franzén *et al.*, 1993; Suzuki *et al.*, 1994; Ten Dijke *et al.*, 1994a; Ten Dijke *et al.*, 1994c). ALK1 can bind TGFβ and activin, although no biological responses are known (Attisano *et al.*, 1993). ALK4 signals in response to activin, whereas ALK2 can bind TGFβ, activin, BMP2 and BMP7, but not BMP4 (Attisano *et al.*, 1993; Ebner *et al.*, 1993; Tsuchida *et al.*, 1993; Carcamo *et al.*, 1994; Ten Dijke *et al.*, 1994c). However, with respect to the signaling capacity of ALK2 in response to these ligands, conflicting data have been reported. Activin can induce fibronectin and PAI-1, but these responses could not be relayed by ALK2 (Carcamo *et al.*, 1994). Activin can use ALK2 to stimulate 3TPlux, a reporter construct containing the plasminogen activator inhibitor-1 (PAI-1) promoter. However, a kinase deficient ALK2 was unable to exert a dominant negative effect on the activin-induced 3TPlux response (Tsuchida *et al.*, 1993; Carcamo *et al.*, 1994). A 3TPlux response to BMP2 or BMP7 was also observed when cells were cotransfected with BMPRII and ALK2 (Liu *et al.*, 1995). In addition, incubation with BMP2 or expression of constitutively active ALK2 in *Xenopus* animal cap explants resulted in similar phenotypes, i.e. ventral mesoderm markers but no dorsal markers were induced (Suzuki *et al.*, 1997). Furthermore, a low level of ALK2 was found to overrule the dorzalizing effect of ALK4 when constitutively active ALK2 and constitutively active ALK4 were cotransfected (Armes and Smith, 1997). Although ALK2 seems to bind activin as well as BMP2 and BMP7, ALK2 only transduces BMP signals but not activin signals, in the context of *Xenopus* embryos. The ubiquitous tissue expression pattern of several type I receptors (Dewulf *et al.*, 1995; Verschueren *et al.*, 1995) suggests that they might be shared with other members of the TGFβ family. For these reasons, we have studied whether the type I receptors identified so far (ALK1-ALK6) might function as

an AMH type I receptor, instead of attempting to clone new type I receptors.

The ability to inhibit FSH-induced aromatase promoter activity was studied using constitutively active type I receptors. Of the six type I receptors studied, only constitutively active ALK2-Q204D and ALK6-Q203D were able to inhibit FSH-induced aromatase promoter activity. In addition, wild type ALK2 and wild type ALK6 were able to relay the AMH signal in the presence of AMHRII, with ALK2 signaling resulting in a significantly stronger inhibition of aromatase promoter activity than ALK6 signaling. In agreement with the observed AMH-induced inhibition of basal aromatase activity in cultured granulosa cells (Di Clemente *et al.*, 1994a), the constitutively active ALK2-Q204D and ALK6-Q203D, and also wild type ALK2 and ALK6 upon AMH incubation, suppress basal aromatase promoter activity. The amount of AMH used to suppress FSH-induced aromatase (1.8 µg/ml) corresponds to the concentration of AMH used in previously reported studies on aromatase inhibition, such as 2.25-3 µg/ml for cultured fetal ovaries and 0.2-7.5 µg/ml for cultured postnatal granulosa cells (Vigier *et al.*, 1989; Di Clemente *et al.*, 1994a).

The inhibition of FSH-induced aromatase activity was dependent on the presence of AMHRII and was specific for AMH. The constitutively active forms of BMP or activin type I receptors, ALK3 and ALK4 respectively, were unable to suppress FSH-induced aromatase promoter activity. Furthermore, activin signaling through an ActRII/ALK2 complex or BMP signaling through ActRII/ALK2, ActRII/ALK6, BMPRII/ALK2, or BMPRII/ALK6 complexes, also did not inhibit FSH-induced aromatase promoter activity. This lack of inhibition was not the result of inactive ligands, since activin and BMPs were able to induce the 3TPlux reporter with the proper type I and type II receptors (results not shown). These results suggest that activin, BMP2 and BMP7 are unable to signal through ALK2 in the aromatase promoter assay.

An essential requirement for an AMHRI, is its correct spatial and temporal expression in the target sites of AMH, i.e. the Müllerian ducts, and the fetal and adult gonads. ALK2 and ALK6 mRNAs were expressed in the genital ridges of both male and female fetuses at all time points studied, although mRNA expression of ALK6 was much lower compared to ALK2

mRNA expression. In contrast to AMHRII mRNA expression, which is lost during male fetal development (Baarends *et al.*, 1994), mRNA expression of both ALK2 and ALK6 remained present in male urogenital ridges. This may point to additional functions for ALK2 and ALK6.

Müllerian duct regression involves a change in mesenchymal-epithelial interaction, resulting in destruction of the epithelial structure (Trelstad *et al.*, 1982). Incorporation of [^{3}H]-thymidine into separately cultured epithelial and mesenchymal ductal cells in the presence of AMH showed that DNA synthesis in epithelial cells was not changed, whereas the labeling index was significantly reduced in the mesenchymal cells. This suggests that the effect of AMH on the epithelial cells of the Müllerian ducts is via the surrounding mesenchymal cells (Tsuji *et al.*, 1992). This suggestion was supported by the localization of AMHRII mRNA expression in the mesenchymal cells (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994b). Therefore, expression of an AMH type I receptor in the mesenchymal cells of the Müllerian ducts is a prerequisite for proper AMH signaling. Using *in situ* hybridization, ALK2 mRNA expression in the urogenital ridge was shown to be present in the mesenchymal cells surrounding the Müllerian ducts but not in the cells near to the Wolffian ducts. In contrast, ALK6 mRNA expression in the urogenital ridge has been located in the epithelial cells, of both Müllerian and Wolffian ducts (Dewulf *et al.*, 1995). Thus, ALK2 is the type I receptor that fulfills all requirements of an AMHRI: it functions as AMHRI *in vitro*, and it is expressed in the urogenital ridge at the right time in the right cells.

Although the role of AMH in the gonads is less clear, some effects have been described. The phenotype of *AMH* or *AMHRII* knockout mice included the presence of Leydig cell hyperplasia, whereas transgenic mice overexpressing AMH developed Leydig cell hypoplasia (Behringer *et al.*, 1990; Behringer *et al.*, 1994; Mishina *et al.*, 1996). Fetal ovaries do not produce AMH, but AMHRII mRNA is expressed in these ovaries. Ovaries of transgenic mice overexpressing AMH develop testis cord-like structures and have decreased aromatase activity (Behringer *et al.*, 1990; Baarends *et al.*, 1994; Di Clemente *et al.*, 1994b), indicating the presence of a functional AMH receptor complex. Therefore, the candidate AMH type I receptor is expected to be expressed in both fetal testis and ovaries. Indeed, ALK2

mRNA expression was found in the fetal gonads of both sexes, at all time points studied during development. In contrast, ALK6 mRNA expression could not be detected in the fetal gonads. Postnatally, ALK6 mRNA expression was found only in the ovary but not in the testis, whereas ALK2 mRNA is expressed in gonads of both sexes. The fetal gonadal expression of ALK2 mRNA further supports our conclusion that ALK2, but not ALK6, is an AMH type I receptor. Ultimate proof that ALK2 can function as an AMH type I receptor may come from conditional knockout experiments, in which ALK2 function is specifically abrogated in the target sites of AMH.

The identification of ALK2 as a functional candidate AMH type I receptor, will enable us to study the downstream components of AMH. In this respect, a recently identified member of the Smad family, Smad8, is of interest (Chen *et al.*, 1997b). Smad8 is specifically phosphorylated by a constitutively active ALK2. Furthermore, Smad8 is able to rescue the expression of genes blocked by a dominant-negative ALK2. These results suggest that Smad8 may function as a downstream factor of ALK2 (Chen *et al.*, 1997b).

The expression pattern of ALK2 mRNA is not restricted to the target sites of AMH, which suggests that ALK2 may also function as a type I receptor for other ligands. A relatively high level of ALK2 mRNA was observed in fetal liver and adrenal. Since ALK2 has been suggested to serve as an activin type I receptor, ALK2 may be involved in the anti-proliferative effects of activin on hepatocytes and fetal adrenal cells (Spencer *et al.*, 1992; Hully *et al.*, 1994; Coerver *et al.*, 1996; Mesiano and Jaffe, 1997). In addition, ALK2 has been suggested to function as a BMP type I receptor, and several BMPs are expressed in the fetal liver and adrenal, in which they are suggested to play a role in growth and differentiation (Özkaynak *et al.*, 1992; King *et al.*, 1994; Song *et al.*, 1995; Knittel *et al.*, 1997). Recent data suggest that, in *Xenopus* embryos, ALK2 may indeed act as a BMP type I receptor (Armes and Smith, 1997). Downstream factors in the BMP signaling pathway may, therefore, also be of interest for AMH signaling. Smad1 and Smad5 were shown to act downstream of BMP2 (Yamamoto *et al.*, 1997). Interestingly, Smad5 expression was detected in the urogenital ridge (Meersseman *et al.*, 1997).

It will be of interest to study, which of the Smads acts downstream of ALK2 in response to AMH.

Acknowledgements

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Chapter Seven

Anti-Müllerian hormone action on Müllerian duct regression involves apoptosis

Chapter Seven

Anti-Müllerian hormone action on Müllerian duct regression involves apoptosis

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in preparation

Abstract

Anti-Müllerian hormone (AMH), a member of the transforming growth factor β (TGFβ) family, induces Müllerian duct regression in mammalian male fetuses. AMH elicits its effect on the epithelial cells of the Müllerian ducts via the surrounding mesenchymal cells. However, the molecular mechanism by which AMH induces Müllerian duct regression is not known, although it has been suggested that induction of apoptotic cell death is involved. Since members of the TGFβ family are able to induce apoptosis in various other cell systems, we investigated whether Müllerian duct regression is associated with this mechanism of programmed cell death. Indeed, using the TUNEL assay, apoptotic nuclear bodies were only detected in the Müllerian ducts of male fetuses during the critical period of Müllerian duct regression, but not in the Müllerian ducts of female fetuses. In addition, apoptotic bodies were absent from the Müllerian ducts of AMH-deficient male fetuses.

The AMH type II receptor and ALK2, recently identified as a strong candidate AMH type I receptor, are both expressed by the mesenchymal cells surrounding the Müllerian ducts. We investigated whether AMH is capable of inducing apoptosis through signaling by an AMHRII/ALK2 complex, in cultured cells. To this end, HEK-293 cells were cotransfected with AMHRII and ALK2. These transfected cells did not undergo apoptosis upon AMH exposure. In view of limited biological activity of AMH in different cell/tissue culture systems, an additional experiment was carried out. Transfection of ALK2 carrying a mutation that renders the type I receptor constitutively active resulted in a markedly increase in apoptotic cells. Cotransfection of green fluorescent protein showed that the apoptotic nuclei were only present in transfected cells.

The results described in this paper show that AMH action on Müllerian duct regression may involve induction of apoptosis, and that in cultured cells, the constitutively active candidate AMHRI is capable of induction of the apoptotic pathway. However, additional experiments are necessary to elucidate the exact molecular mechanism of Müllerian duct regression.

