Targeted Disruption of the *Insl3* Gene Causes Bilateral Cryptorchidism

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The sexual dimorphic position of the gonads in mammals is dependent on differential development of two ligaments, the cranial suspensory ligament (CSL) and the gubernaculum. During male embryogenesis, outgrowth of the gubernaculum and regression of the CSL result in transabdominal descent of the testes, whereas in the female, development of the CSL in conjunction with failure of the gubernaculum development holds the ovaries in a position lateral to the kidneys. Several lines of evidence suggest that regression of the CSL and induction of gubernaculum development are mediated by testosterone and a yet unidentified testicular factor, respectively. The *Insl3* gene (orginally designated *Ley I-L*), a member of the insulin-like superfamily, is specifically expressed in Leydig cells of the fetal and postnatal testis and in theca cells of the postnatal ovary. Here we show that male mice homozygous for a targeted deletion of the *Insl3* locus exhibit bilateral cryptorchidism with free moving testes and genital ducts. These malformations are due to failure of gubernaculum development during embryogenesis. In double-mutant male mice for *Insl3* and *androgen receptor* genes, testes are positioned adjacent to the kidneys and steadied in the abdomen by the CSL. These findings demonstrate, that the Insl3 induces gubernaculum development in an androgen-independent way, while androgen-mediated regression of the CSL occurs independently from Insl3. (Molecular Endocrinology 13: 681–691, 1999)

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INTRODUCTION

Since the appearance of Jost's theory that male sexual differentiation in eutherian mammals is regulated by two fetal testicular hormones (1), several lines of evidence have demonstrated the role of anti-Müllerian hormone, also known as Müllerian-inhibiting substance (MIS), from the Sertoli cells for the regression of Müllerian ducts and of androgen from the Leydig cells for the differentiation of Wolffian ducts into vas deferens, epididymis, and accessory glands. In female fetuses, the absence of MIS and androgen leads to the development of the Müllerian ducts' derivatives and the passive regression of the Wolffian ducts. However, the molecular mechanism underlying the sexual dimorphic position of the gonads in mammals was not included in Jost's theory and has received limited attention, although the respective positions of ovary and testis are of utmost importance for fertility. Over the last century, numerous theories have been proposed to explain the process of testis descent. Controversies between these theories are often centered upon the targeted structures and factors that are involved in this process (2, 3).

The genital mesentery of the internal genital tract is a retroperitoneal structure that connects the gonads and genital ducts to the abdominal wall. The differential development of two parts of the genital mesentery, the cranial suspensory ligament (CSL) and the caudal genital ligament, also called gubernaculum, during male and female development has been determined and proposed to be responsible for a sexual dimorphic position of testis and ovary (2, 3). In mammals, the

process of testis descent has been divided into two functional phases (3). During the first or transabdominal phase, occurring between days 15.5 and 17.5 postcoitum (dpc) in murine development, the development of the gubernaculum and regression of the CSL result in the transabdominal movement of the testis into the inguinal region. In the female embryo, development of the CSL and developmental impairment of the gubernaculum keep the ovary near the kidney (Fig. 1). During the second or inguinoscrotal phase of testis descent, occurring in the mouse between postnatal weeks 2 and 3, the testis descends from the inguinal region to the scrotum while the gubernaculum is inverting or regressing.

To date, a substantial amount of data has accumulated to indicate the relevance of testicular factors in the differential growth of both ligaments during male embryogenesis. The prevention of outgrowth of the fetal CSL is an androgen-dependent process. Prenatal exposure of females to androgen prevent development of the CSL (4). The persistence of the CSL in completely androgen-insensitive male mice that contain a mutation in the gene encoding the androgen receptor (*Ar*), and in male rats that were exposed to antiandrogen during fetal life, further supports the role of androgen in suppression of the CSL development (5–7). Development of the male-like gubernaculum in bovine freemartin, a female fetus exposed to the blood of a male twin by chorioallantoic anastomosis, and in female rabbit fetus that has been grafted with a fetal testis, demonstrated the participation of fetal testicular factors in gubernacular development (8–10). Because a proportion of human males with persistent Müllerian duct syndrome (PMDS) and human males with complete androgen insensitivity syndrome have undescended testes, a potential role of both MIS and androgen in testicular descent was suggested (3, 11). However, recent data showed a normal testicular descent in the homozygous *MIS* and *MIS* type II receptor mutant mice, and the outgrowth of the gubernaculum in male mice with testicular feminization rule out the direct action of MIS and androgen in induction of the gubernacular outgrowth during the transabdominal descent of the testis (5, 12, 13). These data suggested a role of a third fetal testicular factor in gubernacular development (10, 14, 15).

