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5 Mouse Models to Study the Pituitary-Testis Interplay Leading to Regulated Gene Expression

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INTRODUCTION

The past decade has seen the astounding development of transgenic animal technology, which has become the most powerful tool for the study of gene function and dysfunction in vivo. All fields of biology, including endocrinology, oncogenesis, neuroscience, and embryogenesis, have greatly advanced because of the ease in generating genetically modified animals in an increasing number of research laboratories worldwide. The capacity to explore the function of one specific gene in the living animal has particularly enriched our view of complex physiological systems, such as the neuroendocrine axis. In various cases, mutant mice have been developed to verify the presumptive function of previously studied molecules. In others, the generated mutation has revealed unexpected actions of the targeted gene. This chapter focuses on some mutations affecting the reproductive axis, as these reveal the high complexity of the system and the interplay between the regulation of gene expression and pituitary signaling. The aim of this chapter is not to provide an exhaustive list of all mice presenting defects in gametogenesis, but to present a selected number of representative examples.

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A large number of transgenic mice present alterations in the differentiation of germ cells. In many cases, the phenotype observed is the result of random insertion of the transgene in the genome, with the consequent inactivation of one or more genes affecting gametogenesis function. Since 10% of all transgenic insertion mutations are associated with male infertility *(1)*, this suggests that a significant proportion of cases of human male infertility may have a genetic origin, and that a large number of genes must be implicated in the process of germ-cell differentiation. The advent of targeted mutagenesis techniques has allowed a precise analysis of the physiological role of gene dysfunction. The use of homologous recombination is now a popular approach. For example, from 1989 to 1995, over 327 genes have been "knocked-out" and the number is growing exponentially every year *(2)*. Tables 1 and 2 summarize some of the knockout mouse models with reproductive defects either only in males or in both males and females.

Tables 1 & 2

Gametogenesis is a complex and highly regulated process, during which stem cells undergo multiple steps of differentiation (*see* Chapter 7). This program is under tight control from multiple hormones, many of which originate from the hypothalamo-pituitary axis *(3)*. In the male, spermatogenesis occurs in the seminiferous epithelium as a finely tuned, cyclic process that can be divided into three phases: spermatogonial multiplication, meiosis, and spermiogenesis *(4,5)*. Histological, physiological, and biochemical studies have provided a wealth of information on the mechanisms controlling spermatogenesis, but many components of this differentiation cascade still remain obscure.

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Several lines of evidence indicate that highly specialized transcriptional mechanisms ensure stringent stage-specific gene expression in the germ cells. Specific checkpoints correspond to the activation of transcription factors; these regulate gene promoters with a restricted pattern of activity, in a germ-cell-specific fashion *(6)*. There is also evidence that general transcription factors may be differentially regulated in germ cells. For example, TBP (TATA-binding protein) accumulates in early haploid germ cells at much higher levels than in any other somatic-cell type. It has been calculated that adult spleen and liver cells contain 0.7 and 2.3 molecules of TBP mRNA per haploid genome-equivalent, respectively, while adult testis contain 80–200 molecules of TBP transcript per haploid genome-equivalent *(7)*. In addition to TBP, TFIIB and RNA polymerase II were also found to be overexpressed in testis. These remarkable features are consistent with the potent transcriptional activity that occurs in a coordinated manner during the differentiation of germ cells.

PITUITARY HORMONES

Spermatogenesis is under the hormonal control of the hypothalamo-pituitary axis *(3)*. Gonadotropin-releasing hormone (GnRH) is released from the hypothalamus into the hypothalamo-pituitary vein, and stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the gonadotroph cells of the anterior pituitary. FSH and LH bind to receptors located respectively on the somatic Sertoli and Leydig cells of the testis. Pituitary gonadotropins (FSH and LH) and thyroid-stimulating hormone thyrotropin (TSH) are heterodimers composed of a common α-subunit (α-GSU) and a unique β-subunit.

Disruption of the gene for the α-subunit has resulted in mice that did not produce FSH, LH, or TSH. The homozygous mutant mice are hypogonadal, and suffer from severe hypothyroidism resulting in dwarfism *(8)*. The α-subunit-deficient male mice are infer-

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Table 1

Knockout Models with Reproductive Defects Only in Males

Knockout mouse model	*Major reproductive findings*	*Refs*
Acrosin	Delayed fertility, normal binding and penetration of ZP by sperm	*(85)*
Ahch (Dax1)	Infertile; progressive degeneration of the testicular germinal epithelium	*(86)*
Angiotensin-converting enzyme	Reduced fertility caused by decreased ability of sperm to fertilize ova	*(87)*
Apaf-1	Only 5% of the mutants survive to adulthood; males infertile; spermatogonial degeneration	*(88)*
Apolipoprotein B heterozygotes	Reduced fertility; spermatozoa fertilization defects	*(89)*
Bax	Infertile; spermatogenesis block at premeiotic stage	*(61)*
Bclw	Infertile; spermatogenesis block during late spermatogenesis; eventual loss of all germ cells and Sertoli cells	*(90)*
Bone morphogenetic protein 8A	Progressive infertility; germ-cell degeneration, spermiogenesis defects, and epididymis degeneration	*(91)*
Bone morphogenetic protein 8B	Infertile; germ-cell proliferation/depletion defects	*(92)*
BRCA-1, p53 double mutant	Infertile; meiotic failure	*(93)*
Calmegin	Infertility due to impairment of sperm binding to ZP	*(94)*
Casein kinase II alpha'	Infertile; oligospermia and globozoospermia	*(95)*
c-ros tyrosine kinase receptor	Infertile; defect in volume regulatory mechanism in mature sperm, sperm flagellar angulation	*(96)*
CREM	Infertile; block at first stage of spermiogenesis	*(75,76)*
Cyclic GMP-dependent kinase 1	Reduced fertility, failure of corpora cavernosa to relax on activation of the NO/cGMP signaling	*(97)*
Cyclin A1	Infertile; block of spermatogenesis before first meiotic division, increased germ-cell apoptosis	*(65)*
Cyritestin	Infertile; failure of sperm to bind to the ZP	*(98)*
Desert hedgehog	Infertile; defects in germ cell development	*(99)*
Fertilin b	Infertile; defects in sperm-egg adhesion, fusion and ZP binding	*(100)*
Fragile X mental retardation 1 (FMR1)	Normal fertility, macroorchidism due to increased embryonic Sertoli-cell proliferation	*(101)*
GDNF	Infertile; depletion of stem-cell reserves	*(102)*
Hormone-sensitive lipase	Infertile; vacuolated epithelial cells in tubules, oligospermia	*(103)*
Hoxd-13	Defects in formation of the seminal vesicles, ventral and dorsal prostate, and bulbourethral gland	*(104)*
HR6B Ubiquitin-conjugating enzyme	Infertile; possible defect in histone poly ubiquitination and degradation during spermatogenesis	*(105)*