Introduction

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor β (TGFβ) family of growth and differentiation factors (Massagué, 1990). In contrast to other family members, which have a broad range of effects, the biological function of AMH is very restricted. AMH mRNA is expressed by preantral and small antral follicles in the adult ovary and may play a role in ovarian folliculogenesis (Baarends *et al.*, 1995b). Furthermore, the fetal and adult testes express AMH mRNA (Baarends *et al.*, 1995a), where AMH seems to be involved in the regulation of Leydig cell function, since AMH-deficient mice develop Leydig cell hyperplasia, and transgenic mice, which overexpress AMH develop Leydig cell hypoplasia (Behringer *et al.*, 1990; Behringer *et al.*, 1994). In the AMH-overexpressing mice and in cultured fetal ovaries, AMH was also shown to decrease aromatase activity (Behringer *et al.*, 1990). Nevertheless, AMH is best known for its specific role during sex differentiation. In the male fetus, AMH induces the regression of the Müllerian ducts, the anlagen of the female urogenital tract that, in female fetuses in the absence of AMH, will differentiate into the uterus, oviducts and upper part of the vagina (Münsterberg and Lovell-Badge, 1991). Regression of the Müllerian ducts occurs during a critical period of sensitivity to AMH, in the mouse around E13-15 (days *post coitum*). In the rat, exposure of female fetuses to AMH after E16 does not result in Müllerian duct regression (Tsuji *et al.*, 1992). Morphologically, regression of the Müllerian ducts is characterized by a whorl of condensed mesenchymal cells surrounding the Müllerian duct (Dyche, 1979). Electron microscopy demonstrated the dissolution of the basement membrane prior and during Müllerian duct regression, allowing direct contact between epithelial and mesenchymal cells and the possibility of the epithelial cells to enter the mesenchymal cell population (Trelstad *et al.*, 1982). Based on the observation that macrophages and apoptotic cells are present in the regressing ducts, it was suggested that apoptosis (programmed cell death) plays a role in Müllerian duct regression (Price *et al.*, 1977; Catlin *et al.*, 1997). However, with fluorescent labeling techniques in male alligator fetuses, and with the use of chick/quail chimeras, it was demonstrated that the epithelial cells migrated out of the Müllerian duct and were incorporated into the mesonephric

tubules (Hutson *et al.*, 1984; Austin, 1995). Nevertheless, since Müllerian duct regression may involve both apoptosis and cell migration, the results from these studies do not necessarily exclude one another.

From *in vitro* experiments it became clear that AMH elicits its effect on the epithelial cells of the Müllerian ducts via the surrounding mesenchymal cells (Tsuji *et al.*, 1992). Accordingly, the mesenchymal cells are also the cells that express the AMH type II receptor (AMHRII) mRNA during the period that the Müllerian ducts respond to AMH (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994b). However, the molecular mechanism by which AMH induces Müllerian duct regression is not known yet.

AMHRII is a member of the transmembrane serine/threonine kinase receptor family through which the members of the TGFβ superfamily exert their actions. Based on their structure, this receptor family can be subdivided into type I and type II receptors. The type I receptors, also known as activin receptor-like kinases (ALKs), are characterized by a glycine/serine-rich motif (GS box) that is located N-terminal of the kinase domain, whereas the type II receptors contain a longer serine-rich C-terminus (Ten Dijke *et al.*, 1994a; Massagué and Weis-Garcia, 1996). Upon ligand binding by the type II receptor, the type I receptor is recruited into the complex, and serine residues in the GS box become phosphorylated by the type II receptor (Wrana *et al.*, 1994). Replacement of these serines in the GS box by valine residues rendered the receptor unable to transduce signals. In addition to phosphorylation of residues in the GS box, two additional phosphorylated threonines, located near the start of the kinase domain, play a role in receptor activation. Replacement of Thr204 with aspartic acid results in a TGFβ type I receptor with constitutive activity that signals in the absence of ligand and type II receptor (Wrana *et al.*, 1994).

Although the signaling mechanism of several members of the TGFβ family has been well studied, little is known about AMH signaling, also because the type I receptor for AMH had not been identified. Binding studies with AMH proved to be difficult, since radiolabeling of AMH seems to abolish its ability to bind to the receptor (Donahoe *et al.*, 1977; Di Clemente *et al.*, 1994b). To avoid difficulties in binding assays with radiolabeled AMH, we have developed a cell culture system based on the inhibitory action of AMH on the FSH-induced aromatase promoter activity.

Using this *in vitro* model, it was shown that inhibition of FSH-induced aromatase activity by AMH was transduced through an AMHRII/ALK2 receptor complex. Furthermore, ALK2 mRNA expression was found in the mesenchymal cells surrounding the Müllerian ducts, colocalizing with AMHRII at the site where AMH elicits its effects. Therefore, it was proposed that ALK2 is a strong candidate AMH type I receptor (Visser *et al.*, submitted).

The present experiments concern the mechanism of AMH action and the role of ALK2 in Müllerian duct regression. We have investigated whether apoptotic cells are present in the Müllerian duct during the period of Müllerian duct regression. Furthermore, it was studied whether a constitutively active ALK2 can induce apoptosis *in vitro*. The results described herein may contribute to the understanding of molecular and cellular aspects of the mechanism of AMH signaling.

Materials and Methods

TUNEL assay

Apoptotic cells were detected *in situ* using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method (Gavrieli *et al.*, 1992). FVB mice were kept under standard animal housing conditions in accordance with NIH Guidelines for the Care and Use of Experimental Animals. Vaginal plug detection was considered day 0 (E0) of pregnancy. Control and AMH-deficient (Behringer *et al.*, 1994) pregnant mice were sacrificed by cervical dislocation at E13 and E15 of gestation. To determine the sex of the fetuses, PCR reactions using placental genomic DNA (Hogan *et al.*, 1986) were performed as described by Mitchell *et al.* (1991), using primers for the mouse genes *Sbx* and *Sby* (Kay *et al.*, 1991; Mitchell *et al.*, 1991). Fetuses were isolated, fixed overnight in 4% neutral buffered formalin, embedded in paraffin and sectioned transversally at 5 μm.

Tissue sections were rehydrated and incubated with proteinase K (7.5 μg/ml) in PBS for 15 min at room temperature, rinsed in distilled water, and incubated with 3% (v/v) H_2O_2 in PBS for 5 min to block endogenous peroxidase activity. Sections were pretreated with terminal

deoxynucleotidyl transferase (TdT) buffer (0.1 M sodium-cacodylate, 1 mM $CoCl_2$, 1 mM DTT, 5% (w/v) BSA) for 5 min before incubation with TdT-buffer containing 0.01 mM Biotin-16-dUTP (Boehringer Mannheim, Almere, The Netherlands) and 0.3 U/μl TdT-enzyme (Promega, Leiden, The Netherlands) for at least 30 min at 37 C. Enzyme reactions were terminated in TB-buffer (300 mM NaCl, 30 mM sodiumcitrate) for 15 min. Before and after incubation in PBS containing 2% (w/v) BSA, slides were rinsed in water and PBS for 5 min each. Next, slides were incubated with streptavidin-biotinylated horseradish-peroxidase complex (1:200, DAKO; Glostrup, Denmark) for 30 min. After rinsing in water and PBS, sections were stained with 0.075% DAB (3,3'-diaminobenzidine; Fluka, Buchs, Switzerland) in PBS for 5 min. Finally, sections were rinsed in water and counter-stained with hematoxylin.

Transfections

cDNAs encoding wild type mouse ALK2 and the constitutively active mouse ALK2-Q204D (J. de Winter, unpublished) were subcloned into the EcoRI and the BamHI site of pSG5, respectively (Green *et al.*, 1988). HEK-293 (human embryonic kidney) cells were maintained in DMEM-F12 (Life Technologies, Breda, The Netherlands) and 10% fetal calf serum (SEBAK, Aidenbach, Germany) at 37 C in a 5% CO_2 in air atmosphere. Cells were split into 6 well plates and transiently transfected overnight with 1 μg of control expression vector, wild type ALK2 or ALK2-Q204D, using the calcium phosphate method (Chen and Okayama, 1987) at 35 C and 3% CO_2. In some experiments, cells were cotransfected with 1 μg AMHRII and 1 μg ALK2. In addition, the β-galactosidase expression plasmid pCH110 (1 μg/ml precipitate; Hall *et al.*, 1983) was added to measure the number of transfected cells in time, or a vector expressing enhanced green fluorescent protein (pEGFP; 1 μg/ml precipitate; Clontech, Westburg, Leusden, The Netherlands) was added to visualize transfected cells. Cells transfected with AMHRII and ALK2 were incubated with vehicle, or with 1.8 μg/ml AMH obtained from a HEK-293 cell line that constitutively expresses rat AMH (Nachtigal and Ingraham, 1996). Cells were lysed and β-galactosidase activity was measured (Blok *et al.*, 1992) 1, 12, 24 or 36 h after

transfection. At the same time points cells were studied for the presence of apoptotic nuclei.

To visualize apoptosis in unfixed monolayers, cells were incubated for 30 min with the fluorescent DNA binding dyes Hoechst 33342 and propidium iodide (PI) at a final concentration of 1 μg/ml and 3.5 μg/ml, respectively. Hoechst freely crosses the intact and damaged plasma membranes, and leads to a blue staining of the DNA, whereas PI can only penetrate cells with damaged membranes, resulting in pink nuclear staining.

Results

Detection of apoptosis during Müllerian duct regression

To determine whether the AMH-induced regression of the Müllerian ducts might be associated with apoptosis, urogenital ridges of E13 and E15 fetuses were studied for the presence of apoptotic cells. The TUNEL assay was used as an *in situ* apoptosis detection method. With this assay, cells in which DNA fragmentation has occurred are labeled using incorporation of Biotin-16-dUTP with terminal deoxynucleotidyl transferase, and the labeled cells are visualized with streptavidin-biotinylated horseradish-peroxidase complex.

Apoptotic cells were observed in the urogenital ridges of both male and female fetuses at E13 and E15 (Figures 7.1 and 7.2). In male fetuses, apoptotic cells were specifically located in the epithelial compartment of the regressing Müllerian ducts (Figure 7.1A and B, Figure 7.2A and B), whereas in female fetuses apoptotic cells were present in the epithelial part of the Wolffian ducts. The latter result is in agreement with degeneration of the Wolffian ducts in female fetuses, due to absence of testosterone (Figure 7.1C and D). In female fetuses and in male AMH-deficient fetuses no AMH is present. Accordingly, hardly any or no apoptotic bodies were found in the Müllerian ducts of these fetuses (Figure 7.1C-F).

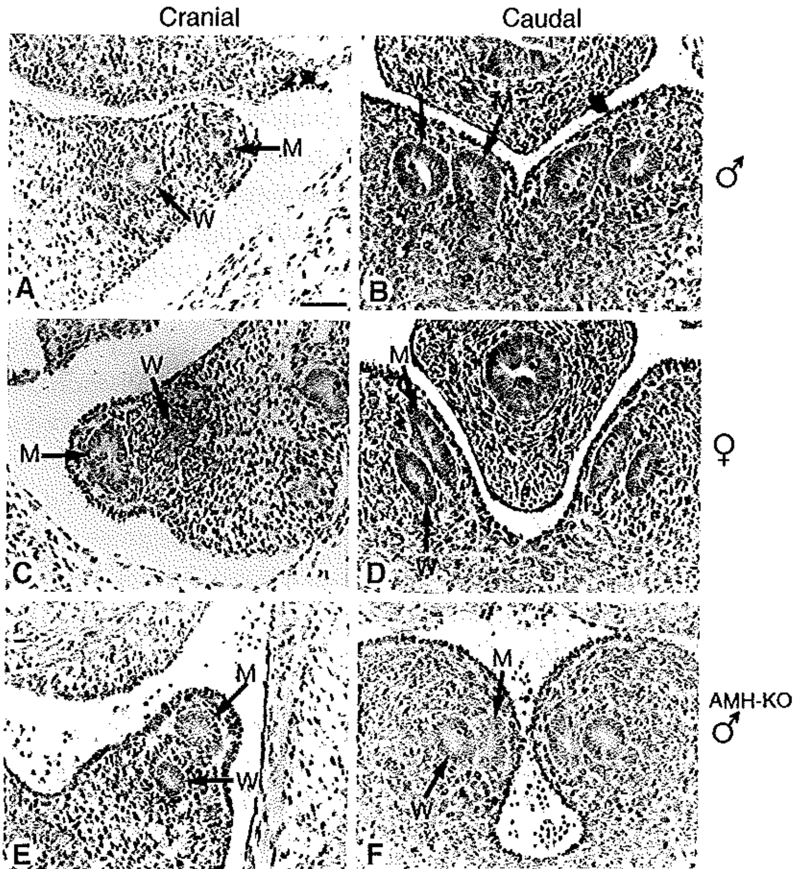

Figure 7.1. **Detection of apoptosis in the reproductive tract of control fetuses and AMH-deficient male fetuses at E13 using the TUNEL assay.**

Apoptotic cells were detected along the whole cranial-to-caudal axis of the Müllerian duct of male fetuses, before regression of the duct is morphologically visible (A and B). In female fetuses, little or no apoptotic cells were detected in the Müllerian duct but apoptotic cells were present in the degenerating Wolffian duct (C and D). In AMH-deficient male fetuses (AMH-KO), in which the Müllerian ducts do not regress due to the absence of AMH, little or no apoptotic bodies were detected along the whole axis of the Müllerian ducts (E and F). Apoptotic bodies are indicated with small arrowheads. Müllerian duct (M) and Wolffian duct (W) are indicated with large arrows; in the caudal sections both left and right ducts are present. Scale bar represents 50 µm.