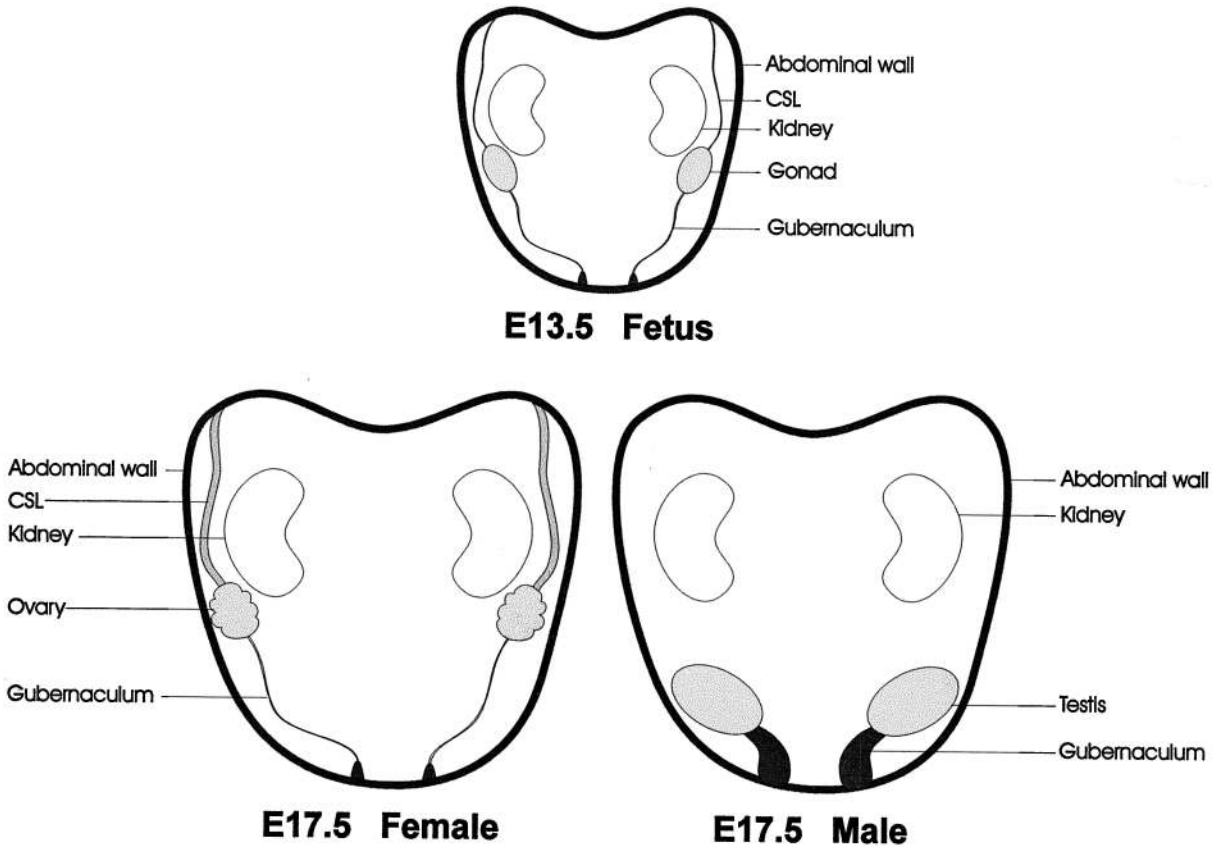

Fig. 1. Schematic Representation of Differential Development of the Cranial Suspensory Ligament (CSL) and the Gubernaculum during Sexual Differentiation

At embryonic day 13.5, position of the developed gonad is identical in male and female fetuses and attached to the abdominal wall by the CSL and the gubernaculum. Between embryonic days 15.5–17.5, development of the gubernaculum and regression of the CSL in the male embryo result in the transabdominal descent of the testis into the inguinal region. In the female embryo, further development of the CSL and developmental impairment of the gubernaculum lead to sustain the ovary near the kidney.

We have previously characterized a novel member of the insulin-like hormone superfamily, *Insl3*, which is specifically expressed in Leydig cells of the fetal and adult testis and in the theca cells of the postnatal ovary (16, 17). The *Insl3* gene is expressed at high levels in the adult testis and at much lower levels in the adult ovary. Analyses of *Insl3* transcripts in testis and ovary throughout the pre- and postnatal life of the mouse revealed a sexual dimorphic pattern of *Insl3* expression during development. No *Insl3* transcripts were detected in female embryos of any stage, whereas in male embryos transcripts were first detected at 13.5 dpc. After birth, the level of *Insl3* transcription in testis remains constant during the first 3 weeks, increases at the time at which the first wave of round spermatids undergoes spermiogenesis, and reaches the highest level in adult testis (18). These results led us to suggest that the Insl3 factor plays an essential role in differentiation and maintenance of the male phenotype and spermatogenesis (16, 18). In the female, expression of *Insl3* is first detected in the ovary at day 6 after birth. This, taken together with the distinct expression pattern of *Insl3* during the estrous cycle and pregnancy, implies a functional role of Insl3 during follicular development (18).

To determine the role of Insl3 in sexual differentiation and gametogenesis, we have generated mice containing a targeted disruption of the *Insl3* gene. Morphological abnormalities were only observed in male *Insl3*$^{-/-}$ mice, which exhibited bilateral cryptorchid testes located high in the abdomen. To investigate the role of Insl3 in the process of the testis descent, we have histologically analyzed gubernacular development during transabdominal descent of the testis in the wild-type and the *Insl3* mutant males. To address the question of whether androgen and Insl3 function independently in the development of CSL and gubernaculum, we have generated double-mutant male mice in which the action of both factors is eliminated. Finally, we have surgically descended the testes of the *Insl3*$^{-/-}$ mice in the inguinal canal to determine the role of Insl3 for male germ cell development.

RESULTS

Generation of *Insl3*-Deficient Mice

To elucidate the potential role of the *Insl3* gene, we deleted the gene in mice through homologous recombination. A replacement targeting vector was designed to delete the two exons encoding the Insl3 factor and replaced them with the neomycin phosphotransferase (*neo*) gene under the control of the phosphoglycerate kinase promoter. Introduction of a negative selection marker, the herpes simplex virus thymidine kinase (*tk*) gene, at the 3′-end of the construct (Fig. 2A) enabled us to use positive and negative selection (19).

MP1 ES cells were transfected with the targeting vector and selected for homologous recombination events (20). Drug-resistant clones were selected, and DNA was isolated and screened by Southern blot analysis using an external probe (data not shown). Three recombinant clones had undergone homologous recombination. One clone produced germ line-transmitting chimeras after aggregation with morula derived from CD1 females. These chimeras were bred with CD1 and 129/Sv females to establish the *Insl3*-deleted allele on a CD1 $\times$ 129/Sv hybrid and a 129/Sv inbred genetic background. Southern blot analysis on DNA isolated from tail biopsies was used to determine the genotype of the offspring. Hybridization with the 3′-external probe (Fig. 2A) visualized a 10-kb *Bam*HI fragment in the case of a wild-type allele and a 14- kb fragment for a targeted allele (Fig. 2B). Both male and female mice heterozygous for the *Insl3* mutation appeared normal and fertile. Heterozygous animals were mated, and approximately 25% (76 of 302) of the offspring were homozygous for the null allele. Northern blot analysis of RNA derived from testes of these mice revealed that the *Insl3*$^{-/-}$ mice failed to produce detectable *Insl3* mRNA (Fig. 2C). These results confirm that the introduced mutation results in a complete loss of *Insl3* mRNA in testis of *Insl3*$^{-/-}$ mice. The phenotypes associated with the homozygous mutation that are described below were on a mixed (CD1 $\times$ 129/Sv) genetic background, but were not different from that on an inbred (129/Sv) genetic background.