(continued)

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Table 2

Knockout Models with Reproductive Defects in Both Sexes

Knockout mouse model	*Major reproductive findings*	*Refs*
α-inhibin	Infertility in females; secondary infertility in males; granulosa-/Sertoli-cell tumors; cachexia-like syndrome	*(27,30)*
Activin receptor type II	Infertility in females; delayed fertility in males; small gonads	*(125)*
A-myb	Male infertility; pachytene stage arrest of germ cells; nursing defects in females due to underdevelopment of mammary glands	*(126)*
Ataxia telangiectasia (Atm)	Male and female infertility; complete absence of germ cells	*(127,128)*
β1,4-Galactosyltransferase	Male and female infertility caused by abnormal glycoprotein hormone glycosylation	*(129)*
Centromere protein B	Males hypogonadal, decreased sperm number strain-dependent uterine defects in females, disrupted luminal and glandular epithelium in the uterus, reduced fertility	*(130,131)*
Cyclin D2	Female infertility secondary to a block in folliculogenesis; males fertile but with decreased testis size	*(64)*
Cyp 19	Progressive infertility, spermiogenic defects, Leydig-cell hyperplasia in males, females infertile, increased follicular atresia prior to ovulation, defects in mammary gland development	*(132,133)*
Dazla	Male and female infertility; loss of germ cells and complete absence of gamete production	*(134)*
Dmc1	Arrest of spermatogenesis at zygotene stage in males; no oocytes in the adult ovary	*(135,136)*
Emx2	Accelerated degeneration of Wolffian-duct and mesonephric tubules without the formation of the Müllerian duct	*(137)*
ERβ	No defect in male fertility, prostate hyperplasia in old males; decreased fertility in females	*(50)*
Estrogen receptor α (ERα)	Uterine/ovarian defects in females; small testes, reduced number of spermatozoa in males	*(47,49,51)*
Estrogen receptor α/β double knockout	Male phenotypes similar to ERα mice, sex-reversal at the gonad level in females	*(51)*
Follicle0stimulating hormone β subunit	Female infertility; folliculogenesis block prior to antral follicle stage; males fertile but decreased testis size	*(9)*
Glycoprotein hormone α-subunit	Infertile; hypogonadal and hypothyroid	*(8)*
Hoxa 11	Partial homeotic transformation of vas deferens to epididymis; failure of testicular descent; absence of uterine stromal, decidual, and glandular cells in females	*(138)*

(continued)

Table 2 *(continued)*

Knockout mouse model	*Major reproductive findings*	*Refs*
Hoxa10	Variable infertility in males and females caused by cryptorchidism and preimplantation embryonic loss, respectively	*(139)*
Insulin-like growth factor (IGF-1)	Hypogonadal and infertile; pre-antral block in folliculogenesis in females	*(140)*
MLH1 DNA mismatch repair enzyme	Male and female infertility; Defective meiosis at pachytene stage (males) and failure to complete meiosis II (females)	*(141)*
Msh5	Male and female infertility; defects in zygotene stage in both sexes, characterized by impaired and aberrant chromosome synapsis, apoptotic cell death	*(142)*
Neuronal helix-loop-helix 2 (Nhlh2)	Males infertile; females fertile only in presence of males; hypothalamic defect	*(143)*
$p27^{Kip1}$ CDK inhibitory protein	Female infertility; corpus luteum defects; males fertile and increased testis size	*(144–146)*
Prolactin receptor	Female infertility caused by multiple abnormalities including irregular estrous cycles and implantation defects; males infertile or subfertile of unknown origin	*(147)*
Rho GDIalpha	Male and female infertility; impaired spermatogenesis with vacuolar degeneration of seminiferous tubules in males; postimplantation defects in females	*(148)*
Telomerase	Progressive infertility in males and females; increased apoptosis in testicular germ cells, and reduced testis size; decreased number of oocytes and uterine abnormalities	*(149)*
TIAR	Infertility; complete absence of primordial germ cells by E 13.5 leading to absence of spermatogonia and oogonia	*(150)*
Zfx	Reduced germ-cell number in both sexes resulting from defective proliferation	*(151)*

tile and exhibit prepubertal external genitalia. The testes are severely reduced in size, but the epididymis and vas deferens are present. The presence of normal prepubertal genitalia support the hypothesis that the differentiation of these structures from the Wolffian duct is testosterone-dependent. LH stimulates testosterone secretion from Leydig cells, and serum-testosterone concentrations are severely reduced in α-$GSU^{-/-}$ mice. However, these low concentrations of testosterone are still sufficient to induce sexual differentiation. Histological examination of α-$GSU^{-/-}$ testis showed that the seminiferous tubules are reduced in diameter, and that spermatogenesis is blocked at the first meiotic division. Therefore, these results indicate that gonadotropins are necessary for postnatal testicular differentiation, but that testis development proceeded normally during the fetal period. This view has been validated by other mice models.