It has been observed that, morphologically, Müllerian duct regression progresses in a cranial to caudal direction (Visser *et al.*, 1998). At E13, the cranial parts of the Müllerian ducts in male fetuses are narrowed and a whorl of mesenchymal cells is visible, but these morphological features of Müllerian duct regression are not present in the caudal part of the male Müllerian ducts, at the same stage of development. However, apoptotic cells were present in both the cranial and caudal regions of the Müllerian ducts of male fetuses at E13 (Figure 7.1A and D), suggesting that the apoptotic process of Müllerian duct regression precedes the morphological characteristics. At E15, the morphological features of Müllerian duct regression are found along the axis of the Müllerian ducts of male fetuses (Figure 7.2A and B), and at this time point an increase in apoptotic cells is found compared to the number found at E13. In female fetuses at E15, apoptotic bodies are found at the midline of the caudal part of the Müllerian ducts (Figure 7.2D). This is of interest, because the caudal part of the left and right Müllerian ducts fuse to form the upper part of the vagina. The presence of apoptotic bodies at the midline of the Müllerian ducts may reflect this fusion process.

Constitutively active ALK2 induces apoptosis in vitro

In a previous study, ALK2 was identified as a candidate AMH type I receptor (Visser *et al.*, submitted). Therefore, we studied the ability of ALK2 to induce apoptosis *in vitro*, using a constitutively active ALK2 mutant (ALK2-Q204D). HEK-293 cells were transiently transfected with ALK2 or ALK2-Q204D, together with either pEGFP or pCH110, and the transfected cells were studied subsequently for the presence of apoptotic nuclei at different time points after transfection.

To detect intact cells, cells undergoing apoptosis, or dead cells resulting from apoptotic and/or necrotic processes in the same sample at the same time, cells were stained with Hoechst and PI. Normal cells are characterized by a faint blue staining of the intact nucleus, whereas apoptotic cells stain bright blue due to nuclear fragmentation and condensation. Dead cells are red fluorescent due to the uptake of PI. Green staining can be observed in cells that express the green fluorescent protein.

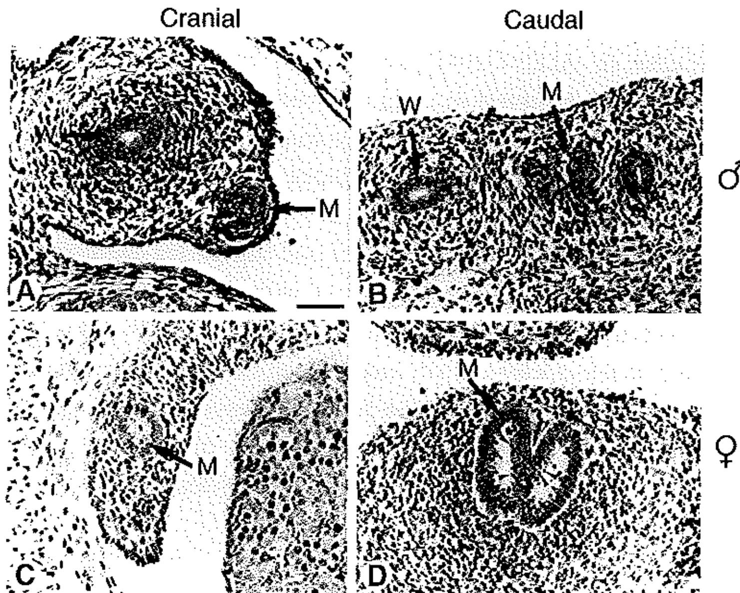

Figure 7.2. Detection of apoptosis in the reproductive tract of control fetuses at E15 using the TUNEL assay.
In male fetuses, many apoptotic cells are detected in the regressing Müllerian duct, but only few in the Wolffian duct (A and B). In the female fetuses, the Wolffian duct has degenerated (C and D). Apoptotic cells are found in the midline of the caudal region of the Müllerian ducts (D) Apoptotic bodies are indicated with small arrowheads. Müllerian duct (M) and Wolffian duct (W) are indicated with large arrows; in the caudal sections both left and right ducts are present. Scale bar represents 50 µm.

No apoptotic nuclei were observed in cells transfected with the wild type ALK2 at any time point up to and including 30 h after transfection (Figure 7.3A-C). Cells transfected with the control vector gave similar results. For HEK-293 cells transfected with ALK2-Q204D, the first apoptotic cells were observed 24 h after transfection (Figure 7.3D). Apoptosis is characterized by nuclear fragmentation, and apoptotic cells were found to round up and detach from the dish (Figure 7.3D, F). The apoptotic effect of the constitutively active ALK2 was probably a cell-autonomous effect, since

apoptosis was only observed in cells that expressed pEGFP, as indicated by the green staining (Figure 7.3E). At 30 h after transfection, the number of apoptotic nuclei in ALK2-Q204D transfected cells had increased, and apoptosis was also observed in cells that did not express pEGFP (results not shown). This latter effect may be explained by the loss of GFP from leaky apoptotic cells, since in late apoptotic cells GFP staining was less bright.

To obtain a quantitative estimation of cell death, we determined the β-galactosidase activity in cells transfected with ALK2 or its constitutively active counterpart (Figure 7.4). With time after transfection, a similar increase in β-galactosidase activity was measured in cells transfected with either the control vector or wild type ALK2. However, for cells transfected with ALK2-Q204D, the β-galactosidase activity at 24 and 30 h after transfection was much lower, compared to cells transfected with wild type ALK2 (Figure 7.4).

In an *in vitro* system based on the inhibitory action of AMH on FSH-induced aromatase activity, ALK2 was identified as a candidate AMH type I receptor (Visser *et al.*, in preparation). Therefore, HEK-293 cells were transiently transfected with ALK2 and AMHRII, and incubated with or without AMH, to study the ability of ALK2 to relay the apoptotic signal of AMH. Unexpectedly, apoptotic cells were not observed upon AMH stimulation (results not shown).

Since ALK2 may also function as a type I receptor for other ligands (Attisano *et al.*, 1993; Armes and Smith, 1997), induction of apoptosis in response to bone morphogenetic protein (BMP) 2, BMP7 or activin was studied in the present cell culture system. HEK-293 cells were transfected with the activin type II receptors (ActRIIA or ActRIIB) or bone morphogenetic protein type II receptor (BMPRII), together with ALK2, and transfected cells were incubated with activin, BMP2, or BMP7. However, no induction of apoptosis was observed with all receptor combinations (results not shown).

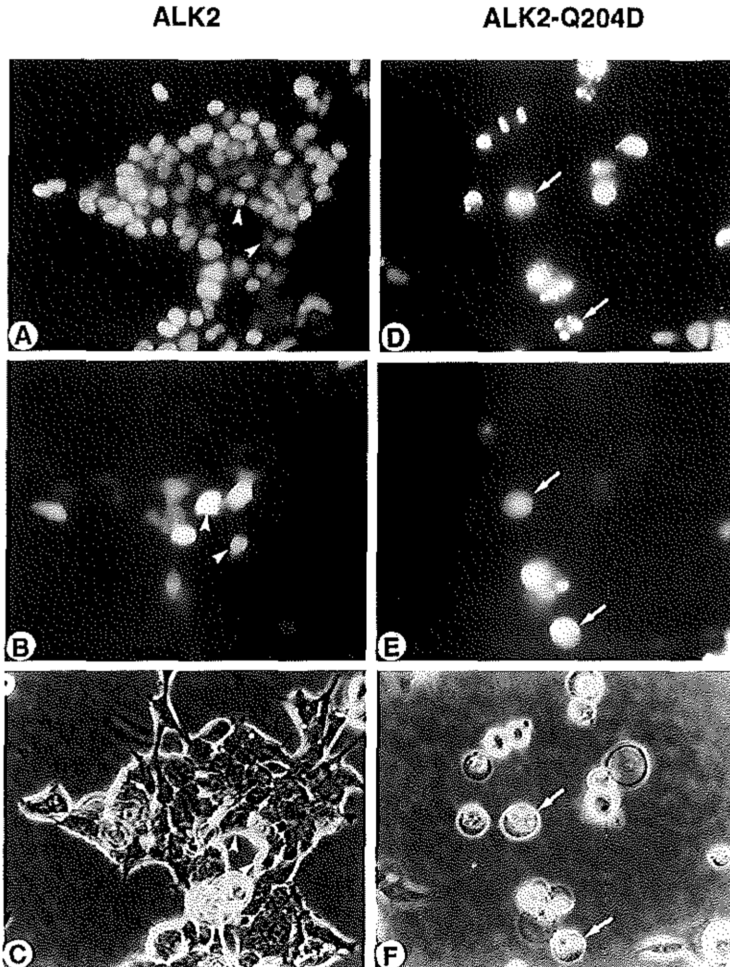

Figure 7.3. Constitutively active ALK2 induces apoptosis *in vitro*.
HEK-293 cells were transfected with ALK2 (A-C), or the constitutively active ALK2-Q204D (D-F), together with pEGFP. Nuclei were stained 30 h after transfection with Hoechst/propidium iodide, and observed using a fluorescent microscope (A and D). GFP staining indicates transfected cells (B and E), and confirmed that nearly all cells transfected with ALK2-Q204D undergo apoptosis (E). Normal cells remained attached to the culture dish (C), whereas apoptotic cells rounded up and detached (F). Arrows indicate examples of apoptotic cells and arrowheads indicate examples of normal cells.

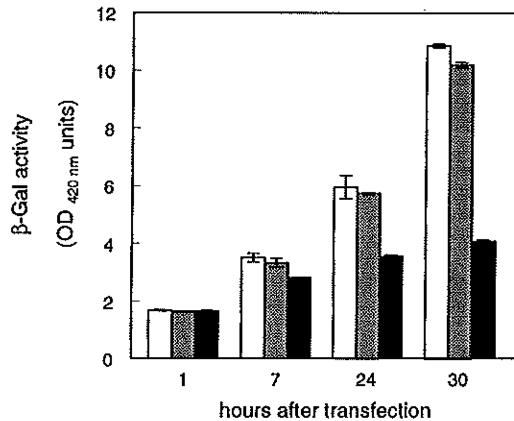

Figure 7.4. **Constitutively active ALK2-induced apoptosis increases in time.**
HEK-293 cells were transfected with pSG5, ALK2, or ALK2-Q204D, together with a vector expressing β-galactosidase (β-Gal). β-Gal activity was measured at 1, 7, 24 and 30 h after transfection. In ALK2-Q204D transfected cells, no increase of β-Gal activity was observed 24 h after transfection. pSG5, open bars; ALK2, grey bars; ALK2-Q204D, closed bars.

