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Normal Sexual Development and Fertility in *testatin* Knockout Mice

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The *testatin* gene was previously isolated in a screen focused on finding novel signaling molecules involved in sex determination and differentiation. *testatin* is specifically upregulated in pre-Sertoli cells in early fetal development, immediately after the onset of *Sry* expression, and was therefore considered a strong candidate for involvement in early testis development. *testatin* expression is maintained in the adult Sertoli cell, and it can also be found in a small population of germ cells. Testatin shows homology to family 2 cystatins, a group of broadly expressed small secretory proteins that are inhibitors of cysteine proteases in vitro but whose in vivo functions are unclear. *testatin* belongs to a novel subfamily among the cystatins, comprising genes that all show expression patterns that are strikingly restricted to reproductive tissue. To investigate a possible role of testatin in testis development and male reproduction, we have generated a mouse with targeted disruption of the *testatin* gene. We found no abnormalities in the *testatin* knockout mice with regard to fetal and adult testis morphology, cellular ultrastructure, body and testis weight, number of offspring, spermatogenesis, or hormonal parameters (testosterone, luteinizing hormone, and follicle-stimulating hormone).

The testis and the ovary arise from a common bipotential gonad during mammalian embryogenesis. During fetal life, at 11 days postcoitum (dpc) in the mouse, the indifferent gonad develops as a narrow band of tissue close to the kidney. The testis-determining gene *Sry*, located on the Y chromosome, acts dominantly to trigger differentiation of testes from the indifferent gonads that would otherwise develop as ovaries (22, 29, 48). Once the gonads begin to differentiate as testes, they secrete factors, notably anti-Müllerian hormone and testosterone, which determine further sexual development and are required for normal reproductive function in the adult individual. Disturbances in the initial sex-determining switch or in the subsequent differentiation of the testis will lead to incomplete sexual development in XY individuals who would otherwise develop as males.

In addition to the master switch in sex determination, *Sry*, several genes have been identified that are involved in the formation of the indifferent gonad or subsequent differentiation of the testis. These include genes encoding, e.g., the transcription factors Sf1, Wt1, Emx1, Lhx9, M33, and Dmrt1 and the signaling molecules Fgf9, Wnt7a, Wnt4, and Dhh (reviewed in references 7 and 50). Despite the characterization of these genes, it is clear that key factors in gonad and testis development are lacking, and no genes that are directly regulated by the transcription factor *Sry* have been characterized. As an illustration of this in the human, mutation analysis of known candidate genes provides a molecular diagnosis in only a minority of patients with disturbed gonadal development.

We have previously reported the isolation of the *testatin* gene in a screen focused on finding novel genes involved in sex determination and differentiation (53). *testatin* was identified using a modified form of differential display, designed to detect genes encoding proteins containing signal peptides, in RNA from XY compared to XX mouse embryonal gonads at 13.5 dpc. *testatin* is specifically upregulated immediately after the onset of *Sry* expression, in pre-Sertoli cells, making it a strong candidate for involvement in early testis development (53). Expression of *testatin* is maintained in the adult Sertoli cell (53), and *testatin* transcripts have also been found in 20 to 25% of fetal germ cells and adult spermatogonia (26).

Testatin shows homology to family 2 cystatins, a group of small secretory proteins (10 to 14 kDa) that are reversible competitive inhibitors of C_1 cysteine proteases such as plant papain and the mammalian cathepsins B, H, and L in vitro (54). Most cystatins are broadly expressed and, therefore, thought to have housekeeping functions. Although it is well established by in vitro studies that cystatins are potent inhibitors of specific cysteine proteases, their in vivo functions are less clear. They have been suggested to play important roles in normal body processes such as prohormone processing and bone resorption (16, 38) as well as in pathological conditions such as tumor progression and inflammation (8, 32).

Among the family 2 cystatins, a new subfamily has emerged during recent years, comprising genes that all display expression patterns that are strikingly restricted to reproductive tissues (13). *testatin* belongs to this novel subfamily together with six additional mouse genes. The first-described and best-characterized member is the gene encoding the cystatin-related epididymal spermatogenic protein (Cres) (15). Other members

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are *cystatin T* (47), *cystatin SC* (34), *cystatin TE-1* or *Cres3* (24, 34), *Cres2* or *cystatin E1* (24, 33), and *cystatin E2* (33).

To investigate a possible role of testatin in sex determination, testis development, or male reproduction, we have generated a mouse with targeted disruption of the *testatin* gene by homologous recombination in mouse embryonic stem (ES) cells. The *testatin* knockout mice have been characterized with regard to fetal and adult testis morphology, cellular ultrastructure, body and testis weight, number of offspring, spermatogenesis, and hormonal parameters (testosterone, luteinizing hormone [LH], and follicle-stimulating hormone [FSH]).

MATERIALS AND METHODS

Construction of the *testatin* gene targeting vector. Two genomic clones that together contain the three exons of the *testatin* gene were isolated from a Lambda Fix II mouse genomic library (Stratagene) using the mouse *testatin* cDNA (53) as a probe. A 2.8-kb fragment containing the 5′-flanking region of the *testatin* gene was subcloned in a directional manner into the BglII/KpnI sites of the pKO-v905 vector (Lexicon Genetics). Thereafter, a 10-kb NotI fragment containing exon 3 and the 3′-flanking region of the *testatin* gene was filled in with T4 DNA polymerase (Promega) and subcloned into the SmaI site of the v905 targeting vector. Finally, a 3.7-kb fragment containing the first two exons, including the translation start site, of the *testatin* gene was replaced with a 1.6-kb neomycin resistance gene that was excised out from the vector pKO 800 (Lexicon Genetics) and subcloned into the AscI site of the v905 targeting vector.

Generation of testatin-deficient mice. The HpaI-linearized *testatin* targeting vector was electroporated into mouse ES cells derived from the 129/Sv mouse strain, and neomycin (G418)-resistant colonies were selected. Genomic DNA from individual neomycin-resistant colonies was digested with XbaI and analyzed by Southern blotting using an 0.5-kb 5′ probe flanking the targeting vector (see Fig. 1B). Of 700 clones screened, five ES cell clones were obtained that had incorporated the targeted vector by homologous recombination. Four clones were microinjected into C57BL/6 blastocysts, which were subsequently transferred into pseudopregnant foster mice to generate chimeras. Two clones (3BE6 and 6AC10) generated chimeric males that were mated with C57BL/6 females that passed the mutant allele to their offspring. To generate animals with complete (homozygous) deficiency of the *testatin* gene, heterozygous offspring were intercrossed, and genomic DNA was isolated from tail biopsy samples of 3-week-old animals, digested with XbaI, and analyzed by Southern blotting using the 5′-flanking probe or by PCR using primers for amplification of both a *testatin* gene-specific 612-bp fragment (primers P_1 [5′-CAGAGTCTCAGGACATAG TC] and P_2 [5′-GTTCCATCCTGTAGGCAT]) and a *neo* gene-specific 349-bp fragment (primers P_1 [5′-CAGAGTCTCAGGACATAGTC] and P_{neo} [5′-CGC ATTGTCTGAAGTAGGT]). PCR genotyping was also done on genomic DNA isolated from tail biopsy samples from mouse embryos. Determination of genetic sex was done by PCR using primers specific for *Sry* (upstream, 5′-GGTTGCA ATCATAATTCTTCC; downstream, 5′-CACTCCTCTGTGACACTTTAG). The animals were treated in accordance with Swedish law and regulations of the Karolinska Institutet. They were housed under specific-pathogen-free conditions in a 12-h-light/dark-cycle facility with free access to food and water. The research protocol was approved by the Swedish Ethical Board.