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Mice carrying a targeted mutation in the FSHβ subunit gene have been generated *(9)*. Mutant females are infertile because of a block in folliculogenesis prior to antral-follicle formation. Importantly, and in contrast to the classical view of the FSH requirement for spermatogenesis and Sertoli-cell growth, FSHβ-deficient males are fertile, despite their small testes. The critical role played by FSH signaling is illustrated by the effect of FSH-R mutations in humans *(10–12)*. An inactivating mutation (Ala189Val) found in females with pure ovarian dysgenesis leads to a disease characterized by normal karyotype, high gonadotropins, and streaky gonads associated with primary amenorrhea. More recently, additional mutations have been described (Asp224Val and Leu601Val) that are associated to a similar pathological condition *(13)*. These mutations lie either in the extracellular domain (Ala189Val and Asp224Val) or in the third extracellular loop (Leu601Val) of the FSH-R, and are believed to modify protein folding. Importantly, males with the Ala189Val mutation display various degrees of spermatogenic failure, without azoospermia or absolute infertility *(14)*. Thus, the same inactivating mutation differentially influences reproduction in males or females.

A more recent approach, aimed at altering FSH signaling at the target tissue, has been to mutate the gene encoding the FSH receptor *(15)*. Similar to the FSHβ mutant mice, FSH-R-deficient males display small testes, partial spermatogenic failure, and reduced fertility. Thus, it appears that FSH signaling is not essential for initiating spermatogenesis, but is required to sustain adequate viability and motility of the sperm. Again, the phenotype of mutant females is much more severe. These display thin uteri and small ovaries, and are sterile as a result of a block in folliculogenesis prior to antral-follicle formation. Drastic changes have been found in pituitary hormone levels, especially FSH, which is increased 15- to 20-fold in females and about threefold in males. This dramatic increase in FSH levels verifies the classical view of FSH signaling retroinhibition, underscoring the apparent simplicity of the system in which no alternative retroinhibitory routes seem to be activated by the lack of FSH signaling *(15)*.

Additional hormonal changes include a significant decrease in the levels of testosterone in the males. This result indicates that low testoterone levels are sufficient to sustain sex accessories, and indicates a link between FSH signaling and testosterone production. This link could involve an intracellular communication pathway that would be compromised, despite normal LH levels, in the FSH-R mutants. At the level of the pituitary gland, there is a moderate but significant enlargement in the anterior lobe, accompanied by a drastic increase of FSH-positive cells. These animals have been considered as possible models for the study of the physiological link between gonads and pituitary, and hypergonadotropic ovarian dysgenesis and infertility.

A mouse model to study aberrant LH signaling has not yet been developed. However, mice with a targeted mutation in the LH-receptor gene would constitute an invaluable tool to explore the link between pituitary-hormonal signaling and sexual differentiation. In this respect, it is important to note that homozygous missense mutations in the LH-R gene in humans are tightly associated with male pseudohermaphroditism *(16)*.

TRANSFORMING GROWTH FACTOR-β FAMILY

Müllerian-Inhibiting Substance

During mammalian embryogenesis, the Müllerian ducts have the potential to differentiate into the oviducts, uterus, and upper vagina of the female reproductive tract, while

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the Wolffian ducts differentiate into the vas deferens, epididymis, and seminal vesicles in the male. During male development, the Sertoli cells of the testis produce Müllerian-inhibiting substance (MIS), a protein that actively represses the differentiation of the Müllerian ducts and prevents the development of female reproductive organs *(17)*. Subsequently, testosterone produced by the Leydig cells induces the differentiation of the Wolffian ducts into male external genitalia. The MIS protein is a member of the transforming growth factor-b (TGFb) gene superfamily which also includes the activin and inhibin genes.

The MIS gene has been mutated in the mouse by homologous recombination. The testis of $MIS^{-/-}$ males descend normally, their size is normal, and the Wolffian duct system differentiates properly *(18)*. Histological analysis of the testis shows no obvious anomalies, and there is no apparent difference in the spermatogenesis from wild-type and $MIS^{+/-}$ males. However, these males also develop Müllerian-duct-derived tissues such as a uterus, oviducts, and a vagina. About 85% of the males are infertile. Although MIS is not necessary for normal germ-cell development, the infertility of $MIS^{-/-}$ males probably results from a diversion of the sperm from its normal pathway. Finally, MIS appears to play an antitumor role in the testis, because about 25% of MIS-deficient males develop Leydig-cell hyperplasia and neoplasia.

Activin and Inhibin

Activins and inhibins are members of the TGFβ gene superfamily. Inhibins were initially isolated from mammalian follicular fluid on the basis of their ability to inhibit the release of FSH from anterior pituitary cells *(19)*. Side fractions that activated FSH release led to the isolation of activins. Activins and inhibins are protein dimers composed of a unique α-subunit and common but homologous βA or βB subunits. In adult animals, the highest levels of activin and inhibin transcripts are found in the Sertoli cells of the testis and the granulosa cells of the ovaries *(20–22)*. Interestingly, recent evidence points to the regulation of inhibin a gene expression by some isoforms of CREM *(23)*. In the testis, activin stimulates spermatogonial proliferation and androgen biosynthesis by Leydig cells, whereas inhibin has the opposite activity. Activin interacts with type I and type II cell-surface receptors, which have serine/threonine kinase activity. Two type II activin receptors—activin-receptor type IIA (ActRIIA) and activin-receptor type IIB (ActRIIB)—have been identified *(24)*. The ActRIIA isoform has been detected in specific populations of male germ cells, suggesting that this hormone can act as a gonadal paracrine and/or autocrine regulator *(20,25,26)*.

Research indicates that α-inhibin functions as a tumor suppressor with gonadal specificity. Male mice with a targeted mutation of the α-inhibin gene initially appeared healthy, and had normal external genitalia. However, male mutants were sterile, and analysis of the testis showed the presence of mixed or incompletely differentiated gonadal stromal tumors *(27)*. Spermatogenesis was initially active in the first 5–7 wk of life, but regression was evident with the enlargement of the tumor masses. Serum FSH concentrations were elevated two- to threefold in the homozygous mutants as compared to wild-type littermates, which confirmed the inhibitory role of inhibin on FSH release. A regulatory mechanism between activin and inhibin has been described by showing that $\alpha\text{-inhibin}^{-/-}$ mice have a 200-fold overexpression of the activin βA subunit and a threefold reduction of ActRIIA mRNA transcript in the testis *(28)*. These results suggest that inhibin is not necessary for the normal differentiation of embryonic gonads and sper-

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matogenesis, but plays an important autocrine and/or paracrine function as a tumor suppressor in the gonads.