Insl3 Homozygous Mutant Male Mice Are Sterile and Have Bilateral Cryptorchidism

The pattern of *Insl3* expression in ovaries at various stages of the estrous cycle and during pregnancy showed a correlation with follicular development (18). However, homozygous mutant females underwent normal estrous cycles, as indicated by the cytology of vaginal smears, and after mating with wild-type or heterozygous male mice, they became pregnant and produced litters of normal size [9.1 $\pm$ 0.6 (n = 18) *vs.* 9.8 $\pm$ 0.9 (n = 14) in control females]. Normal folliculogenesis was observed in the ovaries of the *Insl3*-deficient females (data not shown), suggesting that the Insl3 factor is not essential for female germ cell development or folliculogenesis.

Morphological abnormalities were only observed in male *Insl3*$^{-/-}$ mice, which were infertile despite normal sexual behavior toward female mice and production of copulation plugs. Anatomical examination of the male *Insl3*$^{-/-}$ mice revealed that the Wolffian duct derivatives had differentiated normally into vas deferens, epididymis, and accessory glands and no Müllerian duct derivatives were present (Fig. 3, A and C). However, all *Insl3*$^{-/-}$ males exhibited bilateral cryptorchid testes located high in the abdomen (Fig. 3, A and C). The testicular arteries originated in the abdominal aorta below the renal arteries and ran just below the kidneys in an ovarian vasculature-like fashion. No tight attachment of the testis and epididymis to the inguinal region was found. Therefore, gubernacular

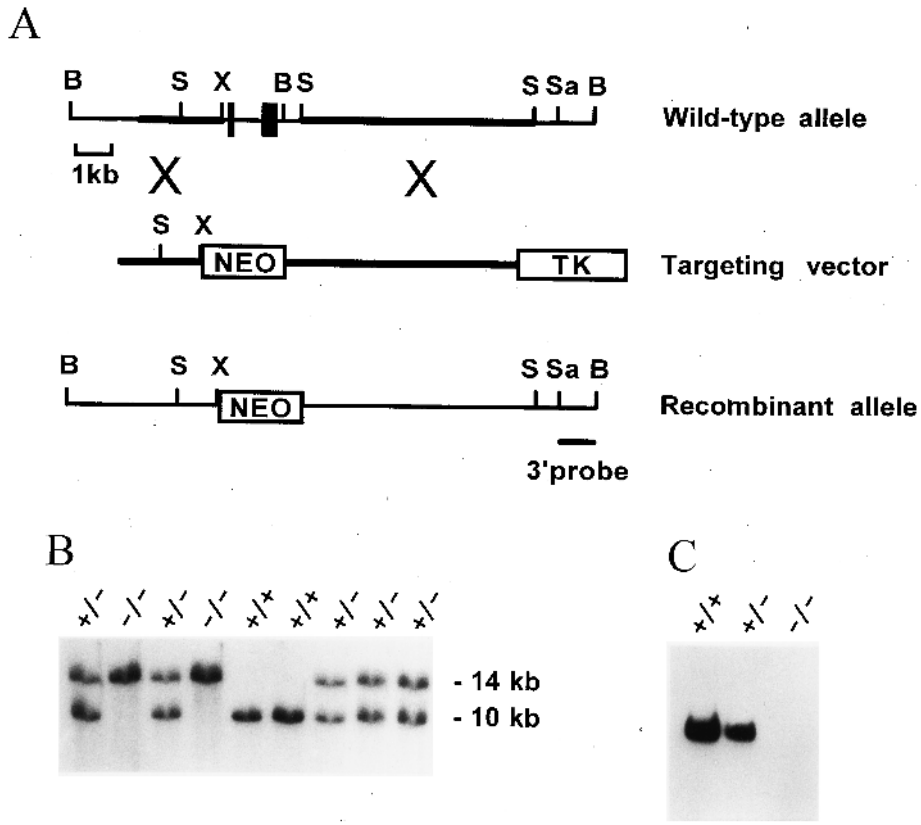

Fig. 2. Targeted Disruption of *Insl3* Gene, and RNA and DNA Analysis of Generated Mice

A, Structure of the wild-type allele, targeting vector, and targeted allele are shown together with the relevant restriction sites. A 1.8-kb *Xho*I-*Sst*I fragment containing both exons of the gene was replaced by a *pgk-neo* selection cassette (NEO). TK, *Thymidine kinase* cassette; B, *Bam*HI; S, *Sst*I; Sa, *Sal*I; X, *Xho*I. B, Southern blotting of *Bam*HI-digested DNA from F_2 mice, hybridized with a 3′-external probe, revealing a 10-kb wild-type and a 14-kb mutated fragment. C, Northern blotting of testicular RNA from $Insl3^{+/+}$, $Insl3^{+/-}$, and $Insl3^{-/-}$ adult mice, hybridized with the mouse *Insl3* cDNA probe, revealing a 0.9-kb mRNA prominent in $Insl3^{+/+}$, reduced in $Insl3^{+/-}$, and absent in $Insl3^{-/-}$ testes.

development could be affected in these mutant mice. Torsion of the vas deferens and testicular artery and localization of the right testis in the contralateral position did occur in some $Insl3^{-/-}$ mice, presumably due to the absence of tight attachment of the testes to the inguinal region in combination with regression of the CSL.

Roles of Androgen and Insl3 in Development of the CSL and the Gubernaculum Are Independent

The structural abnormalities contributing to cryptorchidism in $Insl3^{-/-}$ male mice are different from those observed in male mice with testicular feminization. The development of CSL and gubernaculum in *Ar*/Y mice disrupts normal testis descent. Consequently, testes of these mice are located at an intermediate position of ovaries and testes in wild-type mice (Fig. 3D). To address the question of whether androgen and Insl3 function independently in the development of both ligaments, we have generated double- mutant male mice in which the action of both factors is eliminated. Testes in *Ar*/Y $Insl3^{-/-}$ mice are completely undescended (Fig. 3E), and tight attachment of the testes to the inguinal region is absent. The male external and internal genitalia are not virilized, and Müllerian and Wolffian duct derivatives are absent. In contrast to $Insl3^{-/-}$ mutant mice, the testes are situated adjacent to the kidneys in a comparable position as ovaries in wild-type mice (Fig. 3, B and E) and are attached to the dorsal abdominal wall via well developed CSLs.