RT-PCR and RNA in situ hybridization. Total RNA was extracted from testes of wild-type, heterozygous, and homozygous null animals using GeneElute (Sigma-Aldrich) following the manufacturer's protocol. Reverse transcription (RT) analysis was performed as previously described (53) using primers specific for *testatin* (upstream, 5′-ATGTTCTCATCACTCCTGTC; downstream, 5′-TTCA GACCATGGCTCTCCTG) and *Hprt* (upstream, 5-CCTGCTGGATTCCATTA AAGCACTG; downstream, 5′-GTCAAGGGCATATCCAACAACAAAC) cDNAs. *Hprt* was included as an internal control for efficiency of each RT reaction. RNA in situ hybridization was performed on 8-μm-thick paraffin sections from newborn wild-type and testatin-deficient mouse testes using a medium-stringency protocol with hybridization at 60°C overnight, as recommended by the manufacturers of the Discovery instrument (Ventana). The digoxigenin-labeled antisense and sense riboprobes for *testatin* were prepared from linearized plasmids containing full-length *testatin* cDNA.

Histology, immunohistochemistry, and electron microscopy. Testes were dissected from male embryos that were collected by dissection of pregnant mice that were sacrificed by CO_2 asphyxiation on day 15.5 or 17.5 of pregnancy. Testes from embryos and newborn pups were immediately fixed in fresh 1% paraformaldehyde in phosphate-buffered saline for 1 h on ice and thereafter equilibrated in 0.5 M sucrose on ice, embedded in Tissue-Tek (Sakura), and frozen at −75°C. Frozen testes were cryosectioned in 8-μm sections at −20°C and mounted on Superfrost glass (Menzel-Glaser). The slides were stored at −20°C, taken to room temperature, and left to dry in the open air for 10 min followed by hematoxylin and eosin staining and immunohistochemical analyses. Immunofluorescence analysis was performed as described previously with an additional 10-min postfixation in 1% phosphonoformic acid directly on the slides prior to blocking (39). Primary antibodies were rabbit polyclonal antiserum directed against the germ cell marker Mage-b4 (39) diluted 1:250 to 500, WT1 (Santa Cruz Biotechnologies) diluted 1:50, human P450scc (patient serum kindly provided by O Kämpe, Uppsala University, Sweden) diluted 1:500, fluorescein isothiocyanate-conjugated monoclonal anti-smooth muscle α-actin (Sigma-Aldrich) diluted 1:250, and rat anti-mouse laminin α-1 (19), undiluted. The secondary antibodies were fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulin G (IgG; Sigma-Aldrich) diluted 1:100 and tetramethyl rhodamine isocyanate-conjugated rabbit anti-rat immunoglobulin G (Sigma-Aldrich) diluted 1:100. Whole testes from adult mice were fixed, embedded in plastic, sectioned, and stained with toluidine blue as described previously (44). For electron microscopy the gonads were prepared as previously described (20). Briefly, the testes were fixed by immersion in 5% glutaraldehyde (Fluka) in 0.16 mol/liter sodium cacodylate-HCl buffer (pH 7.4) and postfixed with potassium ferrocyanide-osmium fixative (27). The tissues were embedded in epoxy resin (Glycidether 100; Merck) and sectioned. Ultrathin sections were stained with uranyl and lead citrate (Reichert Ultrostainer; Leica) and examined in a Jeol JEM-100XS and -1200EX electron microscope (JEOL).

Assessment of fertility. Five to six *testatin*$^{-/-}$ and *testatin*$^{+/+}$ males were put alone in big cages overnight. The next day three C57BL/6J females were put in each cage. The male and females were housed together until all females became pregnant (4 to 8 weeks). Pregnant females were sacrificed by CO_2 asphyxiation at 10 to 18 days of pregnancy. Embryos were immediately killed through cervical dislocation, counted, and put in cold phosphate-buffered saline. Sex typing was done by *Sry* PCR using genomic DNA from tail snips as well as, in embryos older than 12.5 dpc, morphological examination of the gonads. Most embryos were counted at 15.5 dpc.

Preparation and analysis of epididymal spermatozoa. Male mice were sacrificed by CO_2 asphyxiation. The lower abdomen was opened in linea alba, and the genital apparatus on each side was freed and lifted forward. Each cauda epididymis was identified and sectioned at the vasal end and at the border to the corpus epididymis. Each testis and cauda epididymis were weighed, the caudae were transferred into prewarmed sperm preparation medium, and the organ was minced in small pieces followed by repeated pipetting to obtain a homogeneous mixture of sperm suspension. The percentage of motile spermatozoa was assessed in duplicate droplets as described previously (31), and at least 200 sperm were assessed. Sperm numbers were counted in duplicates according to the Nordic Association for Andrology-European Society of Human Reproduction and Embryology manual for semen analyses (31), and at least 400 spermatozoa were counted.

Hormone measurements. Serum levels of testosterone, LH, and FSH and intratesticular testosterone levels were determined at the Institute of Biomedicine, Turku University, as described previously (45, 57).

Bioinformatics. Signal peptides were predicted using Phobius (25). Signal peptides were removed prior to protein sequence alignment with Kalign (T. Lassmann and E. L. Sonnhammer, unpublished data). Resulting output was visualized using the Belvu (http://www.cgb.ki.se/cgb/groups/sonnhammer/Belvu.html) and Boxshade (http://www.ch.embnet.org/software/BOX_form.html) tools. Alignments were correlated with the Mafft program (28) to confirm phylogenetic tree shape.

Protein sequence accession numbers. The accession numbers for each of the protein sequences are as follows: NP_034109 (testatin), XP_130534 (cystatin E2), AAL30841 (cystatin SC), AAL30843 (cystatin TE-1/Cres3), NP_034108 (Cres), NP_081300 (cystatin T), AAL51004 (Cres2/cystatin E1), NP_034106 (cystatin C), AK003744 (cystatin E/M), and AF031826 (cystatin F).