Activin-βA-deficient mice develop to term, but die within 24 h. These mice present multiple cranio-facial deformities, such as a lack of whiskers and lower incisors *(29)*. Mice with a mutation in the ActRcIIA gene have also been generated *(24)*, and were expected to possibly mimic the phenotype of the activin-$\beta A^{-/-}$ mice. Although some ActRcIIA$^{-/-}$ mice suffered from cranio-facial anomalies, most of them developed into adults. The ActRcIIA-deficient mice suffered from a complete suppression in FSH synthesis, and their reproductive ability were altered. Male ActRcIIA$^{-/-}$ mice reached puberty later than wild-type animals, but their stages of spermatogenesis were seemingly normal. Seminiferous-tubule diameter and volume were reduced, which could result from an overall decrease in the number of Sertoli cells. Since activin stimulates FSH synthesis in the pituitary, the small testis of the ActRcIIA$^{-/-}$ animals may result from the decreased levels of FSH, although though LH levels were normal. Mice with a double mutation of either the α-inhibin and activin βB genes or of the α-inhibin and ActRcIIA genes also develop testicular tumors *(30)*.

Disruption of the common activin/inhibin βB subunit gene produces mice deficient in activin B, activin AB, and inhibin B *(31)*. Homozygous mutants present defects in eyelid development, and $\beta B^{-/-}$ females manifest a profoundly impaired reproductive ability. However, the reproductive organs from $\beta B^{-/-}$ males appeared normal, and these mice bred normally.

MIS and Inhibin

The gonadal tumors of inhibin-deficient mice are first detected around 4 wk of age. It could be hypothesised that MIS, which is synthesized in the same gonadal cells as inhibin, may act as a tumor suppressor at this early stage. It has been demonstrated that inhibin/MIS double-mutant male mice develop testicular tumors at an even earlier age. These tumors are different from those observed in either α-inhibin$^{-/-}$ or MIS$^{-/-}$ mice *(32)*. They grow more rapidly, are faster, less hemorrhagic, and produce less estradiol as compared to the tumors of inhibin-deficient male mice. These results suggest that inhibin and MIS synergize to function as gonadal-tumor suppressors.

NUCLEAR RECEPTORS

Retinol Receptors

Vitamin A, or retinol, is absolutely essential for spermatogenesis. Rats fed a vitamin A-deficient diet become sterile and show a drastic reduction in testis weight *(33,34)*. In animals on a vitamin A-deficient diet, only Sertoli cells and spermatogonia are apparent, while meiotic and postmeiotic germ cells degenerate.

There are two known classes of retinol receptors, retinoic-acid receptors (RARα, β, and γ) that bind 13-*cis*-retinoic acid, and all-*trans*-retinoic acid *(35,36)*, and the retinoic X receptors (RXRα, β and γ), which have a high affinity for 9-*cis*-retinoic acid *(37)*. All retinoid receptors are member of the nuclear-receptor superfamily. The RARα, RXRα, and RXRβ isoforms are widely expressed in the embryo and adult tissues *(38–41)*, while the expression of the RARγ gene is restricted to the skin. These receptors can homodimerize and heterodimerize with a wide variety of nuclear receptors, and are believed to be responsible for a wide variety of signaling pathways *(42)*.

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The RARα gene encodes two isoforms. The major isoform, RARα1, is expressed ubiquitously, while the RA-inducible isoform RARα2 has a more restricted expression pattern *(43)*. Knocking out the RARα2 gene has no apparent deleterious effect since RARα2-deficient mice are healthy and fertile. However, high postnatal lethality is observed in mice homozygous for a mutation of the entire RARα gene *(44)*. Although most RARα$^{-/-}$ mutants survive birth, the survival rate at 24 h of age is 40%, and only 12% of homozygous animals are alive after 1–2 mo. Mice surviving over 2 mo of age appear normal, but none of the males are able to sire any litters. Histological analysis of the testis has showed severe degeneration of the germinal epithelium, although some of the tubules appear normal. Vacuolization of the Sertoli cells is evident, and cytoplasmic expansion of these cells often partially fills the lumen. The epidymal duct appears normal, but contains very few spermatozoa. The degeneration observed in RXRα$^{-/-}$ testis is almost identical to that observed in animals kept on a vitamin A-deficient diet. This observation suggests that retinoic acid, and not retinol, is required for the maintenance of spermatogenesis. This hypothesis is supported by the observation that high doses of retinoic acid can restore the germ-cell degeneration induced by the vitamin A-deficient diet *(45)*.

The RXRβ gene is expressed mostly in Sertoli cells. Targeted mutation of the RXRβ gene *(46)* reveals that approx 50% of RXRβ$^{-/-}$ mutants die in utero or shortly after birth, for unknown reasons. Homozygote females are fertile, but RXRβ-deficient males are sterile. Histological analysis of the epididymis of males RXRβ$^{-/-}$ mice shows low levels of spermatozoa, most of which (95%) remains immotile. A majority of spermatozoa from RXRβ-deficient mice exhibit a coiling of the tail, and 30% have an acrosome that is indented or partially detached from the nuclear envelope. It has been suggested that the high frequency of such defects in mutant spermatozoa results from an impaired attachment of the acrosomal membrane to the nucleus. The diameter of the seminiferous tubules is normal, and the proportion and length of stages of the cycle is apparently normal. However, some of the late spermatids fail to align at the luminar side of the tubules. Moreover, remnants of spermatid heads are located inside the cytoplasm of the Sertoli cells. In young animals, lipid droplets are apparent in the cytoplasm of Sertoli cells—droplets which become more and more apparent as the animal gets older. In 6-mo-old RXRβ$^{-/-}$ animals, the lipid droplets are larger than the Sertoli-cell nuclei, and the tubule have a reduced diameter with variable degrees of cell loss. By the age of 12 mo over one-half of the tubules are replaced by tubular ghosts consisting of a thickened and convoluted basement membrane filled with lipids. These results demonstrate that disruption of RXRβ gene results in alterations of Sertoli-cell function and underscore the crucial role played by retinols in the germ-cells differentiation process.