Insl3 Is Required for Normal Development of the Gubernaculum during Transabdominal Descent of the Testis

The transabdominal descent of the testis coincides with the regression of the CSL, the shortening of the gubernacular cord, and the outgrowth of the gubernacular bulb including the differentiation of its outer mesenchymal layer into myoblasts (4, 21, 22). Analysis of E17.5 wild-type males by scanning electron microscopy reveals that the gubernaculum shows swelling (Fig. 4A), whereas the gubernaculum of the $Insl3^{-/-}$ male and control female displays a small bulb and an

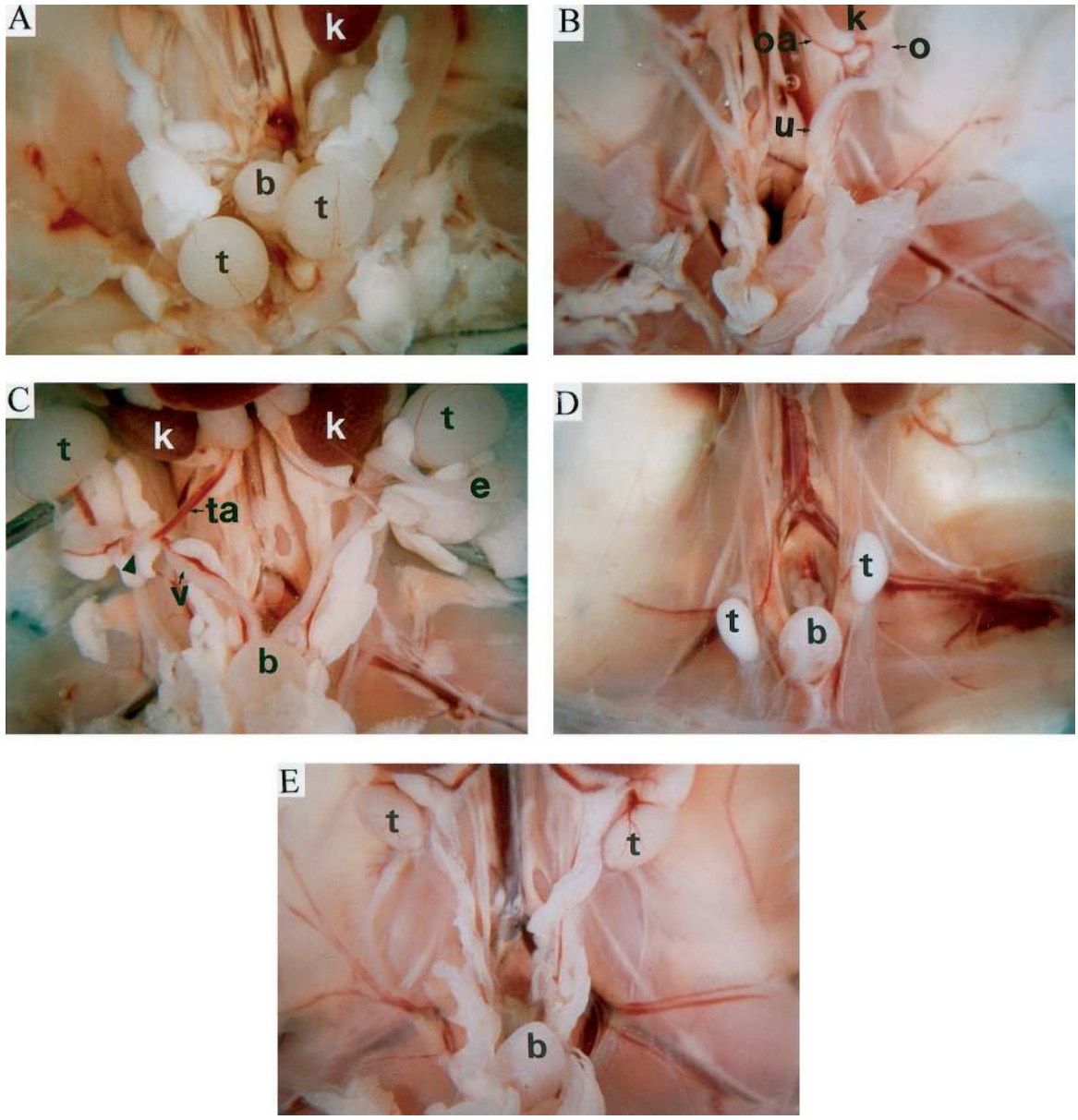

Fig. 3. Testicular Position in *Insl3*$^{-/-}$, *Ar*/Y, and *Ar*/Y *Insl3*$^{-/-}$ Male Mice

A, Dissected abdominal region of a 4-week-old wild-type mouse shows the testes (t), which were already descended into the scrotal sac, adjacent to the bladder (b). B, Genital tract of a 4-week-old wild-type female shows the position of ovaries (o) adjacent to the kidneys (k), uterine horns (u), and ovarian arteries (oa). C, Free moving genital tract in the abdomen of 4-week-old *Insl3*-deficient male. The Wolffian duct derivatives are normally differentiated into epididymis (e), vas deferens (v), and accessory glands (not shown). Note the torsion (*arrowhead*) of the vas deferens and testicular artery (ta). D, Testes of 3-week-old *Ar*/Y mouse located above the bladder and attached to the abdominal wall with cranial suspensory ligament and gubernaculum. E, Testes of 4-week-old *Ar*/Y *Insl3*$^{-/-}$ male situated adjacent to the kidneys in a comparable position as ovaries in wild-type mouse (B).

elongated cord (Fig. 4, B and C). To investigate whether the cryptorchidism found in the *Insl3*$^{-/-}$ male mice may result from an affected development of the gubernaculum, we have analyzed transverse sections from fetuses at stages before (E15.5) and during (E17.5) the transabdominal descent of the testes. At E15.5, the gubernacular bulb of wild-type males and females and *Insl3*$^{-/-}$ males is similar in size and contains loose mesenchymal cells (data not shown). At E17.5, the gubernacular bulb in wild-type males is enlarged and well developed into mesenchyme in the center and myoblasts in circumferential layers (Fig. 4D). In contrast, the gubernacular bulb in the *Insl3*$^{-/-}$ males and in the wild-type females is poorly developed, as indicated by the lack of structural organization into outer and inner layers (Fig. 4, E and F). These observations suggest that Insl3 stimulates gubernacular development in male mice.