RESULTS

Generation of testatin-deficient mice. *testatin* knockout mice were generated by homologous recombination with a targeting vector in mouse ES cells (Fig. 1A). A neomycin cassette replaced the major coding exons 1 and 2, including the translation start site of the *testatin* open reading frame. The targeting vector was electroporated into ES cells, and two successfully

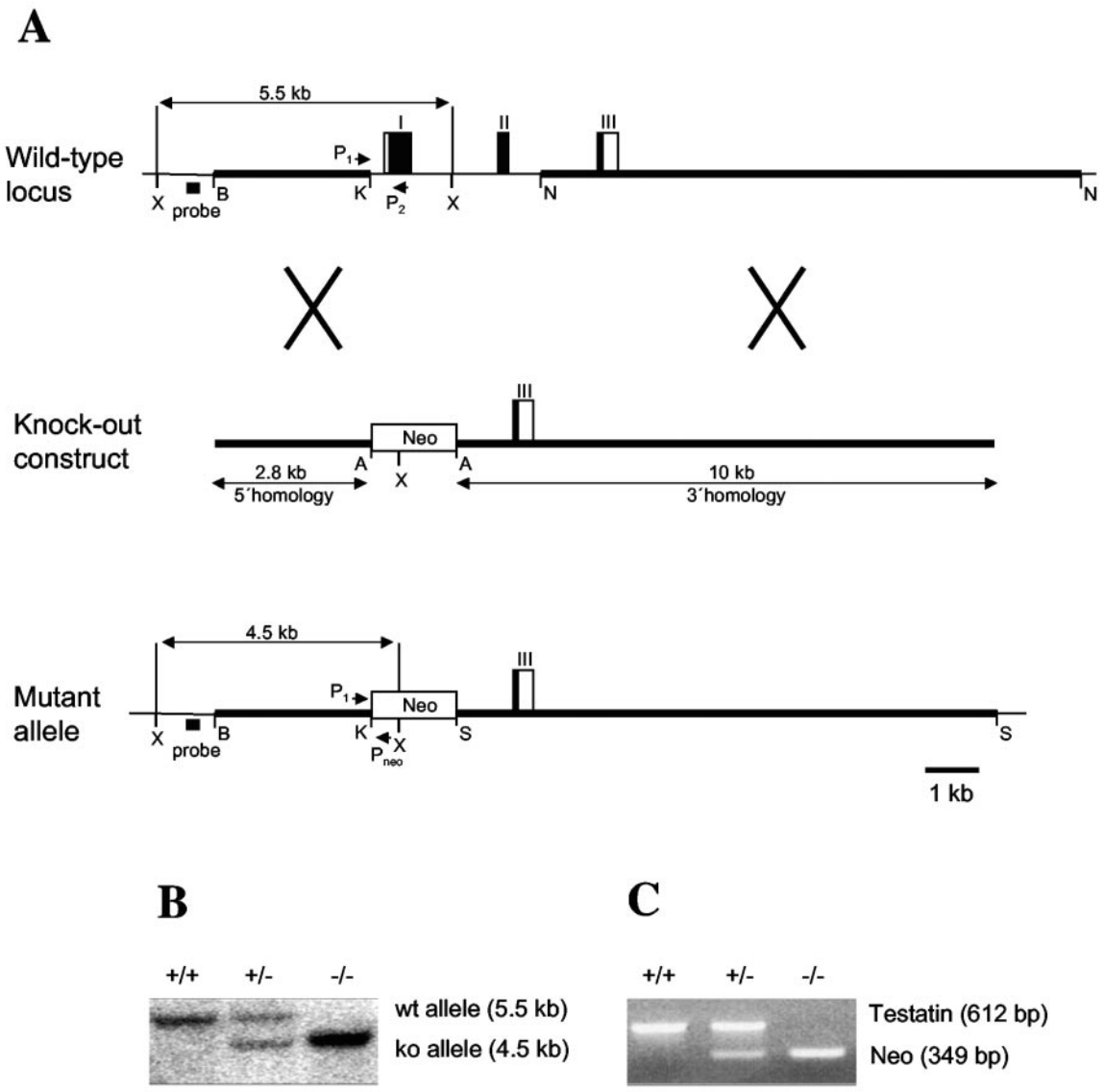

FIG. 1. Targeted disruption of the *testatin* gene by homologous recombination in mouse ES cells. (A) Organization of the wild-type *testatin* locus, the targeting construct, and the disrupted *testatin* allele. The numbered boxes (I, II, and III) denote the exons, the first in-frame ATG codon being located in exon I. The black boxes mark the open reading frame. Relevant restriction sites are as follows: A, AscI; B, BglII; K, KpnI; N, NotI; S, SmaI; X, XbaI. The *neo* cassette was inserted into the AscI sites, replacing exons 1 and 2 of the *testatin* gene. The position of the 0.5-kb 5′ probe used in Southern blotting is indicated, as well as the positions of the primers (P_1, P_2, and P_{neo}) used for PCR genotyping. (B) Southern blot genotyping of wild-type (+/+), heterozygous (+/−), and *testatin* null (−/−) mice using XbaI-digested tail DNA and the indicated 5′ probe. The 5.5-kb fragment of the wild-type *testatin* allele shifts to a 4.5-kb fragment in the cells where homologous recombination successfully has introduced the recombined mutant allele. wt, wild type; ko, knockout. (C) PCR genotyping of genomic tail DNA from the F_2 progeny. The wild-type allele generates a fragment of 612 bp using primers P_1 and P_2 whereas the mutant allele is specifically amplified with primers P_1 and P_{neo}, generating a 349-bp fragment.

targeted clones were used to generate chimeric mice that transmitted the mutated *testatin* allele through the germ line. Heterozygous mating produced offspring with a normal Mendelian distribution of wild-type, heterozygous, and homozygous mutants, indicating no embryonic-lethal effect of testatin deficiency. Deletion of the *testatin* allele was confirmed by Southern analysis (Fig. 1B) and allele-specific PCR (Fig. 1C). RT-PCR analysis and RNA in situ hybridization confirmed the absence of *testatin* transcripts in the −/− mice (Fig. 2B and C). Testatin-deficient mice were viable and did not show any obvious abnormalities.

Testatin-deficient mice show no sex reversal, and their testes develop normally. *testatin* was originally isolated based on its highly specific expression pattern in the testis, with upregulation immediately after the onset of *Sry* expression in early development (53), making it a strong candidate for involvement in sex determination/testis differentiation. To assess the presence of sex reversal in testatin-deficient mice, genetic sex was determined in offspring of heterozygous mice and compared to the anatomical sex. There was an equal distribution of males and females among the offspring, and no cases of ambiguous genitalia or sex reversal were found. To investigate