Estrogen Receptors

Two estrogen receptors exist, ERα and ERβ. These receptors hare significant homology, and both belong to the steroid-receptor superfamily *(47,48)*. These receptors bind 17β-estradiol, the female-sex steroid that plays a critical role in female sexual development. Targeting of the ERα has been achieved, while a full description of the anatomical and physiological features of mice mutated for ERβ is ongoing *(49,50)*. Surprisingly, inactivation of ERa gene affects male fertility. The mutant males exhibit impaired sexual behavior, including decreased intromission and ejaculation. ERα$^{-/-}$ males have low fertility with reduced testis size, and a 90% reduction in sperm number. In contrast to the severe reproductive phenotypes of ERα-deficient male mice, ERβ-deficient male mice

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are fertile and have no testicular defects. Older males demonstrate epithelial hyperplasia in the prostate and the bladder *(50)*. Double-mutant male mice that are deficient in both ERα and ERβ are infertile *(51)* and essentially demonstrate the ERa-deficient phenotypes, i.e., infertility and reduction in sperm number and motility.

PROLIFERATION AND APOPTOSIS

The p53 Protein

In contrast to the great body of information on the regulation of the mitotic-cell cycle, much less is known about the molecular mechanisms involved in the regulation of meiosis. Yet, the stringent control in the differentiation and proliferation timing of the germ cells indicates the presence of critical checkpoints. An analysis of cyclin-dependent kinases (cDKs) has revealed that their expression occurs at specific steps of the meiotic-cell cycle, and suggests a role in the differentiation program *(52)*. Normally, a proportion of germ cells undergo apoptosis in the seminiferous epithelium. This number increases dramatically in some pathological conditions, including idiopathic infertility caused by spermatogenic arrest in human males. Interestingly, somatic-cell-cycle regulation by the Cdk family of genes is modulated by the pleiotropic action of the tumor suppressor p53 *(53)*.

The activity of the p53 protein has been associated with both apoptosis and cell differentiation *(53–55)*. The pattern of expression of the p53 gene has been studied in mice by developing transgenic animals in which the bacterial gene encoding chloramphenicol acetyltransferase (CAT) is under the control of the p53-gene promoter *(56)*. This study showed that the testis is the organ with the highest levels of p53-gene expression in the adult. In the testis, p53-gene expression is mostly restricted to primary spermatocytes at the pachytene phase of meiosis, just before they develop into haploid spermatids. A giant cell testicular degeneration syndrome is occasionally seen in transgenic mice carrying a p53-promoter-CAT fusion, which results in decreased p53 levels in the testes *(57)*. These mice seem to recapitulate a degenerative syndrome, probably resulting from the inability of the tetraploid primary spermatocytes to complete meiotic division. The severity of this syndrome is variable from one strain to another, and can be correlated to the reduction in p53 levels. The role of p53 in the regulation of the cell cycle and in the apoptotic pathway is further emphasized by the finding of the robust overexpression of wild-type p53 protein in most testicular tumors *(58)*.

When the mouse p53 gene is knocked out in ES cells, p53-deficient mice are generated *(59)*. Mutant animals initially appear healthy and fertile. However, most p53-deficient animals die by 3–6 mo of age from multiple neoplasms. Spermatogenesis appears normal, although giant cells can be found in some tubules. Testicular section of p53-deficient mice of the 129 background exhibit a high incidence of seminomas and undifferentiated teratocarcinomas. In fact, this strain of mice seems to have a profound effect on the testicular phenotype, because $p53^{-/-}$ mice of pure 129 strain are sterile, whereas p53-deficient mice of other strains and mixed background are fertile *(57)*.

The Bax Protein

A direct connection with the germ-cell apoptotic pathway is provided by the finding that p53 acts as a transcriptional activator of the Bax gene *(60)*. Bax is a dimerization partner of Bcl-2, a protein with the potential to interfere with programmed cell death in

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response to a number of apoptotic stimuli. Importantly, Bax has an opposing function to Bcl-2—its ablility to accelerate apoptosis. The deletion of the Bax gene by homologous recombination results in a complete block of the spermatogenesis (*see* Chapter ????). Bax-deficient male mice are infertile, with atrophied testes and empty epididymis and vas deferens. Histological analysis of the seminiferous tubules shows spermatogenic arrest, accumulation of premeiotic spermatocytes, abnormal mitotic or meiotic figures, and multinucleated giant cells. Round spermatids are rare, and elongated spermatids are completely absent. Electron microscopy has also revealed a disordered maturation scheme. Premeiotic cells have an atypical distribution of decondensed chromatin, and an irregular size and shape not typical of spermatogonia or preleptotene spermatocytes. Flow cytometry of testicular cells shows an elevated number of 2N cells, which reflects the abnormal premeiotic-cell expansion noted in the testicular section. A small proportion of 1N cells representing the round spermatids is present, but the more condensed 1N population represented by elongating spermatids and spermatozoa is completely absent. Multinucleated and pyknotic cells are also present in the testes of Bax-deficient mice, suggesting increased apoptosis in this tissue. Thus, in Bax-null mice, the premeiotic germ cells appear atypical, and instead of differentiating, enter a pathway of programmed cell death *(61)*. Deletion of the genes encoding the Bax partners Bcl-2 and Bcl-x reveals massive cell death in the lymphoid-cell lineage, but does not cause germ-cell aberrations *(62,63)*. Interestingly, Bax-null mice also show lymphoid hyperplasia *(61)*. Thus, the same molecule may act positively or negatively on the apoptotic pathway of different cell lineages.

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Cyclin D2

The D-type cyclins D1, D2, and D3 are critical controllers of the G1 phase of the mammalian cell cycle. The three cyclins are expressed in overlapping, apparently redundant fashion in various proliferating tissues. Homologous recombination at the cyclin D2-gene locus has been achieved in mouse ES cells (64). Male mice carrying a cyclin-D2 mutated gene are fertile but display hypoplastic testes, suggesting a role for this cyclin in the regulation of testis growth. Interestingly, the expression of the cyclin D2 gene, and not of the D1 or D3 genes, was found to be FSH-inducible via the cAMP-dependent pathway. This finding indicates that the regulation of the various cyclin genes is under the control of various intracellular signaling pathways. The hypoplasia observed in testes correlates with the observation that some human testicular tumors display a high cyclin-D2 expression.