Discussion

Anti-Müllerian hormone (AMH) plays an essential role in the induction of Müllerian duct regression in male fetuses. This Müllerian duct regression is morphologically characterized by the whorled pattern of condensed mesenchymal cells surrounding the Müllerian ducts (Price *et al.*, 1977), indicating that the mesenchymal cells are an important target in the action of AMH. Concomitant with the condensation of the mesenchymal cells, the basement membrane is disintegrating, allowing direct contacts between mesenchymal and epithelial Müllerian duct cells (Dyche, 1979; Price *et al.*, 1979; Trelstad *et al.*, 1982). At the electron microscopic level, an increase in the number of lysosomes in epithelial ductal cells was observed, which may be the first morphological event of the removal of epithelial Müllerian duct cells, followed by an invasion of the duct by macrophages from the adjacent mesenchyme. The latter observations have led to the suggestion that Müllerian duct regression is initiated by programmed cell death or apoptosis (Price *et al.*, 1977; Price *et al.*, 1979).

Several members of the TGFβ family have been implicated to play a role in induction of apoptosis in various tissues, and in cultured cells. Activin induces apoptotic cell loss in rodent liver and in hepatocyte cultures (Hully *et al.*, 1994). Furthermore, apoptosis is induced in the liver of α-inhibin-deficient mice, probably due to a relatively high activin level in these animals (Matzuk *et al.*, 1992; Matzuk *et al.*, 1994). In addition, activin signaling leads to apoptosis in myeloma cells, B cell hybridoma cells, and LNCaP (lymph node carcinoma of the prostate) cells (Koseki *et al.*, 1995; Wang *et al.*, 1996; Yamato *et al.*, 1997). BMP4 action is associated with induction of apoptosis in neural crest cells and dental epithelia, and this factor is also required for apoptosis of interdigital tissue in developing chick limbs (Graham *et al.*, 1994; Yokouchi *et al.*, 1996; Zou and Niswander, 1996; Macias *et al.*, 1997; Jernvall *et al.*, 1998). Furthermore, BMP4 induces cell death in fetal lung mesenchyme (Bellusci *et al.*, 1996). TGFβ was shown to induce apoptosis in mink lung cells and in gonocytes in testis of 25-day-old rats (Souchelnytskyi *et al.*, 1996; Olaso *et al.*, 1998).

Since members of the TGFβ family can induce apoptosis, it is an obvious hypothesis that apoptosis in the regressing Müllerian ducts is triggered by AMH. To investigate whether apoptotic cell death plays a role in the series of events that follows AMH action on the Müllerian ducts, Müllerian ducts of fetal mice were studied for the presence of apoptotic bodies during the AMH sensitive period of Müllerian duct regression. Indeed, markers for apoptotic cell death were present in the Müllerian ducts of male fetuses, and were absent in female fetuses and AMH-deficient male fetuses. These results perfectly correlate with the involvement of apoptotic cell death in the events evoked by AMH action on the Müllerian ducts.

Previously, Catlin *et al.* (1997) have demonstrated induction of apoptosis in cultured female urogenital ridges upon AMH exposure, although only few cells were found to be apoptotic at a given time point in their experiments, leaving open the question whether apoptosis is the dominant pathway for male Müllerian duct regression. In the present study, ample numbers of apoptotic cells were observed, and we conclude that apoptosis is the main cellular mechanism of AMH-induced Müllerian duct regression.

Recently, we have reported that, Müllerian duct regression is progressing in a cranial to caudal direction (Visser *et al.*, 1998) and it was expected that also apoptosis would follow this pattern. However, apoptotic cells were detected along the entire cranial-to-caudal axis of the Müllerian ducts at E13, suggesting that the apoptotic signal for regression precedes the morphological signs of Müllerian duct regression.

The apoptotic cells are predominantly found among the epithelial cells of the Müllerian duct, although some apoptotic cells are found in the mesenchyme surrounding the Müllerian ducts. *In vitro* culture experiments with Müllerian duct epithelial and mesenchymal cells revealed an absence of a direct effect of AMH on epithelial cells, suggesting that the disappearance of epithelial cells is mediated via the surrounding mesenchymal cells (Tsuji *et al.*, 1992). In support of these observations, AMHRII mRNA and also mRNA encoding ALK2, a candidate AMH type I receptor, are expressed in the mesenchymal cells surrounding the Müllerian ducts (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994b; Visser *et al.*, submitted). Since the epithelial cells undergo apoptosis, the mesenchymal cells most likely produce an apoptosis-inducing factor in response to AMH action on the mesenchymal cells. Members of the tumor necrosis factor family, such as Fas ligand, might be involved, since these factors can induce apoptosis on neighboring receptor-bearing cells (Nagata and Golstein, 1995).

Next, it was studied whether the AMH-induced apoptosis in the Müllerian ducts can be mediated by ALK2. Many of the HEK-293 cells transfected with constitutively active ALK2-Q204D went into apoptosis, supporting the possible role of ALK2 as an AMH type I receptor. Transfection of HEK-293 cells with constructs encoding other constitutively active mutant type I receptors (ALK1-Q201D, ALK3-Q233D, ALK4-T206D, ALK5-T204D and ALK6-Q203D) did not induce apoptosis (results not shown). The induction of apoptosis by ALK2-Q204D may be a cell-autonomous effect, since apoptosis was restricted to cells that displayed green staining as a result of GFP expression. However, at 30 h after transfection, apoptosis was also detected in non-green staining cells. This result could be explained by the loss of GFP from leaky apoptotic cells, although it may also be suggested that ALK2-Q204D may signal to other,

nontransfected cells to switch on the apoptotic program, reflecting the mesenchymal-epithelial interaction in Müllerian ducts.

In a previous study, we showed that AMH signaling mediated by an AMHRII/ALK2 complex resulted in inhibition of FSH-induced aromatase promoter activity (Visser *et al.*, submitted). That observation led us to propose that AMHRII and ALK2 form a functional AMH type II/type I receptor complex that can transduce the AMH signal. Although HEK-293 cells transfected with cDNAs encoding AMHRII and ALK2 do respond to AMH when the transcriptional activity of the aromatase promoter is used as a response parameter, these cells did not display any characteristics of apoptosis upon exposure to AMH. The absence of AMH-induced apoptosis in HEK-293 cells indicates that AMH-induced effects on the Müllerian ducts *in vivo*, that clearly do involve apoptosis, cannot be mimicked *in vitro*. Possibly, one or several factors are missing from these cells, which are essential for an AMH effect on apoptosis. Alternatively, signals that are conferred by the constitutively active ALK2 may be much stronger than the signal of a ligand-receptor complex. Indeed, although ALK2 may also function as a type I receptor for BMPs and activin (Attisano *et al.*, 1993; Armes and Smith, 1997), TGFβ family members that induce apoptosis in some tissues, HEK-293 cells expressing the type II receptor together with ALK2 also do not display apoptosis upon addition of the relevant ligands.

The importance of a fully activated type I receptor in ligand-induced signaling was demonstrated with a mutant TGFβ type I receptor ALK5. The wild type ALK5 is phosphorylated at multiple serine and threonine residues in and outside the GS box (Wieser *et al.*, 1995; Souchelnytskyi *et al.*, 1996; Willis and Mathews, 1997). In a study by Souchelnytskyi *et al.* (1996) it was shown that mutation of a serine residue at position 165 of ALK5, only abrogates an effect of TGFβ on induction of apoptosis but not other signaling responses of ALK5. One might argue that the signaling responses of type I receptors are dependent on the phosphorylation state of the receptor, and that, in addition to ligand stimulation, cell specific factors play a role in determining the phosphorylation pattern.

In conclusion, the present *in vivo* and *in vitro* observations on apoptosis strongly suggest that the action of AMH on regression of the Müllerian ducts in male fetuses involves apoptosis of the epithelial cells.

Acknowledgement
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Chapter Eight

General Discussion

Chapter Eight

General Discussion

8.1 Introduction

Early in development, the phenotypic sex of the fetus is morphologically not apparent. During this so-called indifferent stage, the formation of the gonadal anlagen and the Wolffian and Müllerian ducts takes place. Following testis determination, the Wolffian ducts in the male differentiate into epididymides, vasa deferentia and seminal vesicles, due to the action of testosterone from the testis. The Müllerian ducts, which form the anlagen of the Fallopian tubes, uterus and upper part of the vagina, appear to develop in the female without a need for action of estrogens; these hormones have their actions mainly during puberty and adult life in the female. The Wolffian ducts in the female fetus do not appear to be removed actively, but rather degenerate as a result of lack of stimulation in the absence of androgens. In contrast, regression of the Müllerian ducts is caused by the presence of the testicular product anti-Müllerian hormone (AMH) acting through its AMH type II receptor (AMHRII) and a type I receptor. Several clinical syndromes characterized by Müllerian anomalies exist, in which impairment and/or dysregulation of AMH action may play a role. Most cases of persistent Müllerian duct syndrome (PMDS), a rare form of male pseudohermaphroditism with structures derived from Müllerian ducts found in 46,XY boys, are caused by inactivating mutations in the *AMH* or *AMHRII* genes (Imbeaud *et al.*, 1994; Imbeaud *et al.*, 1995). Indeed, a similar phenotype has been observed in *AMH* and *AMHRII* knockout mice (Behringer *et al.*, 1990; Mishina *et al.*, 1996). Another syndrome, Müllerian duct aplasia, is characterized by complete absence of Müllerian duct derivatives in women (Shokeir, 1978). A similar phenotype is found in female transgenic mice overexpressing AMH (Behringer *et al.*, 1990), but it is not clear whether dysregulation of AMH action plays a causative role in the origin of Müllerian aplasia in women.

In the next paragraphs, a possible role of dysregulation of AMH signaling in the origin of Müllerian duct anomalies is discussed, in the context of the results described in the previous chapters and data in the literature (paragraph 8.2). Subsequently, several suggestions for future research on the molecular mechanism of AMH signaling are given (paragraph 8.3). These suggestions are partially based on a few unpublished observations, which are included in this chapter.

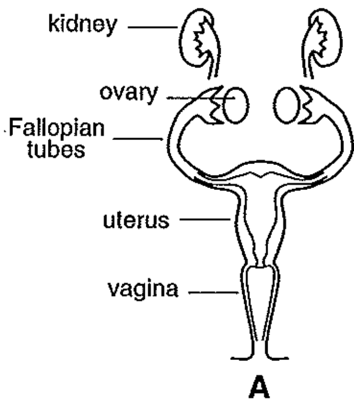
8.2 Müllerian duct aplasia

8.2.1 Clinical and genetic characteristics

Congenital anomalies of the development of the Müllerian ducts are common, occurring with a prevalence of up to 2% (Golan *et al.*, 1989). Several different types of Müllerian duct anomalies have been described. The pair of Müllerian ducts gives rise to the Fallopian tubes and, upon fusion, also to the uterus and upper part of the vagina (Grünwald, 1941). Failure of this fusion results in a septate uterus or a bicornuate uterus, with or without a septate vagina (Figure 8.1 G-I;Sarto and Simpson, 1978). Fusion of the Müllerian ducts occurs during week 9-12 of pregnancy in human and during E15-18 in the mouse (Grünwald, 1941; Sarto and Simpson, 1978). This process of Müllerian duct fusion may be associated with apoptosis, as indicated by the high number of apoptotic cells in the midline of the fusing Müllerian ducts at E15 in female mice (Chapter 7, Figure 7.2).