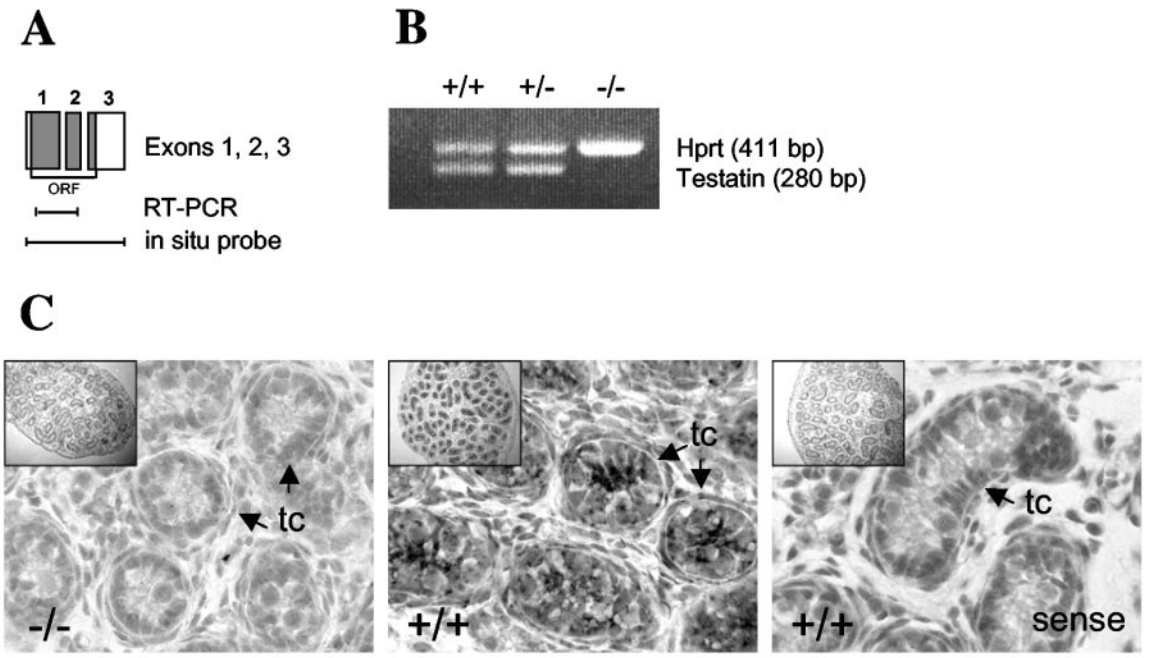

FIG. 2. Lack of *testatin* gene expression in gonads of male testatin-deficient mice. (A) Schematic figure showing the regions covered by the RT-PCR fragment (exons 1 and 2) and the in situ probe (exons 1, 2, and 3). ORF, open reading frame. (B) RT-PCR analysis on total RNA extracted from testes of newborn wild-type (+/+), heterozygous (+/−), and *testatin* null (−/−) mice using primers specific for *testatin* (280-bp fragment) and *Hprt* (411-bp fragment). *testatin* expression was detected in wild-type and heterozygous animals but not in testes from homozygous knockout animals. *Hprt* primers were included as an internal control. (C) RNA in situ hybridization using digoxigenin-labeled antisense and sense *testatin* probes on sections from newborn (day 1) −/− and +/+ mouse testis. *testatin* expression is seen only in the testis cords of +/+ testis. Control hybridization using the sense-strand probe for *testatin* in a wild-type animal gave no significant signal. tc, testis cords. Testis sections were analyzed at magnifications of ×10 (insets) and ×63.

whether testatin deficiency has subtle effects on testis differentiation, testis morphology was examined by histology, immunohistochemistry, and electron microscopy. Figure 3 shows hematoxylin-eosin stains of frozen sections from fetal testes at embryonic day 17.5 (E17.5). Newborn pups and E15.5 embryos were also analyzed (not shown). Cord formation was normal in the homozygous mutant animals, and there was no difference in testis development from that of wild-type littermate controls. We next performed immunohistochemistry on E17.5 testes using antibodies directed at the following cell-type-specific markers (Fig. 4): Mage-b4, which is expressed exclusively in prepachytene germ cells in the testis (39); Wt1, which is expressed by Sertoli cells from E12.5 and onwards (42); smooth-muscle actin, which is expressed by peritubular myoid cells (not shown); and P450scc, which is specific for the steroid-producing Leydig cells. We also included antibodies directed at laminin α-1, which is a component of the basal membrane surrounding the testis cords. We could not detect any differences

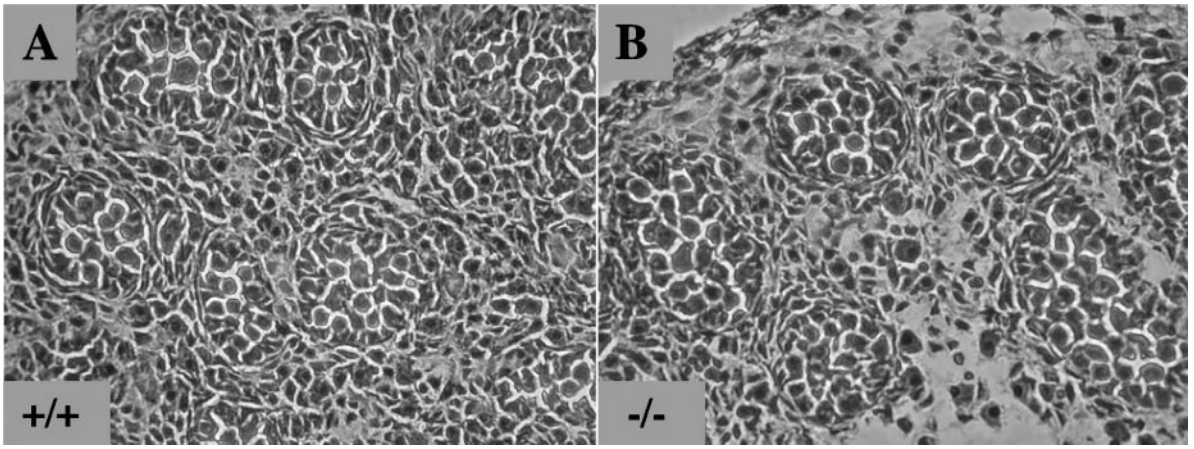

FIG. 3. Hematoxylin-eosin staining of fetal mouse testes at embryonic day 17.5 from control (+/+) (A) and homozygous testatin-deficient (−/−) (B) animals at a magnification of ×40.