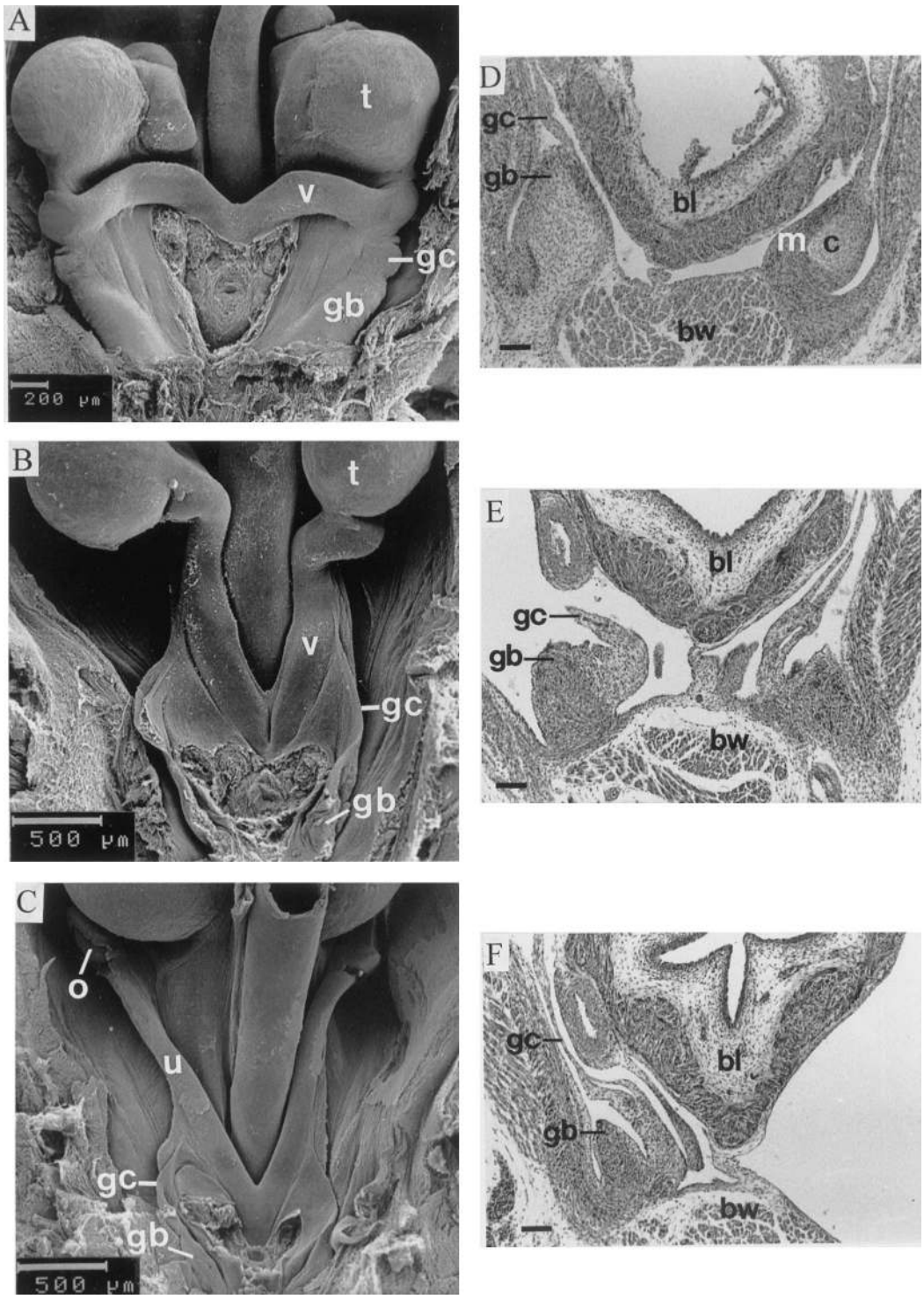

Fig. 4. Impaired Gubernacular Development in E17.5 *Insl3*$^{-/-}$ Male

A–C, Scanning electron microscopy of the reproductive tract revealing a swelled gubernaculum in control male (wild-type or *Insl3*$^{+/-}$) (A), and thin, elongated gubernaculum in *Insl3*$^{-/-}$ male (B) and control female (C). D–E, Histological analysis of inguinal abdomen at E17.5 shows a well developed gubernacular bulb in control male (D), as indicated by marked differentiation into a mesenchymal core surrounded by muscular outer layers; whereas in *Insl3*$^{-/-}$ male (E) and control female (F) the gubernacular bulb is undifferentiated. bl, Bladder; bw, abdominal body wall; c, mesenchymal core; gb, gubernacular bulb; gc, gubernacular cord; m, myogenic outer layer; o, ovary; t, testis; u, uterus; v, vas deferens. Scale bar: D–F, 100 μm.

Insl3 Is Not Essential in Male Germ Cell Development

Histological analyses of the testes of 3-month-old *Insl3*$^{-/-}$ mice revealed abnormal spermatogenesis (Fig. 5, A and B). All seminiferous tubules showed a reduced number of spermatogonia, karyolysis of most of the primary spermatocytes, and vacuolization of Sertoli cell cytoplasm. Most notably, there was a complete absence of postmeiotic cells such as spermatids and spermatozoa. Electron microscopy documented a normal appearance of Leydig cells (data not shown). Furthermore, the testes of *Insl3*$^{-/-}$ mice at 5 days of age showed an intact tubular structure with normal development of Sertoli and spermatogenic cells (Fig. 6, A and B). Clear signs of germ cell depletion were observed at 2 weeks of age when the first wave of spermatogenic cells undergoes meiotic divisions (Fig. 6, C and D). The fact that there is an increase in the testicular expression level of the *Insl3* after the third week of postnatal development raises the question of whether germ cell depletion reflects a primary or a secondary defect. The cryptorchid testes of 3-week-old *Insl3*$^{-/-}$ mice were surgically displaced and fixed in the inguinal canal. Three months after the operation, mating of four *Insl3*$^{-/-}$ male mice with wild-type females failed to produce any offspring, and no spermatozoa were detected in uteri of the females possessing vaginal plugs. Histological examinations of the surgical descended testes of the *Insl3*$^{-/-}$ mice revealed occurrence of normal spermatogenesis in most seminiferous tubules (Fig. 5C) and the presence of sperm in the epididymis (data not shown). Taken together, these observations demonstrate that Insl3 is not essential for male germ cell development. The germ cell depletion in abdominal testes of *Insl3*$^{-/-}$ mice might be attributed to a higher testis temperature, which is known to affect spermatogenesis.