In addition to uterine fusion defects, anomalies are found that reflect partial or complete absence of Müllerian duct derivatives (Figure 8.1 B-F). This form of Müllerian duct anomalies is known as Müllerian aplasia, also referred to as the Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome (Sarto and Simpson, 1978; Shokeir, 1978; Golan *et al.*, 1989).

Figure 8.1. (next page) **Anomalies of the female reproductive tract.**
A: Normal Müllerian duct differentiation into Fallopian tubes, uterus and vagina. B-F are schematic representations of the different forms of Müllerian aplasia, B: absence of the upper part of the vagina; C: rudimentary uterus and absence of the upper part of the vagina; D: complete absence of uterus and upper part of the vagina; E: absence of Fallopian tubes, uterus and upper part of the vagina, described by Shokeir (1978) as *complete* Müllerian aplasia. F: unilateral absence of Fallopian tubes, and complete absence of uterus and the upper part of the vagina. All appearances of Müllerian aplasia may be associated with abnormalities or absence of the kidneys. G-I represent examples of uterine fusion anomalies. G: septate uterus; H: bicornuate uterus; I: bicornuate uterus and septate vagina.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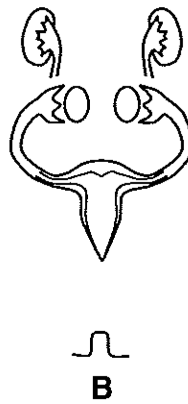
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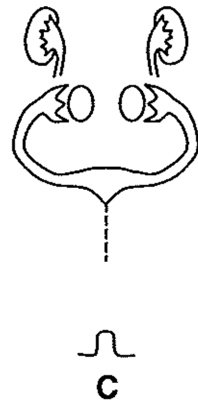
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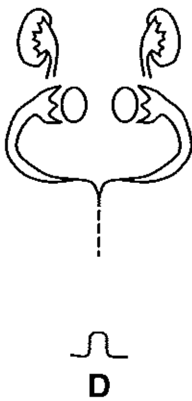
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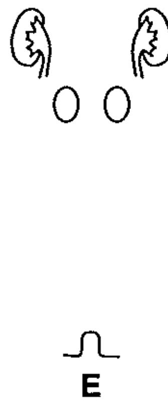
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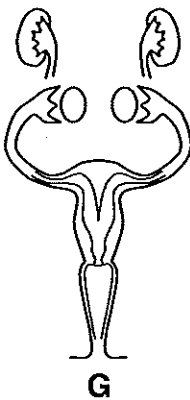
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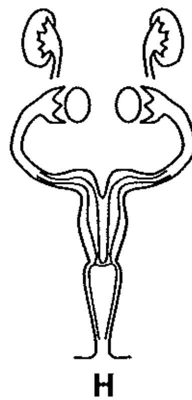
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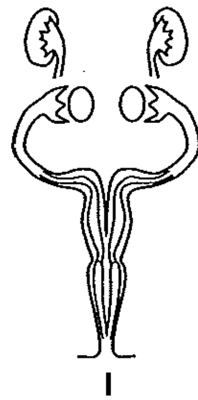
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Müllerian aplasia patients have a 46,XX karyotype, develop normal female external genitalia and secondary sex characteristics develop normally at puberty, which indicates the presence of functional ovaries. Because patients with Müllerian aplasia lack the upper part of the vagina, resulting in a blind ending vagina, they are diagnosed with primary amenorrhea (Shokeir, 1978; Lindenman *et al.*, 1997). The degree at which other Müllerian duct structures (uterus and Fallopian tubes) are absent varies (Sarto and Simpson, 1978; Shokeir, 1978; Golan *et al.*, 1989). Based on this variation, Stelling *et al.* (1997) divided Müllerian aplasia patients in two groups. The first group represents the classic MRKH syndrome, which is characterized by the presence of normal Fallopian tubes attached to symmetrical uterine buds, whereas in the second group, the atypical MRKH syndrome, also the Fallopian tubes are affected. In addition, the atypical form is frequently associated with extragenital abnormalities. One third of the Müllerian aplasia patients have renal abnormalities, such as absent or ectopic kidneys, or horseshoe kidneys (Stelling *et al.*, 1997), and the body side of kidney abnormalities was found to correspond with the side of Müllerian defects (Tarry *et al.*, 1986). When only one kidney is present, the ipsilateral Müllerian duct develops normally or partially, while the contralateral duct is absent or abnormal. In addition, the contralateral ovary was found to be absent in several cases of Müllerian aplasia. When both kidneys are present, the Müllerian defects are symmetrical (Tarry *et al.*, 1986). Furthermore, spinal anomalies, such as asymmetric, fused or wedged vertebrae, and skeletal anomalies, such as absence of digits and syndactyly, are found in some of the Müllerian aplasia patients (Lindenman *et al.*, 1997; Stelling *et al.*, 1997).

According to Shokeir (1978), a distinction in Müllerian aplasia can be made based on the complete or partial absence of the Müllerian ducts. In complete Müllerian aplasia, the uterus and upper part of the vagina are missing and Fallopian tubes are rudimentary or absent, whereas patients with partial Müllerian aplasia also lack the upper part of the vagina but have a rudimentary uterus and normal Fallopian tubes. In addition, renal and urinary malformations are found in patients with partial Müllerian aplasia (Shokeir, 1978). The classifications of Müllerian aplasia patients by

Shokeir (1978) and Stelling *et al.* (1997) do not correspond completely, indicating the complexity of the syndrome of Müllerian aplasia.

Familial analysis of 13 unrelated patients with complete Müllerian aplasia revealed that other female relatives were similarly affected, whereas no abnormalities in male relatives were described. Based on this study, Shokeir (1978) concluded that the pedigree pattern of Müllerian aplasia patients was consistent with a sex-limited autosomal dominant inheritance.

8.2.2 Possible causes of Müllerian aplasia

The variability in the partial or complete absence of Müllerian duct derivatives in patients with Müllerian aplasia, complicates the identification of a direct cause.

The presence of rudimentary Fallopian tubes or uterus in Müllerian aplasia patients, suggests that the Müllerian ducts did not completely fail to develop. Therefore, factors that play a role in early intermediate mesoderm induction and initial induction of formation of Wolffian and Müllerian ducts are not likely to be important. Factors that are involved in the formation of the Müllerian ducts themselves or their regression may be more relevant in this respect. Several hypotheses are discussed below.

AMH

In male fetuses, Müllerian duct regression is induced by AMH. In the female, however, the ovaries do not produce AMH until after birth (Münsterberg and Lovell-Badge, 1991). It has been suggested that mutations in the *AMH* gene promoter, resulting in ectopic expression of AMH also during the female fetal period, might cause complete Müllerian aplasia (Lindenman *et al.*, 1997). Such a mutation would correspond with an autosomal sex-limited inheritance pattern, since the *AMH* gene is located on human chromosome 9 (Cohen-Haguenauer *et al.*, 1987). Ectopic expression of AMH in female mice indeed results in complete absence of Müllerian ducts (Behringer *et al.*, 1990). However, whereas Müllerian aplasia patients have normal ovaries, ectopic AMH expression in transgenic female mice resulted in aberrant ovarian development, associated with meiotic arrest, loss of germ cells, and presence of testis

cord-like structures (Behringer *et al.*, 1990), which would argue against a role for AMH in Müllerian aplasia. It has to be taken into account, that the AMH level in AMH-overexpressing mice is very high (Behringer *et al.*, 1990; Lyet *et al.*, 1995). It is not known whether a low or a normal level of AMH in female fetuses may result in Müllerian duct regression but normal ovarian development.

Aberrant expression of the *AMH* gene in the female fetus might be caused by a mutation in the *AMH* gene promoter, but may also result from changes in the factors that regulate AMH mRNA expression. As discussed in Chapter 1, in an *in vitro* cell culture system, SF-1 and WT1 interact synergistically in the regulation of *AMH* gene expression, and this synergism is abrogated by DAX-1 (Nachtigal *et al.*, 1998). In the fetal ovary, this inhibitory effect of DAX-1 may be essential for suppression of *AMH* gene transcription prior to the downregulation of SF-1, and it has been suggested that the relative levels of SF-1, WT1, and DAX-1 regulate AMH mRNA expression (Chapter 1). Both an increased level of WT1 or a decreased level of DAX-1 may cause upregulation of AMH mRNA expression in the human fetal ovary. Since absence of DAX-1 action not only may cause aberrant expression of AMH, but also has been found to result in adrenal hypoplasia in human (Muscatelli *et al.*, 1994; Zanaria *et al.*, 1994), DAX-1 is not a likely candidate for playing an active role in the development of Müllerian aplasia. *WT1*, a gene also involved in early kidney development (Reddy and Licht, 1996), may be of more interest, because Müllerian aplasia is often associated with kidney abnormalities.

AMHRII

It can be suggested that activating mutations in the *AMHRII* gene could be another cause for Müllerian aplasia (Lindenman *et al.*, 1997). AMHRII is a member of the transmembrane serine/threonine kinase receptor family that consists of type II and type I receptors (Ten Dijke *et al.*, 1994a). As described in Chapter 2, a type II receptor binds the ligand upon which a type I receptor is recruited into the complex. The type II receptor contains constitutive kinase activity but is unable to signal by itself, and signaling is achieved by activation of the type I receptor (Wrana *et al.*, 1994). Therefore, an activating mutation of the type II receptor should mimic the effect of

ligand binding. A somewhat similar mutation is found in the epidermal growth factor receptor (EGFR), a tyrosine kinase receptor that signals upon ligand-dependent receptor dimerization. A natural occurring mutation in the EGFR results in a receptor that lacks a portion of the extracellular ligand-binding domain due to deletion of exons 2 to 7 (Huang *et al.*, 1997). This mutation results in a mutant receptor that contains constitutively activated tyrosine kinase (Huang *et al.*, 1997; Antonyak *et al.*, 1998; Schmidt *et al.*, 1998). Furthermore, this ligand-independent activity of the mutant EGFR is independent of receptor homodimerization (Chu *et al.*, 1997). One could hypothesize that a similar mutation in a serine/threonine kinase type II receptor might result in a constitutively active type II receptor. Unlike tyrosine kinase receptors that form homodimers, S/T kinase receptors act as heterodimers. An activating mutation must therefore activate or unmask a type I association domain in the type II receptor, which normally occurs by binding of the ligand. Alternatively, the mutated type II receptor may signal directly without participation of the type I receptor. It is not known whether any of the type II receptors can signal in an alternative way, and so far no activating mutation in any of the type II receptors has been reported.

The finding of an identical translocation, t(12;14)(q14;q31), in two unrelated women with complete Müllerian aplasia, suggests that a gene on chromosome 12 or 14 might be involved (Taneja *et al.*, 1986; Kucheria *et al.*, 1988). *AMHRII* cannot be excluded as a candidate gene, since it is located at chromosome 12q13 (Chapter 4; Imbeaud *et al.*, 1995; Visser *et al.*, 1995). Because we could not obtain DNA from the women with t(12;14)(q14;q31), we have analyzed the coding sequence and the intron/exon boundaries of the *AMHRII* gene of one of the 13 patients (patient 7149) with complete Müllerian aplasia described by Shokeir (1978). All eleven exons were analyzed by PCR, using primers in the flanking intron regions, followed by sequencing. No DNA alterations were found in the *AMHRII* gene, except for exon 9, in which an A to G change was found at position 1264 relative to the translation start site, which was not present in the genomic DNA of three control subjects. However, the base pair change did not result in an amino acid change, since the codons CGA and CGG both encode for arginine. To confirm that this silent mutation reflects a polymorphism, more control

samples should be analyzed. The polymorphism could be used as a marker in a study of allelic variation in Müllerian aplasia.