CYCLIN A1

There are two mammalian members of cyclin A family—cyclin A1 and cyclin A2. Cyclin A2 exhibits a more widespread expression, and cyclin A2-deficient mice die embryonically. In contrast, cyclin A1 is expressed extensively in the male germ-cell lineage in mice *(65)*. Cyclin A1-deficient male mice are infertile because of a block in spermatogenesis before the first meiotic division. This is accompanied by increased germ-cell apoptosis defects in desynapsis, and a 80% reduction in cdc2 kinase activation at the end of the meiotic prophase *(65)*. The residual cyclin B1/cdc2 activity in the complete absence of cyclin A1 has been found to be insufficient for the progression of meiotic divisions. Thus, cyclin A1 represents a novel male meiotic lineage-specific class of cyclins.

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TRANSCRIPTION FACTOR CREM

A Molecular Master-Switch

Cyclic AMP-dependent signaling is known to play an important role in spermatogenesis *(2)*. The receptors for LH and FSH are coupled to Gs proteins, and activate the adenylyl cyclase, further leading to an activation of the cAMP-dependent protein kinase A (PKA). Many genes that are expressed during spermatogenesis possess cAMP-responsive elements (CRE) in their promoter *(66)*. These sequences are targets for CRE-binding proteins, such as CREB or CREM, that can be phosphorylated and activated by the PKA as well as other signaling pathways *(67)*. CRE-binding proteins are relatively ubiquitous and uninducible *(68)*. However, in adult male germ cells, the activator CREM is expressed at levels that are hundreds of times higher than those in other tissues *(69)*. The CREM activator has been shown to function as a regulator of gene expression in haploid cells. CREM levels are regulated during germ-cell differentiation and by the FSH-signaling pathway *(70)*. CREM proteins are expressed in haploid spermatids, where they activate multiple genes, such as transition proteins, angiotensin-converting enzyme (ACE), calspermin, and cholesterogenic lanosterol 14α-demethylase (CYP51) *(66,71–74)*. A summary of cAMP signaling in the testis and expression patterns of key regulators of this pathway is schematically represented in Fig. 1.

The crucial role of CREM during spermatogenesis has been confirmed by its targeted mutation in the mouse *(75,76)*. In male CREM$^{+/-}$ mice, there is a 50% reduction in the number and motility of spermatazoa. There is also a twofold increase in the number of spermatozoa with an aberrant structure. In CREM$^{-/-}$ animals, the females are fertile, but the males are sterile and produce no spermatozoa. Histological analysis of seminiferous tubules reveals a complete arrest of spermatogenesis at the first stage of spermiogenesis (Fig. 2). A 10-fold increase in apoptotic germ cells is also observed, and in many cases, these apoptotic bodies acquire the shape of multinucleated giant cells *(75)*. Finally, serum concentrations of LH, FSH, and testosterone are not reduced in CREM-deficient males, indicating that the phenotype observed in these animals does not result from hormonal alterations.

ACT, A Testis-Specific Coactivator

Crucial steps in transcriptional activation by factors of the CREB/CREM class are phosphorylation at a specific serine regulatory site and the subsequent recruitment of the coactivator, CBP (CREB-binding protein) *(65,66)*. Thus, the phosphorylation event is considered to be the key event, leading to transcriptional activation in response to induction of a specific signaling route. Surprisingly, CREM is found to be unphosphorylated in male germ cells. Thus, activation by CREM must occur independently of phosphorylation, and therefore of binding of CBP. A yeast two-hybrid screen of a testis-derived cDNA library, using the CREM activation domain as bait, has revealed the presence of a novel protein, activator of CREM in testis (ACT) *(77)*. The distinctive feature of ACT is the presence of four complete LIM motifs and another half motif at the N-terminus. LIM domains comprise a conserved cysteine- and histidine-rich structure that forms two adjacent zinc fingers. This structural motif was first identified in the protein products of three genes, *Lin-11*, *Isl-1*, and *Mec-3*. The LIM domain functions as a protein-protein interaction domain. LIM domains can be present with other functional protein motifs, such as homeobox and kinase domains, but ACT belongs to the class of the LIM-only proteins (LMO), and contains no other structural motif.

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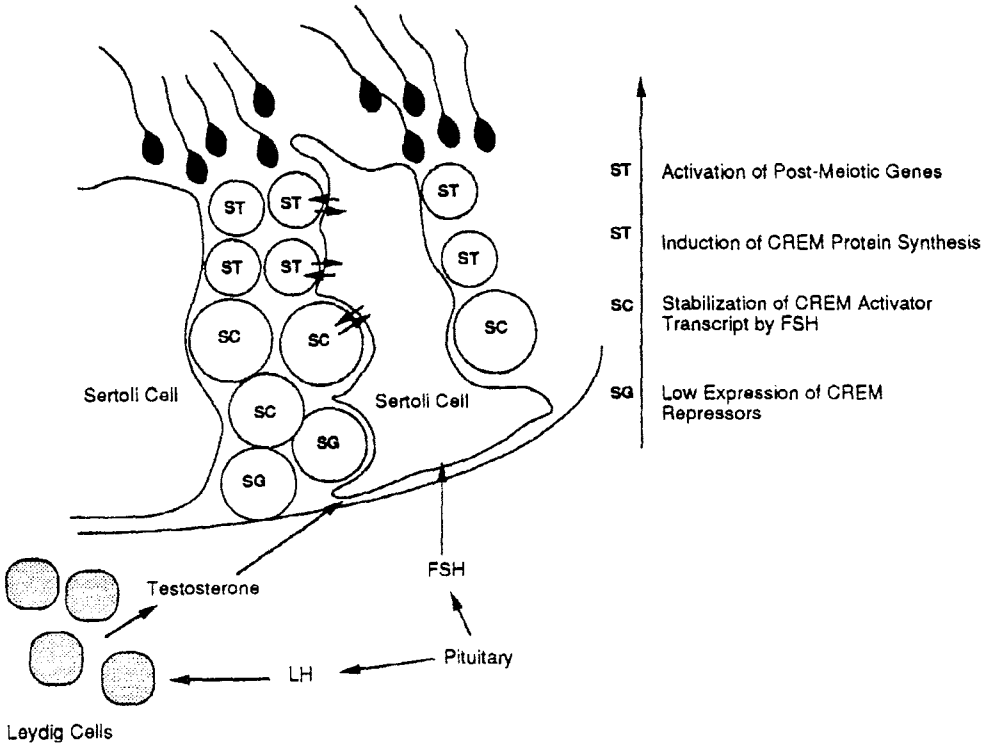