DISCUSSION

To address the biological role of Insl3 in sexual differentiation and fertility, we generated mice lacking the *Insl3* gene. All *Insl3*$^{-/-}$ male mice have bilateral cryptorchid testes and presumably, therefore, are infertile, whereas the *Insl3*$^{-/-}$ female mice are fertile. This striking phenotype was displayed in all *Insl3*$^{-/-}$ male mice regardless of genetic background. Thus, Insl3 plays an essential role in the process of testis descent.

Transabdominal descent of the testis from the posterior abdominal wall to the inguinal region occurs in the fetal mouse as a result of outgrowth of the gubernaculum and regression of the CSL (2, 3). Lack of gubernaculum development and localization of the testis adjacent to the kidney in E17.5 mutant males demonstrate that arrest of the testis descent in the *Insl3*$^{-/-}$ mice takes place during the transabdominal phase. Furthermore, a successful initiation of the early stages of transabdominal descent is evidenced by proliferation of the gubernacular bulb and the differentiation of its outer mesenchymal layer into myoblasts (22). Histological analysis of a E17.5 male mutant showed the lack of structural organization of the gubernacular bulb into an outer layer of myoblasts and an inner mesenchymal layer in both E17.5 male mutants and control females. These observations and the absence of *Insl3* gene expression in female mice during fetal life suggest that Insl3 stimulates the outgrowth and differentiation of the primordium of the gubernaculum in male mice. Whether the Insl3 exerts its role in gubernacular development by direct signaling, through activation of downstream genes that are required for mesenchymal cell proliferation and development, remains to be determined.

The involvement of a third testicular hormone in testis descent has been described by several research

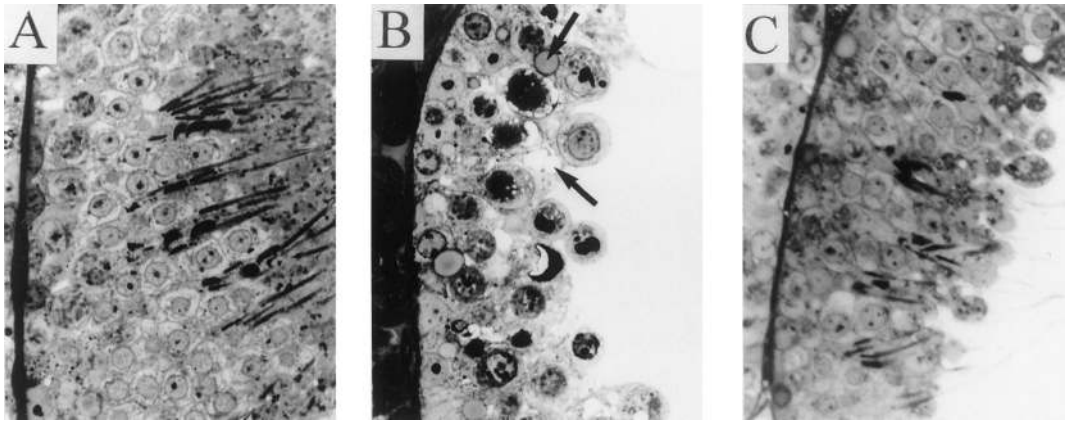

Fig. 5. Spermatogenesis in Cryptorchid and Surgically Descended Testes of *Insl3*$^{-/-}$ Mice

A, Histology of a descended testis from a 3-month-old adult control shows normal spermatogenesis. B, Section through a seminiferous tubule of cryptorchid testis from a 3-month-old *Insl3*$^{-/-}$ male revealing karyolysis of pachytene spermatocytes and the absence of spermatids or spermatozoa. Sertoli cells contain lipid-filled or empty vacuoles (*arrows*). C, Three months after operation, the descended testis from an *Insl3*$^{-/-}$ male shows normal spermatogenesis and presence of mature spermatids in most of the seminiferous tubules. Magnification: A–C, ×490.