ALK2

In addition to AMHRII, dysregulation of signaling by ALK2, the candidate AMH type I receptor (Chapter 6), could also be involved in Müllerian aplasia. ALK2 mRNA is expressed in the mesenchymal cells surrounding the Müllerian ducts, and in the fetal and adult gonads, which are target tissues of AMH (Chapter 6). In addition, ALK2 mRNA expression is found in other tissues, including kidney and bone (Verschueren *et al.*, 1995), which is of particular interest, since Müllerian aplasia is often associated with renal and skeletal abnormalities. The introduction of a constitutively active ALK2 in *Xenopus* animal caps suggests that ALK2 can also function as a BMP type I receptor (Armes and Smith, 1997). It is of interest to note that disruption of the BMP7 signal leads to kidney and bone abnormalities (Dudley *et al.*, 1995). Thus, in addition to the analysis of the *AMHRII* gene, exon 5 of the *ALK2* gene was screened in patient 7149 with complete Müllerian aplasia (see above). Exon 5 encodes the GS box, and it has been shown that replacement of glutamine residue 204 in ALK2 by an aspartic acid residue results in constitutive activity of the kinase domain (Chapters 6 and 7). However, no DNA alterations were found in exon 5 of *ALK2* of patient 7149.

The reasoning that was used against a role for activating mutations in the *AMH* gene in Müllerian aplasia, based on the absence of an effect similar to the effect of ectopic AMH action on ovarian development, can also be put forward to predict the absence of an activating mutation in the *AMHRII* or *ALK2* genes. Since AMH-deficient and AMHRII-deficient mice are exact phenocopies (Behringer *et al.*, 1994; Mishina *et al.*, 1996), indicating that AMH or AMHRII have no independent functions, one would expect that a constitutively activated AMHRII would result in a similar phenotype as observed in mice that overexpress AMH (Behringer *et al.*, 1990). Therefore, it is anticipated that an activating mutation in *AMHRII*, or perhaps also in *ALK2*, might result in ovarian dysfunction (Behringer *et al.*, 1990). However, normal ovarian function was observed in Müllerian aplasia patients (Shokeir, 1978).

An additional argument against a role for AMH signaling in Müllerian aplasia, is the pattern of Müllerian duct regression. The Müllerian ducts were shown to regress, in the mouse, in a cranial to caudal direction (Chapter 5). Furthermore, it was found that AMHRII mRNA expression is lost after the critical period of Müllerian duct regression, even when remnants of the Müllerian ducts are still present, as in DES-exposed male fetuses (Chapter 5). Therefore, an incorrect timing of AMH action in the female fetus may result in incomplete regression of the Müllerian ducts, in particular incomplete regression of the caudal ends. However, in all patients with Müllerian aplasia, the caudal part of the Müllerian ducts (upper part of the vagina) is missing, whereas the variation in Müllerian aplasia is localized in the cranial part of the ducts. This does not correspond with the pattern of Müllerian duct regression as observed in the mouse.

HOX genes

Other candidate genes for Müllerian aplasia may be found among the homeobox (*HOX*) genes. The *HOX* genes are the vertebrate homologs of the *Drosophila* homeotic selector genes that define positional values along the anterior-posterior axis and other developmental body axes (Krumlauf, 1994). In mouse and human, *Hox* genes are organized in four rather similar genomic clusters (Hoxa, Hoxb, Hoxc, and Hoxd), containing closely linked genes. Genes within a cluster are expressed in anterior-posterior domains, with the order of anterior limits of expression reflecting the 3' to 5' order of the genes on the chromosome (Krumlauf, 1994). Gain-of-function mutations or targeted disruption of *Hox* genes in mice have shown that improper expression can lead to altered development or identity of the structure in which the gene is normally expressed (Krumlauf, 1994; Manak and Scott, 1994; Capecchi, 1996). However, sometimes a structure is lost rather than homeotically transformed, implying that *Hox* genes are also needed to stimulate growth, in addition to controlling patterning processes (Morgan and Tabin, 1994; Duboule, 1995; Capecchi, 1996).

The 5' members of the four Hox clusters (*Hoxa 9* - *Hoxa 13*; *Hoxb 9*; *Hoxc 9* - *Hoxc 13*; *Hoxd 9* - *Hoxd 13*) are expressed in posterior domains of the developing mouse embryo, including the intermediate mesoderm

(Izpisua-Belmonte *et al.*, 1991; Taylor *et al.*, 1997), and it has been suggested that the pattern and timing of expression of the *Hoxa 9* - *Hoxa 13* genes are involved in the patterning of the Müllerian duct (Taylor *et al.*, 1997). In the mouse, Hoxa 9 mRNA is expressed in the Fallopian tubes, Hoxa 10 mRNA in the uterus, Hoxa 11 mRNA in the uterus and uterine cervix, and Hoxa 13 mRNA in the upper vagina (Taylor *et al.*, 1997). Indeed, targeted disruption of *Hoxa 10* results in a homeotic transformation of a part of the cranial part of the uterus into an oviduct-like tissue and anterior homeotic transformation of vertebrae (Rijli *et al.*, 1995; Satokata *et al.*, 1995). The same HOXA axis is established in human (Taylor *et al.*, 1997) and mutation of the human gene *HOXA 13* has been found to result in the hand-foot-genital syndrome, in which normal differentiation of the Müllerian ducts is disrupted, resulting in partially or completely divided uterus (Mortlock and Innis, 1997). The link between genital tract and limb abnormalities, resulting from mutation of several posterior Hox genes, as revealed in mice with disruption of *Hoxa 10*, *Hoxa 11* or *Hoxd 13* function (Dolle *et al.*, 1991; Dolle *et al.*, 1993; Hsieh-Li *et al.*, 1995; Satokata *et al.*, 1995) and in humans with a mutation in the *HOXA 13* gene (Mortlock and Innis, 1997), illustrate the strong functional conservation of these posterior genes in evolution (Dickman, 1997). Skeletal malformations are also found in some of the Müllerian aplasia patients (Lindenman *et al.*, 1997; Stelling *et al.*, 1997). This fact and the results discussed above, strongly suggest a role for the posterior expressed *Hox* genes of the four Hox clusters in the origin of Müllerian aplasia. Furthermore, analysis of compound mutants of *Hoxa 13* and *Hoxd 13* demonstrated that these genes are partially redundant in function and act in a dose-dependent manner; removal of an increasing number of copies of these genes resulted in progressively more severe defects (Fromental-Ramain *et al.*, 1996; Warot *et al.*, 1997). If indeed *HOX* genes are involved in the development of Müllerian aplasia, the partial functional redundancy of the *HOX* genes of the four clusters, and the dosage effect of *HOX* gene expression may explain the phenotypic variability of Müllerian aplasia.

Genes involved in Müllerian duct formation

Although dysregulation of *HOX* genes might play an important role in the origin of Müllerian aplasia, this defect might also result from an arrest in Müllerian duct formation. The Müllerian ducts are formed in a cranial to caudal direction (Byskov and Hoyer, 1994; Chapter 5). It can be hypothesized that incomplete Müllerian duct formation can lead to absence of the upper part of the vagina, and depending on the timing of arrest, the absence of uterus and Fallopian tubes. Although the genes that control Müllerian duct formation are not known, it is clear that disorders that affect kidney development often also affect the urogenital tract. Knockout mice for genes that are involved in the induction of intermediate mesoderm, such as *Pax2* and *Emx2*, revealed the absence of kidneys and genital tracts (Torres *et al.*, 1995; Miyamoto *et al.*, 1997). However, both male and female mice were affected. Nevertheless, since the renal system and the genital system both originate from intermediate mesoderm (Saxén and Sariola, 1987), genes that play a role in the differentiation of the intermediate mesoderm, among which *WT1* and *SF-1* (see Chapter 1), might be involved in Müllerian aplasia. It would be of interest to determine whether male relatives of female patients with Müllerian aplasia, might have kidney abnormalities, since such a finding would point in the direction of a subtle failure in intermediate mesoderm induction.

8.3 Downstream factors in AMH signaling

Smads

The identification of ALK2 as a candidate AMH type I receptor (Chapter 6) enables the search for downstream factors involved in AMH signaling. It appears that the recently identified family of Smad proteins transduces the signal of TGFβ family members to the nucleus (Heldin *et al.*, 1997). In some tumors, mutations have been identified in *Smad4/Dpc4*, which abolish the signal of TGFβ and allow enhanced proliferation of the tumor cells (Schutte *et al.*, 1996; Takagi *et al.*, 1996). With respect to the Smad proteins that may transduce signaling through ALK2, experiments by Chen *et al.* (1997b) showed that introduction of Smad8 mRNA in *Xenopus* embryos mimics the effect of a constitutively active ALK2. Whether Smad8 also transduces the

signal of an AMH-activated ALK2, and is expressed in the AMH target tissues, will be next steps to investigate. Another member of the Smad family, Smad5, is expressed at a relatively high level in the defined AMH target cells, which are the mesenchymal cells surrounding the Müllerian duct (Meersseman *et al.*, 1997). Therefore, also Smad5 is a candidate downstream factor in AMH signaling. However, most Smad proteins appear to have a ubiquitous expression pattern, and the fact that a Smad protein may be activated by several ligands [e.g. Smad2 functions in TGFβ and activin signaling (Chapter 2; Baker and Harland, 1996; Macias-Silva *et al.*, 1996)], and the observation that different type I receptors can activate a single Smad [e.g. ALK3 and ALK6 both activate Smad1 (Chapter 2; Hoodless *et al.*, 1996)], suggest that other Smad proteins should not be excluded from AMH signaling.

MAPKs

In addition to the Smads, mitogen-activated protein kinases (MAPKs) have been suggested to play a role in the signaling of TGFβ family members (Chapter 2). Indeed, TAK1 can activate the JNK/SAPK cascade in response to TGFβs and BMPs (see Figure 2.6; Yamaguchi *et al.*, 1995; Wang *et al.*, 1997; Shibuya *et al.*, 1998). In a preliminary study we have investigated whether a constitutively active ALK2 can activate the MAPK cascade. A reporter system was used that measures the activity of ELK1, a member of the large family of Ets domain-containing transcription factors. ELK1 is a substrate for all three distinct classes of MAPKs (ERKs, JNK/SAPKs and p38-MAPK; Gille *et al.*, 1995a; Gille *et al.*, 1995b; Sugimoto *et al.*, 1998; Yang *et al.*, 1998). When the constitutively active mutant ALK2-Q204D was expressed in transfected cells, a five-fold increase in ELK1 activity was measured. Wild type ALK2 did not have such an effect (Figure 8.2; Visser *et al.*, unpublished results). These preliminary results indicate that ALK2 may activate one of the MAPK signaling cascades. Whether ALK2 can induce the MAPK cascade in response to AMH remains to be studied.