Fig. 1. Schematic representation of a section of a seminiferous tubule where the CREM expression patteren is indicated. CREM expression is regulated at multiple levels during spermatogenesis. Premeiotic germ cells spermatogonia (SG) express a low level of CREM repressor isoforms. During meiotic prophase, the pituitary follicle-stimulating hormone (FSH) is responsible for the stabilization of CREM activator transcripts in spermatocytes (SC); CREM protein, on the other hand, is detected only after meiosis in haploid spermatids (ST). Note the strict relationships between the Sertoli and germ cells *(arrows)*. In the haploid spermatids, CREM proteins activate a number of cellular genes expressed specifically during spermtid maturation.

Several lines of evidence point to the coordinated expression of CREM and ACT. ACT is abundantly and exclusively expressed in testis; ACT colocalizes with CREM in spermatids; and ACT and CREM exhibit the same expression pattern during testis development. CREM and ACT efficiently associate; the biological significance of this is that ACT has an intrinsic transactivation capacity and can convert CREM into a powerful transcriptional activator *(77)* (Fig. 3). Most importantly, co-activation through ACT can occur also in yeast, which lacks CBP and TAF130 homologs. Thus, ACT can bypass the need for CREM or CREB phosphorylation. Indeed, ACT converts an inactive CREM mutant (with the serine phosphoacceptor site mutated into alanine) into a transcriptionally active molecule, both in yeast and in mammalian cells. Thus, in male germ cells, ACT provides a novel, tissue-specific phosphorylation-independent route for transactivation by members of the CREB family *(77)*. A general model of CREM interacting with the general transcriptional machinery is depicted in Fig. 4.

Fig. 3

Fig. 4

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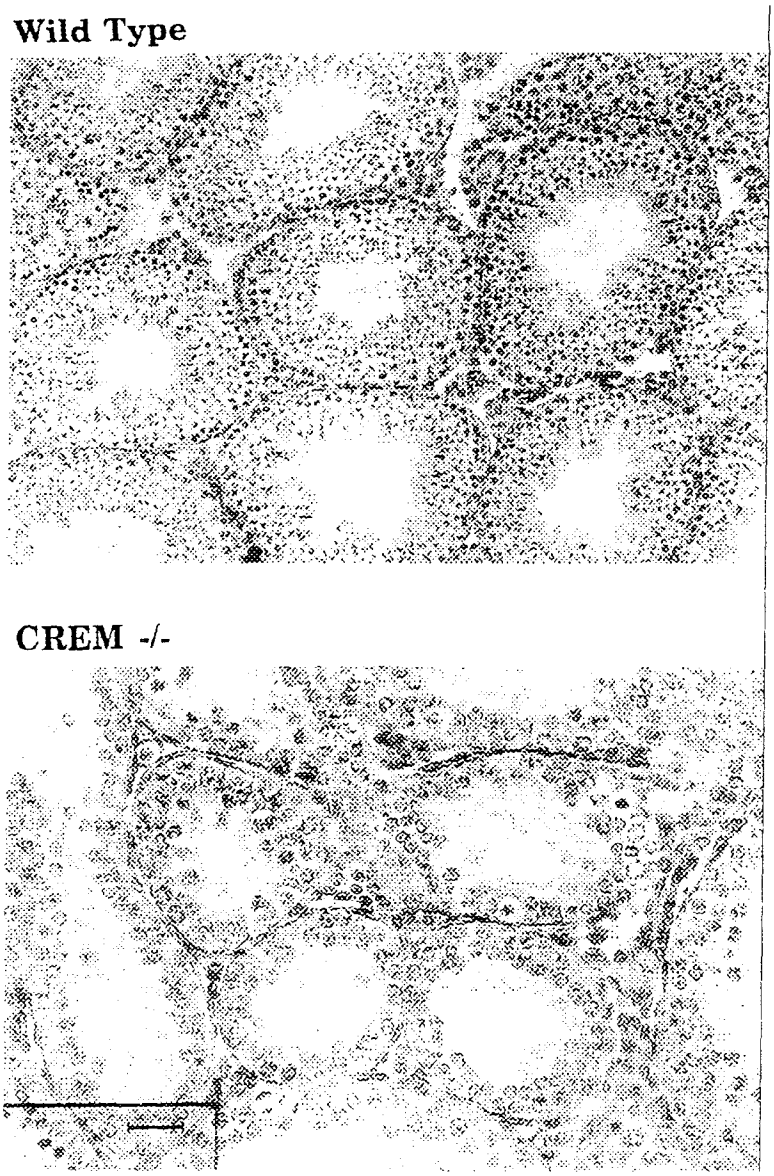

Fig. 2. CREM deficiency causes spermiogenesis arrest and make germ-cell apoptosis. Testes from a 8-wk-old homozygous mutant (–/–) and a wild-type (+/+) mouse littermate. Histological analysis of testis sections. The tubules from the CREM-deficient mice show impared spermatogenesis and some multinucleated apoptotic cells.