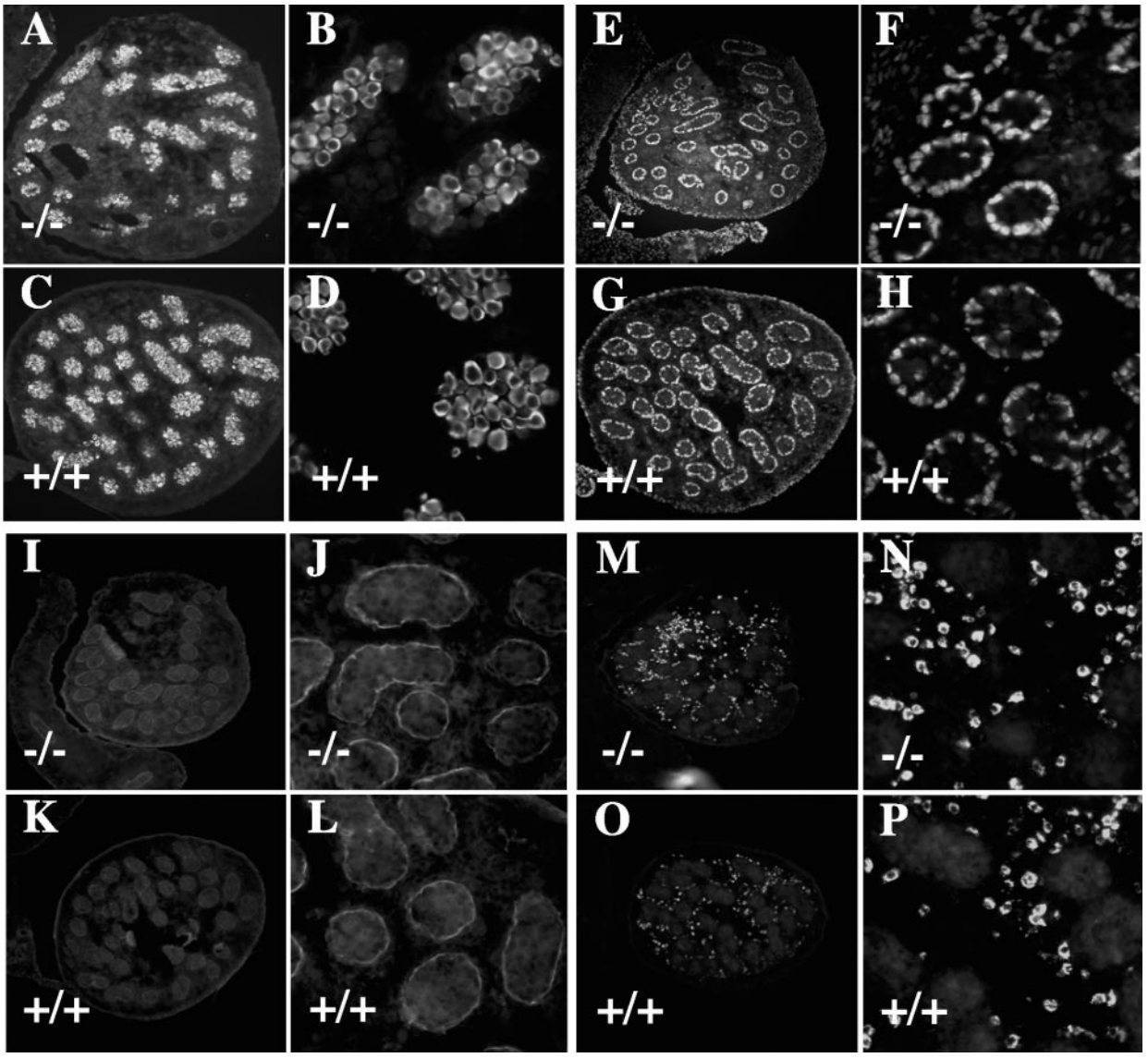

FIG. 4. Immunofluorescence assay using different testis-specific cell markers on sections from E17.5 testis. (A to D) Immunofluorescence assay using rabbit polyclonal antiserum directed against the germ cell marker Mage-b4. (E to H) Immunofluorescence assay using rabbit polyclonal antiserum directed against the Sertoli cell marker Wt1. (I to L) Immunofluorescence assay using rat anti-mouse laminin α-1 antibody, visualizing the basal membrane of the testis cords. (M to P) Immunofluorescence assay using rabbit polyclonal antiserum directed against the Leydig cell marker P450scc. Testes from −/− (A, B, E, F, I, J, M, and N) and +/+ (C, D, G, H, K, L, O, and P) animals were analyzed at magnifications of ×10 and ×40, respectively.

in cell numbers, cord formation, or general tissue organization between homozygous mutants and wild-type controls using any of the above antibodies. Electron microscopy on testes from E15.5 embryos as well as from newborn pups also did not reveal any abnormalities. Figure 5 shows a typical view of the developing testis that is representative of both knockout and control animals on the first day after birth. We also studied spermatogenesis and testis histology in 3-month-old adult animals. We found no differences between *testatin* knockout and wild-type animals (not shown). All stages of mouse spermatogenesis (I to XII) were present. We measured the diameters of the seminiferous tubules, which are known to reflect spermatogenesis. These were similar in knockout animals and controls (Table 1).

Testatin-deficient males are fertile. *testatin* is expressed by fetal and adult Sertoli cells (53) and adult germ cells (26). Sertoli cells are crucial for spermatogenesis, by providing a physical matrix and protection as well as by providing secreted factors essential for the germ cells. We therefore wanted to investigate whether fertility was affected in the testatin-deficient animals. We found no significant difference in litter size between *testatin*$^{-/-}$ and *testatin*$^{+/+}$ animals (Table 1). There was an even sex distribution among the offspring in both cases (not shown).

Sperm production and motility are normal in mice lacking testatin. Knockout mice have been described who have reduced sperm counts despite normal fertility as assessed by counting the number of pups (46). We assessed sperm number recovered from epididymal caudae, sperm motility, and