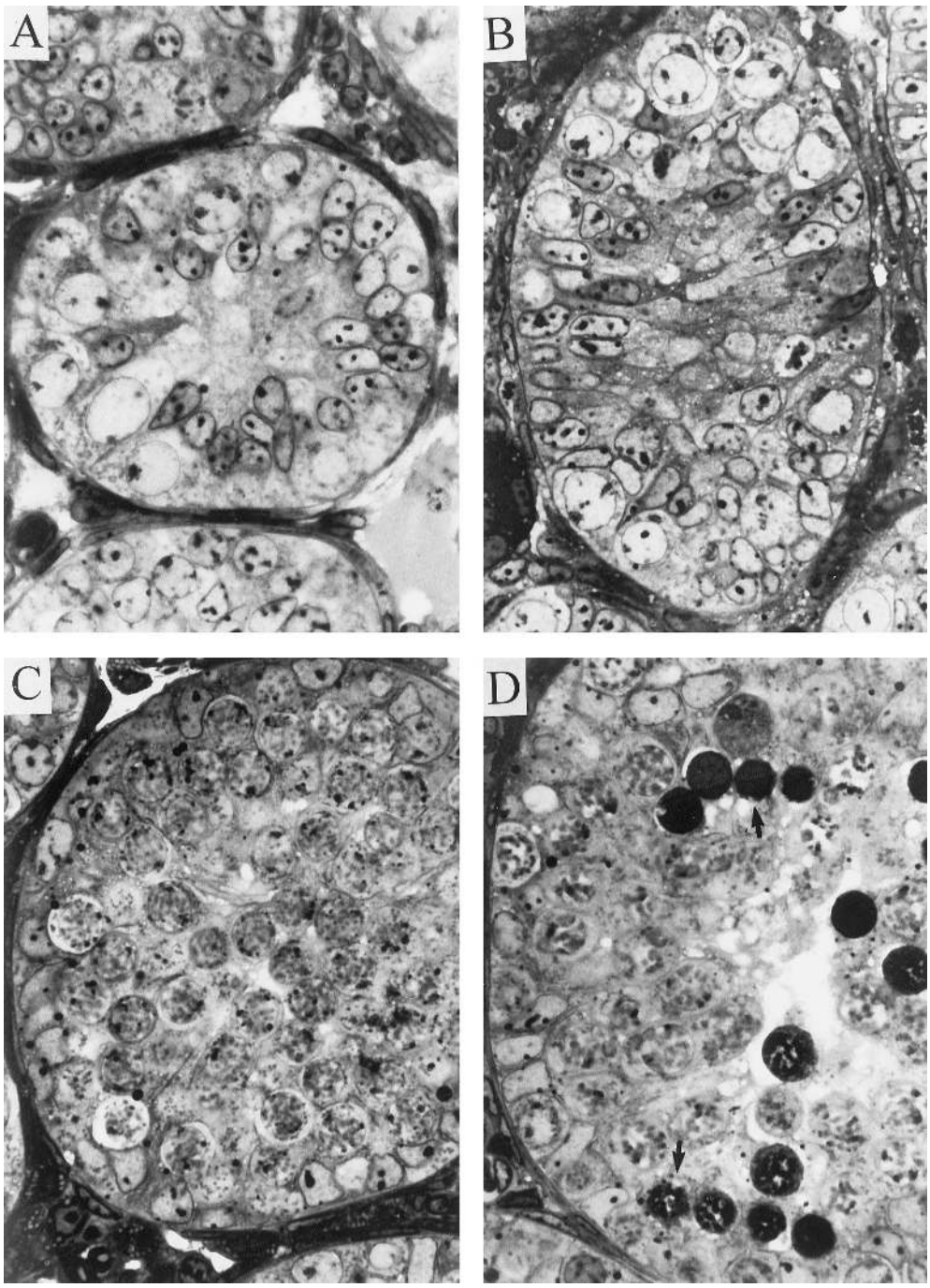

Fig. 6. Testes Histology of Wild-Type and Insl3$^{-/-}$ Mice at 5 and 15 Days Postpartum

A and B, Histology of a testis from a wild-type (A) and an *Insl3*$^{-/-}$ mouse (B) at 5 days of age showing the presence of immature Sertoli cells and spermatogonia. C and D, Section through a testis from a wild-type (C) and an *Insl3*$^{-/-}$ mouse (D) at postnatal day 15 showing a few degenerated spermatocytes with condensed and darkly stained nuclei (*arrow*) in the center of a seminiferous tubule of *Insl3*$^{-/-}$ testis. Magnification: A–D, ×800.

groups (10, 14, 15). In an *in vitro* analysis of testicular hormone action on pig fetal gubernaculum, MIS, inhibin, or androgen could not stimulate the proliferation of gubernacular cells (14). Normal outgrowth of the gubernaculum in *Ar*/Y mice and full descended testes in the homozygous *MIS* and *MIS* type II receptor mutant mice (5, 12, 13) support the idea that neither androgen nor MIS but, rather, a third testicular factor is involved in prenatal development of the gubernaculum. Both androgen and MIS are still potentially involved in postnatal regression/inversion of the gubernaculum during the inguinoscrotal phase (23). We

hypothesize that the Insl3 factor is the as-yet-unidentified testicular factor, which is specifically involved in gubernacular development. Full virilization of the male external genitalia, normal differentiation of the Wollfian duct derivatives into vas deferens, epididymis, and accessory glands, and absence of Müllerian duct derivatives in *Insl3*-deficient mice are a strong indication that failure of gubernacular development in *Insl3* mutant male mice is not due to absence of androgen- and MIS-mediated activities during fetal life.

The ovary-like position of the testes in the *Ar*/Y *Insl3*$^{-/-}$ double-mutant mice, which, similar to wild-type females, lack androgen- and Insl3-mediated activities during prenatal development, demonstrates that the testicular factors androgen and Insl3 are essential for the establishment of the sexual dimorphic position of the gonads via regulation of CSL regression and gubernacular development, respectively. Normal regression of the CSL in the male *Insl3* mutants indicates that the action of androgen on CSL regression does not require Insl3. Furthermore, the development of the gubernaculum in male *Tfm*/Y mice, which lack androgen-mediated activity, demonstrates that the function of Insl3 in gubernacular development is independent from androgen.

Although the pattern of *Insl3* expression during postnatal development of testis and ovary showed a correlation with spermatogenesis and folliculogenesis (18), normal spermatogenesis and follicle development were observed in the surgically descended testes of *Insl3*$^{-/-}$ mice and in ovaries of *Insl3*-deficient mice, respectively. These results suggest that Insl3 is not essential for germ cell development. The germ cell depletion in abdominal testis of *Insl3*$^{-/-}$ mice might be attributed to the higher testis temperature, which is known to affect spermatogenesis (24). The infertility of the Insl3^{-}/$^{-}$ male mice with surgically descended testis may be due to anatomical alteration of the reproductive organs during the operation, which mechanically obstructed the transfer of the sperm along their normal pathway from the epididymis to the uteri of the female mice, which had a vaginal plug.

The insulin-like family ligands are structurally related to each other and mediate many of the biological effects on cellular metabolism, growth, and differentation through binding and activation of their receptors, which are also structurally very similar (25, 26). It is known that insulin can bind to the insulin-like growth factor-1 receptor (IGF-1R), and the insulin-like growth factor-I and II (IGF-I and -II) to the insulin receptor (IR), albeit with lower affinities. The result of targeted mutagenesis of genes encoding members of insulin-like family ligands and receptors exhibit a growth deficiency in mouse embryos carrying a null mutation of the gene encoding IGF-I and II and IGF-1R, while mice homozygous for a null allele of the insulin-1 and -2 and insulin receptor are born with apparently normal intrauterine growth but die within hours after birth as a result of diabetic ketoacidosis (27–31). The striking phenotype of the *Insl3* mutant mice described suggests that the action of Insl3 on gubernacular development is specific and that other members of the insulin-like family do not compensate for the lack of the Insl3 during fetal development of male *Insl3*$^{-/-}$ mice. However, it remains to be investigated whether the action of Insl3 on gubernacular development is mediated through an interaction with its own receptor, which has not yet been identified, or through cross-talk with other members of the insulin-like receptor family located in the gubernacular primordia.