CHAPERONE HSP70-2

Members of the 70-kDa heat shock protein (HSP70) family are chaperones that assist in the folding, transport, and assembly of protein in the cytoplasm, mitochondria, and endoplasmic reticulum *(78)* HSP70-2 is a testis-specific gene that is expressed at high levels in pachytene spermatocytes during the meiotic phase of spermatogenesis *(79,80)*. The developmentally regulated expression of HSP70-2 during spermatogenesis implies

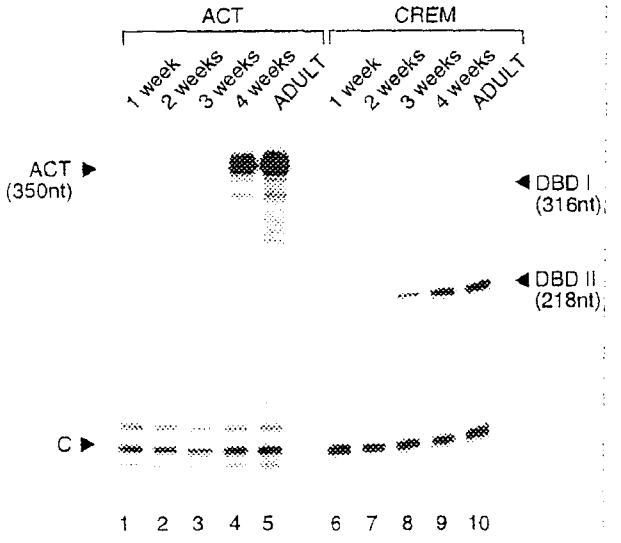

Fig. 3. ACT is exclusively in testis. Co-expression of ACT and CREM druring testis development. RNA was extracted from testes of mice at different ages and analyzed by RNase protection assay, using ACT- and CREM-specific riboprobes. C indicates a b-actin protected frgament used as an intenal control. DBD I and DBD II refer to the two alternative DNA-binding domains of CREM.

that it performs a specialized function during meiosis. This protein has been identified as a component of the synaptonemal complex in prophase nuclei of spermatogenic cells *(81)*.

HSP70-2 mutant males produce no spermatozoa and are infertile *(82)*. Spermatogonia and pachytene spermatocytes, mainly with aberrant structures, are present in HSP70-2$^{-/-}$ testis, but postmeiotic spermatids are completely absent. Pachytene spermatocyte with condensed nuclei are observed, and there is a major increase in the level of apoptotic cells in HSP70-2 mutant testis. Although typical-appearing synaptonemal complexes are observed in pachytene cells from HSP70-2$^{-/-}$ testis, synaptonemal complex development beyond the middle to late pachytene stages is not observed *(82)*. These observations suggest that HSP70-2 is not necessary for synaptonemal complex assembly, but is required during synapsis, which allows progression to the subsequent meiotic divisions.

CONCLUSIONS

The use of genetically modified mice has brought a wealth of information on the genetic control of gametogenesis, yet additional questions have arisen. Much more will be revealed by the homologous recombination approach in reproductive biology as many other animal models will be generated. Importantly, not all gene inactivations believed to influence the germ-cell differentiation program have led to the anticipated sterile phenotype. For example, the normal fertility of the acrosin-mutant mice suggests that this endoprotease is not essential for sperm penetration of the oocyte zona pellucida (ZP) or fertilization *(83)*. Important considerations include the finding that analysis of testicular function is often complicated by deleterious or lethal consequences of a specific gene inactivation. On the other hand, it is evident that many crucial elements are involved in the regulation of gametogenesis, some of which have not been considered.

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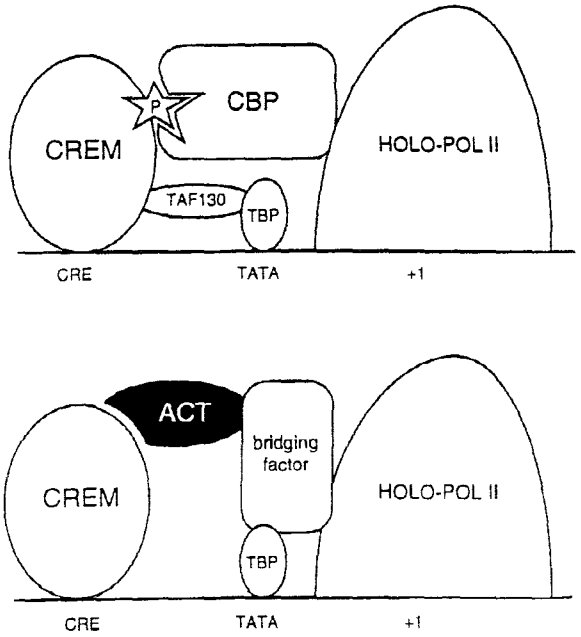

Fig. 4. CREM-mediated transcription is promoted by interaction with different co-activators. (Top) A schematic representation of the classical view by which, through interaction with CBP, activators as CREB and CREM elicit their function. A key event in this scenario is phosphorylation at Ser117 P, since it is required for binding to CBP and subsequent transcriptional activation. Interaction with TAF130 is constitutive, and occurs via the Q2 domain of CREB/CREM. (Bottom) Representation of how ACT may elicit its co-activator function via interaction with CREM. In yeast, CREB and CREM are inactive because of the lack of CBP and TAF130. ACT elicits its function and interacts with CREM, also in the absence of Ser117 phosphorylation. Thus, ACT provides an alternative activation pathway that appears to work in a signaling-independent manner. A hypothetical bridging factor, linking ACT to the basal transcription machinery, is represented.

Special attention should be given to the pathway of programmed cell death of germ cells. Very little is known, as little research has been done on the meiosis cycle as compared to the mitotic cell cycle. The increased proportion of apoptotic bodies in many mutated animals with testicular alterations indicates that apoptosis must play an important, but poorly defined, role in the spermatogenic cascade. Further studies will focus on the precise role played by well-known mitotic apoptosis-related proteins, and possibly on the discovery of novel, meiosis-specific, cell-death molecules. One interesting approach has been the screen of lines of mutant mice created using a retroviral gene-trap system for male infertility *(84)*. This approach has led to the finding that Bclw-deficient mice have testicular degeneration *(84)*.

Analysis of some of the mutant mice suggests previously unrecognized relationships. Of particular interest is the very close testicular phenotype observed in the CREM, HSP70-2, and BAX-deficient mice, suggesting that an interplay of these genes may place them on the same, or related, signaling cascades. Future work will take advantage of multiple mutations, and of conditional homologous recombination, to remarkably improve our understanding of gametogenesis.

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