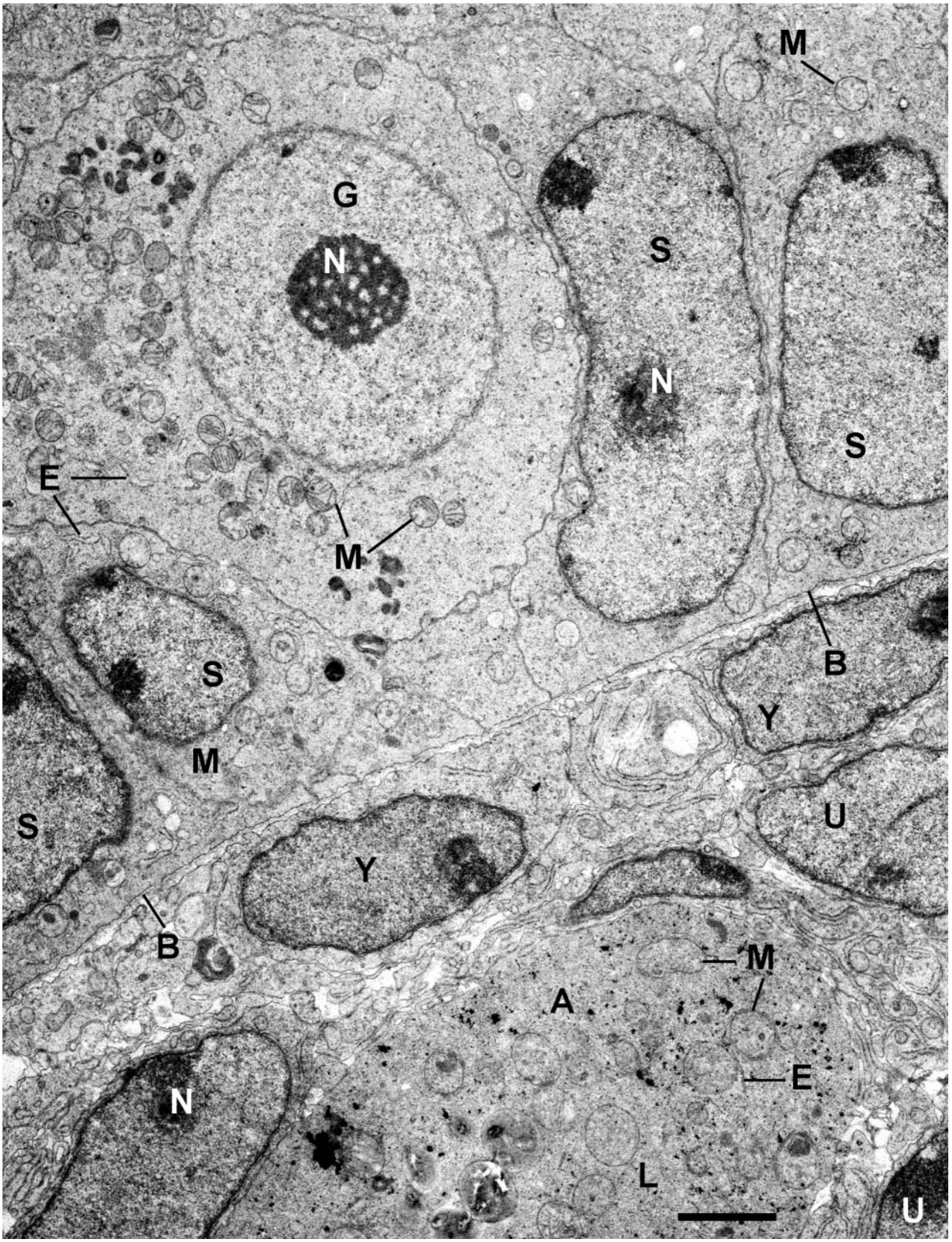

FIG. 5. Electron micrograph of neonatal testis from a *testatin* knockout mouse. The upper part is a portion of a testicular cord outlined by a basement membrane (B), which separates it from interstitial tissue below. The bulk of the solid cords consists of columnar or polymorphic Sertoli cells (S) and spermatogonia (G). The Sertoli cells (S) have free polysomes, granular endoplasmic reticulum (E), and mitochondria (M). The spermatogonia (G) are large spherical cells where the cytoplasm contains free polysomes, several mitochondria (M), and short cisternae of granular endoplasmic reticulum (E). In the interstitium the cells (Y) adjacent to the cords are elongating to become the myoid cells. Undifferentiated mesenchymal cells (U) are seen among the differentiated Leydig cells (L). A portion of a large Leydig cell (L) cytoplasm is seen in the lower part. The cytoplasm is full of agranular endoplasmic reticulum (A), mitochondria (M), and some cisternae of granular endoplasmic reticulum (E). Several cells have large nucleoli (N). Bar, 2 μm.

TABLE 1. Weights and reproductive data for testatin-deficient mice and controls at 3 months of age[a]

Genotype	Wt (g) of:			Litter size (no. of pups)	Sperm count (10^6)	Motile sperm (%)	Seminiferous tubule diam[b] (μm)
	Body	Testis	Cauda epididymis				
testatin$^{-/-}$	33.3 ± 0.8	0.21 ± 0.01	0.028 ± 0.002	9.1 ± 0.55 (6 males, 13 litters)	31.0 ± 3.0	44.6 ± 3.0	172.0 ± 3.9
testatin$^{+/+}$	31.2 ± 1.2	0.23 ± 0.01	0.025 ± 0.002	7.8 ± 0.58 (5 males, 12 litters)	31.1 ± 3.2	51.4 ± 3.2	170.9 ± 6.7

[a] All values are means ± standard errors of the means, $P > 0.05$ (Student's *t* test). For every value except litter size, *n* was 6.
[b] The total numbers of tubules analyzed were 76 for −/− mice and 145 for +/+ mice.

weights of testis and cauda epididymis in testatin-deficient and wild-type male mice. There was no significant difference in total sperm count, percentage of motile spermatozoa, or weight of testis or cauda epididymis when −/− and +/+ males were compared (Table 1).

Normal testosterone, FSH, and LH levels in testatin-deficient mice. Testosterone secreted from the Leydig cells is essential for all aspects of male reproductive function, and gonadotrophins secreted from the anterior pituitary reflect both sex steroid homeostasis and spermatogenic capacity. We measured serum levels of testosterone, LH, and FSH, as well as intratesticular testosterone levels, in testatin-deficient mice and controls. We found no significant difference in any of these parameters (Table 2).

DISCUSSION

The present work describes the first mouse model generated with targeted disruption of one of the members of a recently discovered gene family, the *Cres/testatin* subfamily of genes, which are related to the family 2 cystatins.

Family 2 cystatins belong to a superfamily of cysteine protease inhibitors that consists of three families: the stefins, cystatins, and kininogens (5). Family 1 cystatins (stefins) are 11-kDa intracellular proteins that lack disulfide bonds. Family 3 cystatins (kininogens) are large-molecular-mass secretory proteins that contain three family 2 cystatin domains, two of which possess inhibitory activity. The family 2 cystatins (cystatins C, D, E, F, S, SN, and SA) are 10- to 14-kDa secreted proteins that are found in most tissues as well as biological fluids including saliva, tears, urine, plasma, and cerebrospinal fluid, with the highest concentration (cystatin C) in seminal plasma (2, 52). They are reversible, tight-binding competitive inhibitors of cysteine proteases in vitro. Classical targets are the lysosomal cathepsins B, H, and L.

Cystatins have been implicated in normal body processes such as prohormone processing and bone resorption (16, 38), and disturbances in the balance between cysteine protease and inhibitor function have been implicated in several pathological conditions, such as tumor progression and inflammation (8, 32). Cystatin C has also been shown to exhibit antimicrobial properties in vitro (6, 10), and cystatins have been proposed to target proteolytic enzymes that are essential virulence factors for microorganisms during infection (4). However, firm evidence for a physiological relevance of any of these functions is lacking, and the in vivo roles of the cystatins remain largely unknown. Two human diseases are known to be caused by cystatin mutations: a dominant, gain-of-function mutation in *cystatin C* causes amyloid angiopathy in an Icelandic kindred (21), whereas inactivating, recessive mutations in *cystatin B* give rise to a specific type of epilepsy (43).

TABLE 2. Hormonal data for testatin-deficient mice and controls at 3 months of age[a]

Genotype	Testosterone level (pg/100 μl)		Serum hormone level (ng/ml)	
	Serum	Intratesticular	LH	FSH
testatin$^{-/-}$	555 ± 385	1,575 ± 916	0.484 ± 0.342	46.68 ± 6.46
testatin$^{+/+}$	936 ± 363	1,888 ± 885	0.308 ± 0.096	35.95 ± 5.48

[a] All values are means ± standard errors of the means ($n = 6$, $P > 0.05$, Student's *t* test).