Cryptorchidism is the most common disorder of sexual differentiation in human males, with an incidence of 3.4% in the term newborn, which decreases to 0.8% at 1 yr of age. Severe complications of cryptorchidism are infertility and an increased risk for testicular malignancy (32). The complex process of testicular descent involves a series of hormonal and mechanical factors. Since the *INSL3* gene is also present in human genome (33), INSL3 could be one of these factors, and mutations in the gene encoding INSL3 could be a new etiology of cryptorchidism in humans.

MATERIALS AND METHODS

Construction of the Targeting Vector

The *Insl3*-targeting vector was constructed by using the plasmid pPNT (provided by Dr. R. Mulligan). A 7.5-kb *Sst*I fragment containing a 3′- flanking region of the *Insl3* gene was isolated and ligated with the *Xba*I/*Eco*RI-digested pPNT vector after filling the end with Klenow enzyme (clone *Insl3/1*) (18, 34). Finally, the 2.0-kb *Sal*I/*Xho*I fragment (*Sa/I* site from polylinker of phage clone) containing a 5′-flanking region of the *Insl3* gene was isolated and inserted in the *Xho*I-digested clone *Insl3/1* by blunt end ligation. The resulting 16.5-kb targeting vector was linearized with *Not*I before electroporation.

ES Cell Culture, Generation of Chimeric Mice, and Screening

The ES cell line MPI (provided by Dr. P. Gruss) was cultured as described previously (35). Confluent plates were washed in PBS buffer and trypsinized, and the cells were suspended in the same buffer at 2×10^7/ml. Aliquots of this cell suspension were mixed with 30 μg of linearized targeted vector and electroporated at 250 V and 500 μF using a Gene Pulser apparatus (Bio-Rad Laboratories, Inc., Richmond, CA). Cells were plated into nonselective medium in the presence of G418-resistant embryonic mouse fibroblasts. Selection was applied 36 h later using medium containing G418 at 350 μg/ml and gancyclovir at 2 μM. After 10 days of selection, individual drug-resistant clones were picked into 24-well trays. Three days later, individual recombinant ES clones were replicated into 24-well trays for freezing and isolation of DNA.

Genomic DNA was extracted from ES cells, digested with *Bam*HI, electrophoresed, and blotted onto Hybond N$^+$ membranes (Amersham, Arlington Heights, IL). The blots were hybridized with ^{32}P-labeled 1.3-kb *Sal*I/*Bam*HI fragment (Fig. 2A). To confirm a correct homologous recombination event of the targeted *Insl3* gene and absence of additional random integration of targeted construct, a neomycin fragment was used to probe Southern blots. Hybridization was carried out

at 65 C overnight in the following solution: 5× SSC/5× Denhardt's solution, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. Filters were washed twice at 65 C to final stringency at 0.2× SSC/0.1% SDS.

Chimeric mice from ES cells carrying the disrupted *Insl3* allele were generated by aggregating 10–15 compact ES cells with 2.5-day-old embryos of the CD1 mouse strain as described previously (36). Chimeric animals obtained were mated to CD1 or 129/Sv partners, and F_1 agouti offspring were genotyped by Southern blot analysis. Heterozygous animals were crossed to obtain homozygous mice, which were genotyped by Southern and PCR analyses. PCR was performed according to standard protocols to discriminate wild-type and mutant alleles in the DNA from the mouse tails and from the head of embryos. Primer sequences were as follows: 1 (*Insl3* sense), 5′-CCGCACCTGGGAGAGGACTTC; 2 (*Insl3* antisense), 5′-GTTATCCACGCTTGTCCAACC; 3 (*Pgk* antisense), 5′-TTCCATTGCTCAGCGGTG CTG. Thermal cycling was carried out for 30 cycles, denaturation at 94 C for 1 min, annealing at 58 C for 1 min, and extension at 72 C for 1 min. Animal studies were conducted in accordance with The Endocrine Society Guidelines for the Care and Use of Experimental Animals.

RNA Analysis

Total RNA was extracted from testes of 12-week-old mice using the RNA now Kit (ITC Biotechnologies) according to the manufacturer's recommendation. The RNA was size fractionated by electrophoresis on a 1% agarose gel containing formaldehyde, transferred to a nylon membrane, and hybridized with ^{32}P-labeled *Insl3* cDNA fragment under the same conditions as used for Southern blot hybridization (18).

Generation of *Ar*/Y *Insl3*$^{-/-}$ Mutant Mice

To generate *Ar*/Y *Insl3*$^{-/-}$ double-mutant mice, females *Ta Ar*/$^{++}$, which have *tabby* variegated coats owing to X chromosome inactivation, were mated with *Insl3*$^{+}$/$^{-}$ males. Females *Ta Ar*/$^{++}$ *Insl3*$^{+/-}$ in the progeny were then crossed with *Insl3*$^{+}$/$^{-}$ males. *Ta Ar*/Y *Insl3*$^{-/-}$ mice, which were phenotypic females with tabby coat, were identified by a *Insl3*- and a *Zfy*-specific PCR-based assay (37).

Histological Analysis

Embryos (15.5 and 17.5 dpc) were collected in PBS, fixed in Bouin's fixative, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin-eosin. Testes from 5- and 15-day-old and 12-week-old mice were fixed with 5% glutaraldehyde in 0.2 M phosphate buffer, postfixed with 2% osmium tetroxide, and embedded in epoxy (Epon) resin. Sections at 1 μm were stained with 1% toluidine blue/pyronine.

Scanning Electron Microscopy

After material was preserved for genotyping, the abdominal cavity of the E17.5 was opened, and the gastrointestinal tract and the urinary bladder were removed. After fixation by immersion in 1.5% glutaraldehyde in Locke's solution for 12 h and dehydration in a graded ethanol series, the embryos were critical point dried using ethanol as the transitional and CO_2 as the exchange fluid. The dried specimens were mounted with conducting silver and spattered with gold-palladium to a layer of about 40 nm. Specimens were examined and photographed in a DSM 960 scanning electron microscope (Carl Zeiss, Thornwood, NY).

Surgical Transplantation of the Cryptorchid Testis into the Inguinal Canal (Orchiopexy)

After anesthesia of 3-week-old *Insl3*$^{-/-}$ males, the abdominal cavity was opened by a 4-mm long transversal incision immediately below the umbilicus. The testicular artery was cut, and the testes were mobilized, brought down, and steadied into the inguinal canal by suturing their capsule to peritoneum. These testes retained sufficient vascularity from collateral blood flow through the deferential artery.

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