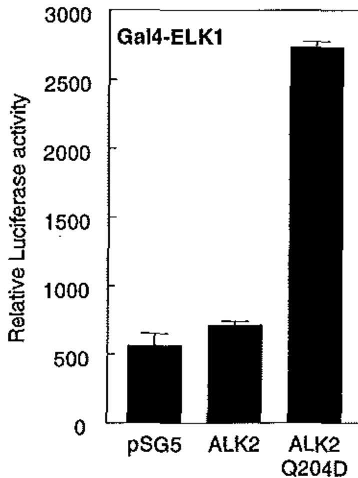
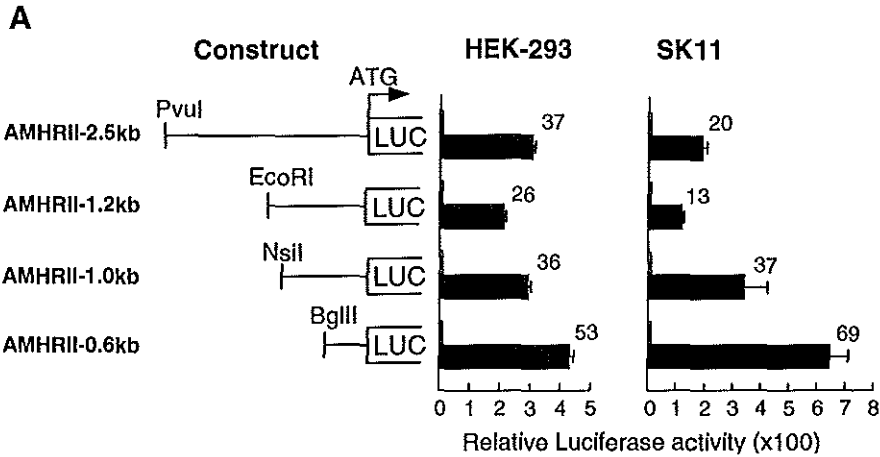


Figure 8.2. **ALK2-Q204D enhances ELK1 activity.**
HEK-293 cells were transfected with Gal4-ELK1 and Gal4-LUC together with the indicated expression vectors. After transfection, cells were kept on serum-free medium for 24 hours, prior to determination of luciferase activity. Luciferase activity was normalized for transfection efficiency.

Induction of the MAPK cascade by ALK2 may be related to induction of apoptosis, since MAPKs have been shown to mediate signals leading to apoptosis, such as the signals generated by TGFβs and BMPs (Shibuya *et al.*, 1998). Several members of the TGFβ family, in particular TGFβs, activins and BMPs, have been shown to induce apoptosis (Graham *et al.*, 1994; Hully *et al.*, 1994; Olaso *et al.*, 1998) and it can be hypothesized that this apoptotic signal is mediated through MAPKs. The finding that ALK2-Q204D induces apoptosis and activates ELK1 *in vitro*, points in this direction. Apoptosis is also involved in the regression of the Müllerian ducts, and the fact that apoptotic cells were not found in the Müllerian ducts of female fetuses and AMH-deficient male fetuses, indicates that the apoptotic signal is induced by AMH (Chapter 7). Since ALK2 can function as an AMH type I receptor, it will be of interest to study whether MAPKs are also involved in mediating AMH-induced apoptosis in the Müllerian ducts.

To study the role of ALK2 in AMH signaling *in vivo*, the generation of ALK2-deficient mice will be an important tool. However, ALK2-deficient mice will show defects in signaling of several ligands, since ALK2 can also serve as a type I receptor for BMPs and perhaps several other TGFβ family members. Furthermore, ALK2 is expressed during early embryogenesis (Verschueren *et al.*, 1995), and therefore ALK2-deficient mice may show lethal defects in early embryogenesis. Since we are interested in the role of ALK2 in AMH target tissues, the generation of a conditional *ALK2* knockout, using the Cre-LoxP system, promises to be a very useful approach. Cre is a DNA recombinase that recognizes LoxP sites, and splices out the DNA between LoxP sites. Two mouse lines need to be generated, one in which LoxP sites are introduced into the gene of interest, using homologous recombination, and a second line that expresses the *Cre* gene driven by a tissue-specific promoter. Cross breeding of the two lines will result in the disruption of the gene only in those cells that express *Cre*, while in other non-expressing cells the function of the gene is not disrupted (Gu *et al.*, 1994; Porter, 1998). To abrogate the ALK2 signal in the mesenchymal cells surrounding the Müllerian ducts and in the gonads, the use of the *AMHRII* promoter might be a good approach, since AMHRII mRNA expression is restricted to these tissues. However, the possibility of interference of this transgenic *AMHRII* promoter with the endogenous *AMHRII* promoter, should be considered.

The mouse *AMHRII* gene was isolated, and luciferase reporter constructs that contain 2.5 kb, 1.2 kb, 1.0 kb or 0.6 kb of the sequences upstream of the translation start site were constructed. All constructs showed basal promoter activity upon transient transfection in HEK-293 cells or SK11 cells, a Sertoli cell line established from H-2K^b-tsA58 transgenic mice (Walther *et al.*, 1996; Figure 8.3). The 0.6 kb *AMHRII* promoter-luciferase reporter construct showed the highest activity (Figure 8.3; Visser *et al.*, unpublished results). However, whether this construct contains the elements that confer tissue-specific expression and can be used to direct Cre expression to all possible AMH target cells, remains to be demonstrated.



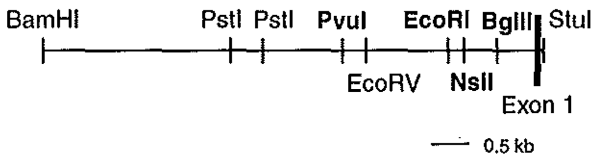


Figure 8.3. **Basal activity of the mouse *AMHRII* promoter.**
A. *AMHRII* promoter-LUC constructs, in which the translation start site of the *AMHRII* gene is replaced by that of the *luciferase* gene, were transiently transfected into HEK-293 or SK11 cells. Open bars: activity of the control luciferase plasmid; closed bars: activity of the *AMHRII* promoter constructs. The induction factor is indicated at the right side of the bars.
B. Partial restriction map of the mouse *AMHRII* promoter.

In addition to further evaluation of temporal and mechanistic aspects of the effect of AMH on the Müllerian ducts, the generation of conditional *ALK2* knockout mice may prove to be useful to study possible AMH actions in the gonads, in particular in the ovary. AMH- and AMHRII-deficient mice did not show pronounced ovarian abnormalities (Behringer *et al.*, 1994; Mishina *et al.*, 1996), although both AMH and AMHRII mRNAs are specifically and strongly expressed in preantral and small antral follicles (Baarends *et al.*, 1995b). Since ALK2 is suggested to function as a BMP type

I receptor and/or an activin type I receptor (Attisano *et al.*, 1993; Armes and Smith, 1997), BMPs and activins may signal through ALK2 in the ovaries of AMH-deficient mice. Abrogation of the ALK2 signal in the ovary may reveal whether ALK2 functions as a specific AMH type I receptor or whether AMH in the ovary is functionally redundant. Similar experiments can be envisaged for the testis.

The identification of ALK2 as a candidate AMH type I receptor is an important step to obtain more information about the exact signaling mechanism of AMH. Such experiments may contribute to improve our understanding of the mechanism of Müllerian duct regression and the functional importance of AMH action in the gonads.

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Summary

Samenvatting

SUMMARY

During early embryonic development, the gonads are bipotential and can develop into testis or ovary. Furthermore, the precursors of both male and female reproductive tracts, the Wolffian and Müllerian ducts, respectively, are present during this early stage of development. In genetically male embryos, the presence of a Y chromosome determines that the undifferentiated gonads will develop into testes, which start to produce two testicular hormones. Testosterone causes the differentiation of the Wolffian ducts into epididymides, vasa deferentia and seminal vesicles. The other hormone, anti-Müllerian hormone (AMH) is responsible for regression of the Müllerian ducts. The Müllerian ducts are sensitive to AMH for a short period, and exposure before or after this period does not result in complete Müllerian duct regression. In the mouse, this critical period of Müllerian duct regression is between E13-E15 (days *post coitum*). In the female fetus, which does not produce AMH and testosterone, the Wolffian ducts degenerate, whereas the Müllerian ducts differentiate into the Fallopian tubes, uterus and upper part of the vagina, apparently independent of ovarian hormones. In the absence of AMH action, due to a mutation in the *AMH* gene, Müllerian ducts do not regress in male fetuses This phenotype is known as persistent Müllerian duct syndrome (PMDS), in which boys have normal internal male structures but also internal female structures.

This thesis focuses on the molecular mechanism and developmental aspects of AMH action.

In the General Introduction (Chapter 1), most of the known regulatory factors of sex determination and differentiation are described. Furthermore, the regulation of AMH mRNA expression, and the role of AMH during pre- and postnatal life are described in this chapter.

AMH is a member of the transforming growth factor β (TGFβ) family of growth and differentiation factors. Members of the TGFβ family signal through a receptor complex, consisting of a type I and a type II transmembrane serine/threonine kinase receptor. In addition to the family members and their receptors, the mechanism of receptor activation and the downstream factors are described in Chapter 2.

A novel type II receptor has been cloned that, based on its mRNA expression in the mesenchymal cells surrounding the Müllerian ducts, and in the gonads, encodes a candidate AMH type II receptor (AMHRII). Chapter 3 describes the generation of transgenic mice carrying a dominant negative ΔAMHRII, under the control of the zinc-inducible metallothionein promoter, to study the role of AMHRII during several developmental stages. Five transgenic mouse lines were generated, and although the transgene ΔAMHRII is expressed in several tissues, including the AMH target tissues, no aberrant phenotype was found that would imply inhibition of AMH action. The possible shortcomings and improvements of our tested model are also discussed in Chapter 3.

In Chapter 4, the structure and chromosomal localization of the human *AMHRII* gene are described. The coding region consists of 11 exons, divided over 8 kb, and the gene maps to chromosome 12q12-q13. The results, described in Chapter 4, can be used for the identification of possible mutations in the *AMHRII* gene in PMDS patients.

Some of the structures that can develop from Müllerian duct remnants, most pronounced in PMDS patients, are also found in man prenatally exposed to the synthetic estrogen diethylstilbestrol (DES). It has been hypothesized that the Müllerian ducts become less sensitive to AMH upon exposure to DES. Chapter 5 describes the effect of prenatal DES exposure on Müllerian duct development and on AMH and AMHRII mRNA expression, during male fetal mouse development. Upon exposure to DES, the formation of the Müllerian duct was delayed by approximately two days. A similar delay was found for the regression of the Müllerian ducts. Thus, it can be suggested that, in DES-exposed male fetuses, the development of the Müllerian ducts is delayed, resulting in an asynchrony in the temporal aspects of Müllerian duct formation, development and sensitivity to AMH, which may result in partial regression. However, prenatal exposure to DES does cause an increase in the mRNA expression of AMH and AMHRII at E13. The latter does not indicate an inhibition of AMH action at the level of ligand-receptor interaction. This raises the possibility that prenatal exposure to DES may inhibit the action of other factors, functioning downstream of AMH/AMHRII. Possible downstream factors, and their families, are described in Chapter 2.

In Chapter 6, the identification of a candidate AMH type I receptor is described. To study AMH signaling, an *in vitro* model is developed based on an inhibitory effect of AMH on induction of aromatase activity. In this model, a constitutively active type I receptor that can signal in the absence of ligand, was used. The constitutively active mutant ALK2 can suppress the induced aromatase promoter activity, and a wild type ALK2 can form a functional complex with AMHRII that signals in response of AMH. Expression of ALK2 mRNA was found in the target tissues of AMH; in the mesenchymal cells surrounding the Müllerian ducts and also in the gonads. These results strongly suggest that ALK2 is a strong candidate AMH type I receptor. However, since ALK2 mRNA is not only expressed in AMH target tissue, but also in other tissues, it may serve as a type I receptor for other ligands as well.

Chapter 7 describes the ability of a constitutively active ALK2 to induce programmed cell death or apoptosis *in vitro*. Since, ALK2 is a candidate AMH type I receptor, and therefore may be involved in the action of AMH on the Müllerian ducts, apoptosis in Müllerian ducts was studied, during the period of Müllerian duct regression. The TUNEL assay was used to detect apoptosis in histological tissue sections. Apoptotic cells were present in the Müllerian ducts of male mouse fetuses. In Müllerian ducts of female fetuses and of AMH-deficient male fetuses, in which the Müllerian ducts do not regress, apoptosis was not detected. This indicates that apoptosis is involved in the regression of the Müllerian ducts, and may be induced by AMH.