The cystatins have two characteristic disulfide bonds in the C terminus, and mutagenesis and X-ray crystallographic studies have revealed three conserved regions that together form a wedge-shaped structure that blocks the active site of the target proteases (54). These are an N-terminal glycine, a glutamine-valine-glycine (Q-X-V-X-G) loop segment, and a C-terminal hairpin loop containing the residues proline-tryptophan (PW). In addition, as shown for the most studied member of the family, cystatin C, the affinity and specificity of the cystatin/cysteine protease interaction depend upon the N-terminal region (1). Cystatin C has also been shown to inhibit the C_{13} family cysteine protease legumain through a different reactive site (3).

With its isolation in 1992 (15), *Cres* was the first described member of a novel subfamily of cystatin-related genes that today comprises seven genes in the mouse. The Cres protein defined a new subgroup within the family 2 cystatins by virtue of its low sequence identity (28%) with cystatin C but its conserved gene structure and cosegregation with the cystatin C gene to the distal region of mouse chromosome 2 (14). These features were shared by the second characterized member, *testatin* (17, 53). Subsequently the genes encoding cystatin T (47), cystatin SC (34), cystatin TE-1 or Cres3 (24, 34), Cres2 or cystatin E1 (24, 33), and cystatin E2 (33) were isolated. These genes all show a moderate degree of sequence homology to the family 2 cystatins, but all contain the four characteristic C-terminal cysteine residues in highly similar positions, they have putative signal peptides and predicted cleavage sites at the same relative position as that of the cystatins, and they are all located in a gene cluster on mouse chromosome 2, indicating a common evolutionary origin.

Although similar in structure, several unique features discriminate these genes from the classical cystatins. They possess only the C-terminal PW site and lack the N-terminal glycine as

well as the Q-X-V-X-G loop segment that is necessary for inhibition of C_1 cysteine proteases, indicating that they are not directed towards the same targets as the classical cystatins. Their N-terminal regions are also poorly conserved. In accordance with this, it was recently shown that Cres did not inhibit the cysteine protease papain or cathepsin B in vitro. Instead, it was a strong selective inhibitor of prohormone convertase 2 (PC2), a serine protease involved in prohormone processing within the neuroendocrine system (11). Another striking difference between the archetypical cystatins and the genes of the novel subfamily regards their patterns of expression. In contrast to the broad expression of the classical cystatins, all members of the novel subfamily are restricted to reproductive tissues. *Cres* is strongly expressed in epithelial cells of the proximal caput epididymis and can also be found in postmeiotic germ cells in the testis (12), in anterior pituitary gland gonadotroph cells of both sexes (49), and in the corpora lutea of the ovary (24). Cres protein has also been found in the sperm acrosome of the mouse (51) and in the human sperm equatorial segment (55). *testatin* was isolated in a screen designed to detect signaling molecules differentially expressed in XX versus XY gonads during early fetal development. *testatin* is present at very low levels in both XX and XY gonads at 11.5 dpc. In males, it increases dramatically in expression at 11.5 to 12.5 dpc, immediately after the *Sry*-induced initiation of testis differentiation, while it decreases to undetectable levels in the female gonad during the same period (53). Expression is maintained in the Sertoli cell throughout adulthood. *testatin* transcripts were also found in a small population of germ cells (26). *cystatin T* is expressed in germ cells (pachytene spermatocytes and round spermatids) (18), and *cystatin SC* is expressed exclusively in Sertoli cells of the testis, varying in intensity among the seminiferous tubules in a stage-dependent manner (34). *cystatin TE-1* or *Cres3* is expressed in epithelial cells of the proximal caput epididymis, in the Sertoli cells of the testis, and in the ovary and prostate (24, 34), and *Cres2* or *cystatin E1* (24, 33) as well as *cystatin E2* (33) is strongly expressed in caput epididymis and can be seen in low levels in the prostate. A low level of *cystatin E2* expression was also seen in the testis; the cell type remains to be clarified.

Based on protein sequence homology, the subfamily can be further subdivided into two groups, one represented by Cres and the other by testatin (Fig. 6A). The Cres cluster contains cystatin T and Cres2/cystatin E1, whereas cystatin E2, cystatin SC, and cystatin TE-1/Cres3 cluster together with testatin. The Cres subgroup seems to be more closely related to the classical cystatins. In fact, the only part of the classical cystatin reactive site that is conserved in the subfamily, the PW motif, is changed to PG in cystatin E2 and to AW in cystatin TE-1/Cres3 (Fig. 6B). This may indicate that the testatin subgroup has come furthest along an evolutionary path towards a specialized function in reproduction.

Sex determination and testis differentiation are developmental processes characterized by intense activities including, e.g., cell proliferation and differentiation, cross talk between different cell types, migration of primordial germ cells into the genital ridge, migration of somatic cells from the underlying mesonephros into the gonad, vascularization, and testis cord formation (reviewed in reference 7). Proteases and protease inhibitors are likely to play crucial roles in several of these processes. Primordial germ cells arrive at the genital ridge at 11.5 dpc, and in the testis they are mitotically arrested to become prospermatogonia while those in the ovary enter meiosis to become oocytes. This sex differentiation of germ cells is directed by the sex of the gonadal somatic cells and not by that of germ cells themselves (35), but the nature of a putative meiosis-inhibiting factor produced by Sertoli cells is unknown. Testis cord formation begins at about 12.0 dpc in the mouse with a clustering of germ cells that become surrounded by Sertoli cells. The cords get enclosed by an extracellular matrix which connects on the outer side to developing peritubular myoid cells. Thus, extensive tissue remodeling takes place and highly regulated proteolytic events are likely to be involved. Proteases and their inhibitors may also be involved in the activation of the chemoattractants and other signaling molecules that are known to play roles in testis development but that have not yet been identified.

Proteases and protease inhibitors are also implicated in many aspects of adult reproductive function. Sertoli cells form the blood-testis barrier. The cysteine protease cathepsin L and its inhibitor cystatin C are both secreted from adult Sertoli cells. It has been suggested that these molecules have interactive roles in the adherence of germ cells to Sertoli cells and subsequent formation of the intercellular junctions (37). Cystatin C and cathepsin L are also thought to interact to promote sperm maturation through modification of sperm surface proteins and soluble proteins in the surrounding fluid (41). Regulated proteolysis has also been implicated in the migration of germ cells during spermatogenesis as well as in the release of spermatids (36). The ability of spermatozoa to acquire the functional capacities of progressive motility and fertility occurs as spermatozoa migrate through the epididymis. This maturation process is thought to require the interaction of spermatozoa with proteins secreted into the luminal fluid by the epididymal epithelium (56). The genes encoding four of the seven members of the Cres/testatin subfamily (*Cres*, *Cres2*/*cystatin E1*, *cystatin E2*, and *cystatin TE-1*/*Cres3*) show highest expression in the epithelial cells of the proximal caput epididymis, strongly suggesting a role in sperm maturation. The finding of Cres protein in the sperm acrosome of the mouse (51) and in the human sperm equatorial segment (55) suggests a role in the fertilization process, further emphasizing a role in adult male reproduction for these members of the subfamily. *testatin*, *cystatin SC*, and *cystatin T*, on the other hand, are not expressed in the epididymis. *cystatin T* is expressed in pachytene spermatocytes and round spermatids (18), whereas *cystatin SC* is expressed exclusively in Sertoli cells, varying in intensity among the seminiferous tubules in a stage-dependent manner in the adult (34). *testatin* is so far the only member of the subfamily that is known to be specifically upregulated during early fetal development.