Abnormalities of the development of Müllerian ducts in the female occur frequently. In Chapter 8, the possible role of abnormal AMH signaling in the origin of Müllerian aplasia is discussed. Furthermore, the implications of ALK2 as an AMH type I receptor are discussed, in relation to the mechanism of AMH signaling, and suggestions for future research are given.

SAMENVATTING

Gedurende de vroege embryonale ontwikkeling zijn de gonaden ongedifferentieerd, om zich vervolgens tot testes of ovaria te ontwikkelen. Tevens zijn in een vroeg stadium zowel de mannelijke als de vrouwelijke genitale tractus in aanleg aanwezig, de zgn. Wolffse en Müllerse gangen. In genetisch mannelijke embryo's bepaalt de aanwezigheid van het Y chromosoom dat de ongedifferentieerde gonaden zich zullen ontwikkelen tot zaadballen (testes), die vervolgens twee hormonen gaan produceren. Het hormoon testosteron zorgt voor de differentiatie van de Wolffse gangen tot de bijballen, de zaadleiders en de zaadblazen. Het andere hormoon, het anti-Müllerse gang hormoon (AMH), is verantwoordelijk voor het ten gronde gaan (regressie) van de Müllerse gangen. De Müllerse gangen zijn gedurende een korte periode gevoelig voor AMH. Wanneer de gangen voor of na deze periode worden blootgesteld aan AMH vindt er geen volledige regressie plaats. Deze kritische periode van Müllerse gang regressie vindt in de muis plaats tussen dag 13 en 15 na de bevruchting. In de vrouwelijke foetus, waarin de ovaria geen testosteron en AMH produceren, verdwijnen de Wolffse gangen, terwijl de Müllerse gangen zich ontwikkelen tot eileiders, baarmoeder en het bovenste deel van de vagina. De differentiatie van de vrouwelijke genitale tractus lijkt niet afhankelijk te zijn van gonadale hormonen. Afwezigheid van AMH in een mannelijke foetus, bijvoorbeeld door een mutatie in het *AMH* gen, zorgt ervoor dat de Müllerse gangen niet verdwijnen. Dit verschijnsel staat bekend als het persistente Müllerse gang syndroom (PMDS), waarbij jongetjes naast inwendige mannelijke structuren tevens inwendige vrouwelijke structuren bezitten.

Het onderzoek, dat in dit proefschrift beschreven is, heeft zich gericht op het moleculaire werkingsmechanisme en ontwikkelingsbiologische aspecten van de werking van AMH.

In de Algemene Introductie (Hoofdstuk 1) worden de meeste van de thans bekende factoren die betrokken zijn bij de regulatie van geslachtsbepaling en geslachtsdifferentiatie beschreven. Verder wordt in dit hoofdstuk aandacht besteed aan de regulatie van AMH mRNA expressie en de functie van AMH voor en na de geboorte.

AMH behoort tot de TGFβ familie van groei- en differentiatiefactoren. Leden van deze familie geven hun signaal door via een receptor complex dat bestaat uit type I en type II transmembraan serine/threonine kinase receptoren. Naast de familieleden en hun receptoren, worden ook het receptor-activatie-mechanisme en de factoren die betrokken zijn bij het doorgeven van het signaal beschreven in Hoofdstuk 2.

In het voorgaande onderzoek werd een gen geïdentificeerd dat codeert voor een nieuw lid van de type II receptor familie. Op grond van mRNA expressie rondom de Müllerse gang en in de gonaden, werd verondersteld dat dit gen codeert voor een kandidaat AMH type II receptor (AMHRII). Hoofdstuk 3 beschrijft het ontwikkelen van transgene muizen waarin een niet-functionele receptor, tot expressie kan worden gebracht. De mutant receptor ΔAMHRII zou de werking van de normale AMHRII dominant negatief moeten remmen. Het experiment werd uitgevoerd om de rol van AMHRII in het doorgeven van het AMH signaal tijdens verschillende ontwikkelingsstadia te kunnen onderzoeken. Vijf transgene muizenlijnen werden gemaakt, en hoewel het transgen ΔAMHRII tot expressie komt in verschillende weefsels, waaronder de AMH doelwitweefsels, werden geen aanwijzingen gevonden die zouden kunnen duiden op een remming van AMH werking. De mogelijke tekortkomingen en verbeteringen van het door ons geteste model worden tevens in Hoofdstuk 3 besproken.

In Hoofdstuk 4 worden de structuur en de chromosomale lokalisatie van het humane *AMHRII* gen beschreven. Het coderende gebied bestaat uit 11 exonen, verspreid over 8 kb, en het gen ligt op chromosoom 12q12-q13. De resultaten, zoals beschreven in Hoofdstuk 4, kunnen worden gebruikt bij de identificatie van mutaties in het *AMHRII* gen bij PMDS patiënten.

Enkele structuren die zich kunnen ontwikkelen uit overblijfselen van de Müllerse gangen, zoals in het meest uitgesproken geval bij PMDS patiënten, worden ook gevonden in mannen die prenataal zijn blootgesteld aan het synthetisch oestrogeen diethylstilbestrol (DES). Er zijn aanwijzingen dat de Müllerse gangen minder gevoelig voor AMH kunnen worden na blootstelling aan DES. Hoofdstuk 5 beschrijft het effect van prenatale blootstelling aan DES op de ontwikkeling van de Müllerse gangen en op de mRNA expressie van AMH en AMHRII, tijdens de foetale ontwikkeling van

mannelijke muizen. Het bleek dat blootstelling aan DES tot gevolg heeft dat de vorming van de Müllerse gangen met ongeveer twee dagen wordt vertraagd. Deze vertraging werd ook waargenomen voor de regressie van de gangen. Verondersteld kan worden, dat in DES blootgestelde mannelijke foetussen, de ontwikkeling van de Müllerse gangen is vertraagd, zodanig dat er een discrepantie ontstaat tussen de temporale aspecten van aanleg, ontwikkeling, en kritische periode van gevoeligheid voor AMH. Dit zou een oorzaak kunnen zijn voor de partiële regressie. Wel was in de DES behandelde foetussen, op het tijdstip 13 dagen na de bevruchting, de mRNA expressie van AMH en AMHRII verhoogd. Dit laatste wijst zeker niet op remming van AMH werking op het niveau van ligand-receptor interactie, ten gevolge van de DES behandeling. Blootstelling aan DES heeft mogelijk een remmend effect op andere factoren, zoals de intracellulaire factoren die betrokken zijn bij het doorgeven van het AMH signaal. De families waartoe deze factoren behoren zijn beschreven in Hoofdstuk 2.

In Hoofdstuk 6 wordt de identificatie van een kandidaat AMH type I receptor beschreven. Een *in vitro* model werd ontwikkeld om de signaaltransductie van AMH te bestuderen. Dit model is gebaseerd op een remmend effect van AMH op inductie van aromatase activiteit. Daarbij werd tevens gebruik gemaakt van een gemuteerde en daardoor constitutief actieve type I receptor, die een cellulair signaal doorgeeft in afwezigheid van het ligand. De constitutief actieve mutant ALK2 kan de geïnduceerde activiteit van de aromatase promoter remmen, en een normale ALK2 kan een functioneel receptor complex met AMHRII vormen in de aanwezigheid van AMH. Expressie van ALK2 mRNA werd gevonden in de doelwitweefsels van AMH; in de mesenchymale cellen rondom de Müllerse gangen en ook in de gonaden. Deze resultaten suggereren zeer sterk dat ALK2 een belangrijke kandidaat is voor de AMH type I receptor. Omdat ALK2 niet alleen in AMH doelwitweefsels maar tevens in andere weefsels tot expressie komt, kan ALK2 mogelijk ook als een type I receptor voor andere liganden functioneren.

In Hoofdstuk 7 wordt beschreven dat een constitutief actieve mutant ALK2 geprogrammeerde celdood (apoptose) kan induceren in een cellijn. Omdat ALK2 een kandidaat AMH type I receptor is, en daarom betrokken

zou kunnen zijn bij de werking van AMH op de Müllerse gangen, werd apoptose in Müllerse gangen onderzocht ten tijde van de regressie van de gangen. Daarbij werd gebruik gemaakt van de TUNEL methode, voor detectie van apoptose in histologische weefselcoupes. Gevonden werd, in muizen, dat apoptotische cellen aanwezig zijn in de Müllerse gangen van mannelijke foetussen. In Müllerse gangen van vrouwelijke foetussen en van mannelijke AMH-deficiënte foetussen, waarin de Müllerse gangen blijven bestaan, werd geen apoptose gevonden. Dit betekent dat apoptose inderdaad betrokken is bij de regressie van de Müllerse gangen, en dat AMH mogelijk apoptose induceert.

Afwijkingen in de ontwikkeling van Müllerse gangen komen frequent voor bij de vrouw. In Hoofdstuk 8 wordt de mogelijke rol van verstoorde AMH werking bij het ontstaan van Müllerse gang aplasie besproken. Ook wordt de betekenis van de identificatie van ALK2 als een AMH type I receptor beschreven, in relatie tot het mechanisme van AMH signaal overdracht, en worden enkele suggesties voor vervolg onderzoek gegeven.

List of publications

Baarends WM, Hoogerbrugge JW, Post M, Visser JA, de Rooij, Parvinen M, Themmen APN and Grootegoed JA. (1995). Anti-Müllerian hormone and anti-Müllerian hormone type II receptor messenger ribonucleic acid expression during postnatal testis development and in adult testis of the rat. Endocrinology 136, 5614-5622.

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Visser JA, McLuskey A, Kramer P, Verhoef-Post M, Nachtigal MW, Ingraham HA, Ten Dijke P, Grootegoed JA and Themmen APN. Identification of ALK2 as a candidate anti-Müllerian hormone type I receptor. Submitted.

Visser JA, McLuskey A, Martens JWM, Nachtigal MW, Ingraham HA, Ten Dijke P, Grootegoed JA and Themmen APN. Anti-Müllerian hormone induced Müllerian duct regression involves apoptosis. In preparation.

Visser JA, Grootegoed JA and Themmen APN. Signaling pathways of TGFβ receptors. (Review). Submitted.

Curriculum Vitae

The author of this thesis was born on the 25th of August 1967, in Hurdegaryp, Fryslân. The secondary education was taken at the Slauerhoff College (VWO) in Leeuwarden. In 1986, she started the study of Animal Science at the Agricultural University in Wageningen. The major subject, Developmental Biology, included a 6 months study entitled "Characterization of the DNA methylation pattern of porcine embryos", supervised by Dr. G. te Kronnie, and a 6 months study entitled "Differentiation of inner and outer cells of bovine embryos after *in vitro* maturation, fertilization, and culture", supervised by Dr. M. Boerjan and Drs. A. van der Schans. Furthermore, six months were spent at the Dept. of Molecular Embryology, IAPGR, Babraham/Cambridge, UK (Dr. W. Reik), to work on a project entitled "Isolation and characterization of candidate genes for genomic imprinting". The Animal Science study was successfully completed in August 1992.

From May 1993 till May 1998, the PhD research, described in this thesis, was performed at the Dept. of Endocrinology & Reproduction, Erasmus University Rotterdam, under the supervision of Dr. A.P.N. Themmen and Prof. dr. J.A. Grootegoed.

In October 1998, she will continue her scientific career with Dr. H.A. Ingraham at the Dept. of Physiology, UCSF, San Francisco, USA, where the interaction between Dax-1 and SF-1 in the regulation of endocrine organs will be studied.

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