We were surprised not to find any abnormalities in our testatin-deficient mice. *Sry* is expressed in pre-Sertoli cells between 10.5 and 12.5 dpc, prior to the appearance of a differentiated testis (23, 30). *Sry* is believed to induce testis formation by triggering somatic cell precursors in the bipotential gonad of the XY embryo to differentiate into Sertoli cells and organize into testicular cords (40). The Sertoli cell is thus the first cell type to differentiate, and Sertoli cells are thought to secrete factors that induce sex-specific differentiation of the

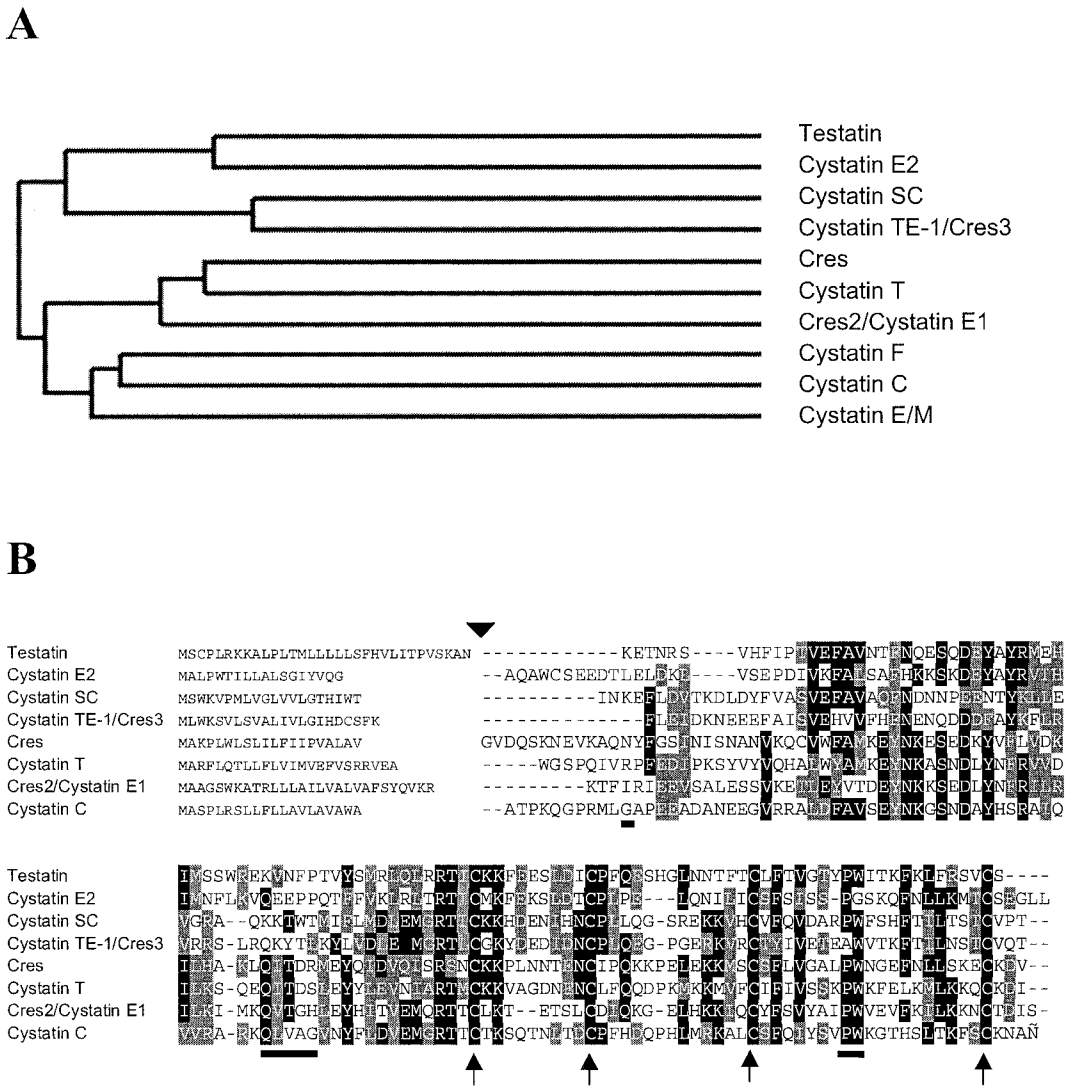

FIG. 6. Relationship of the members of the Cres/testatin subfamily of cystatin-related proteins. (A) Dendrogram showing the evolutionary relationship between the Cres and testatin subgroups and the archetypical family 2 cystatins, cystatins C, F, and E/M. (B) Amino acid alignment of the Cres/testatin subfamily members with mouse cystatin C. Identical residues are boxed in black; conservative changes are shaded. The putative signal sequence cleavage site is indicated by an inverted triangle. The three regions important for inhibition of cysteine proteases by the classical cystatins are indicated by a thick line below the sequence. Vertical arrows point to the four conserved cysteine residues participating in disulfide bond formation.

other cell types in the testis. With its highly restricted expression pattern, being upregulated in the pre-Sertoli cell at 11.5 to 12.5 dpc immediately after the onset of *Sry* expression, and with its characteristics of a secreted protein, testatin represented a strong candidate for a missing link in male sexual development.

It is likely that the loss of *testatin* is compensated for by expression of one or more other genes capable of taking over its functions. Obviously, the most likely candidates for such functional redundancy are the other members of the *Cres*/*testatin* subfamily. Among these, *cystatin SC* and *cystatin TE-1*/*Cres3* resemble *testatin* in that they are expressed in Sertoli cells (24, 34). In addition, *cystatin E2* has been found to be weakly expressed in the testis, although the specific cell type was not determined (33). Interestingly, these are the three genes that cluster together with *testatin* in a group distinct from the *Cres*/*cystatin T*/*Cres2* or *cystatin E1* group (Fig. 6A). *testatin* is so far the only member of the subfamily that has been studied during fetal development. Experiments are currently in progress in our laboratory to clarify the ontogeny of the additional subfamily members, with the aim of identifying the most likely candidates with which *testatin* may have a redundant function.

Another important line of future work is to identify proteins that interact with testatin. Perhaps its putative target protease is the important player exerting one or more of the functions discussed above. The unexpected finding that Cres specifically inhibits the serine protease prohormone convertase 2 in vitro (11) is of high principal significance. Prohormone convertases typically activate propeptides and prohormones by cleaving at

specific residues (9). This activating function is fundamentally different from the functions of the classical cystatins, which largely seem to be protective by regulating unspecific protein degradation that is exerted by lysosomal cysteine proteases. Thus, there is an exciting possibility that the *Cres/testatin* subfamily of genes forms part of a newly discovered regulatory system within the neuroendocrine/reproductive system, the functions of which are only beginning to be unraveled.

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