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Hormonal Control of Gubernaculum Development during Testis Descent: Gubernaculum Outgrowth *in Vitro* Requires Both Insulin-Like Factor and Androgen*

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ABSTRACT

The gubernaculum connects the gonad to the inguinoscrotal region and is involved in testis descent. It rapidly develops in the male fetus, whereas development in the female fetus is lacking. Possible factors involved in gubernaculum development are androgens, anti-Müllerian hormone (AMH), and insulin-like factor (Insl3). Sexual dimorphism in gubernaculum development correlated with the mitotic activity of cells in the gubernacular bulbs from male and female fetuses. Androgen receptor expression was restricted to the mesenchymal core of the gubernacular bulb, whereas skeletal muscle was detected in its outer layer. In an organ culture system devised to further study gubernaculum development *in vitro*, morphology of gubernacular explants grown in the presence of testes was comparable with that of gubernacula developed *in vivo*. Testicular tissue or medium containing R1881, a synthetic androgen, had a growth stimulatory effect on gubernacular explants compared with ovarian tissue or basal medium only. Moreover, $Amh^{-/-}$, $Amh^{+/-}$, and $Insl3^{+/-}$ testes stimulated the growth of gubernacular explants to the same extent as control testes. $Insl3^{-/-}$ testes, however, did not produce such an activity. This study reveals an essential role for both androgen and Insl3 in the gubernaculum outgrowth during transabdominal testis descent. (*Endocrinology* **141:** 4720–4727, 2000)

TESTIS DESCENT IS the process by which the developing testis moves from its initial position high in the abdomen into the scrotum. The process is generally subdivided into two phases (1). During the first or transabdominal phase, which takes place before birth, the testis gains a position at the bottom of the abdomen. The second or inguinoscrotal phase involves movement of the testis from the abdominal bottom to the base of the scrotum. Two ligaments appear to play an important role in determining the position of the gonad: the gubernaculum, which develops below the gonad in the inguinal area of the abdominal cavity, and the cranial suspensory ligament (CSL), which develops between the gonad and the dorsal abdominal wall, near the last rib (Fig. 1) (2, 3). During the transabdominal phase, the male gubernaculum is developing, whereas outgrowth of the CSL is lacking, resulting in a position of the testis close to the bladder neck.

Experimental studies oriented toward understanding the mechanism of testis descent often involve observations on gubernaculum outgrowth and regression (4). In rodents, the gubernaculum can be divided into a cranial part, the gubernacular cord, and a caudal part, the gubernacular cone or bulb. The gubernacular bulb consists of a mesenchymal core with a muscular cover. In larger mammals, including the pig, ungulates, and man, the gubernaculum proper only consists of mesenchymal cells (5). Fetal orchidectomy prevents gubernaculum outgrowth, suggesting the involvement of fetal testicular factors (6). The specific hormone mediating this process appeared not to be androgen, as testosterone was unable to counteract the effects of orchidectomy. This is further supported by the observation that gubernaculum outgrowth normally occurs in both mice and humans with complete androgen resistance (7). Androgens might play a role in the initiation of gubernaculum regression, which occurs during the inguinoscrotal phase (6, 8). A possible role for anti-Müllerian hormone (AMH) in the control of the first phase of testis descent was hypothesized (8), although direct experimental evidence is lacking. A strong argument against a role for AMH in testis descent is the observation that AMH-deficient male mice show normal testis descent (9). Concerning gubernaculum development, involvement of a third testicular factor has been suggested (10–12).

Recently, data became available indicating that insulin-like factor 3 (Insl3), also designated Leydig insulin-like factor or relaxin-like factor, might be an important factor for gubernaculum development in mice (13). Insl3 is specifically expressed in Leydig cells of the pre- and postnatal testis and in thecal cells of the postnatal ovary (14). Insl3 knockout mice, homozygous for a targeted inactivation of the Insl3 gene ($Insl3^{-/-}$ mice), have been generated, and it was observed that male $Insl3^{-/-}$ mice have undescended testes (15). Histological analysis of male $Insl3^{-/-}$ fetuses revealed that the development of the gubernaculum is severely affected. Similar findings were reported by Nef and Parada (16).

Although studies with mutant mouse models have provided more insight into the complicated regulation of gu-

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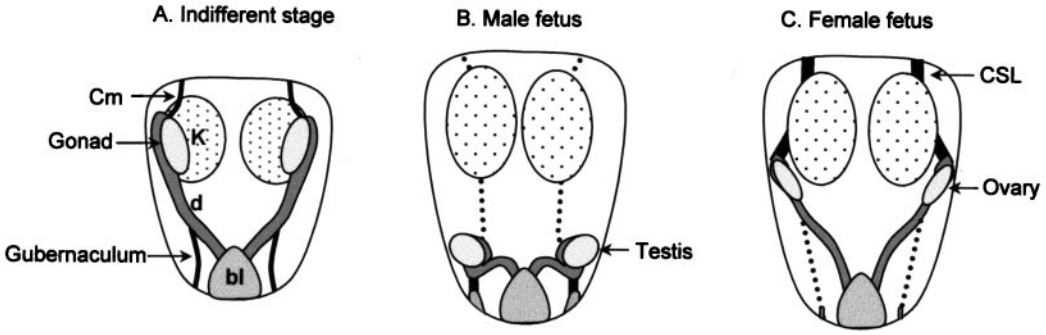

FIG. 1. Transabdominal phase of testis descent in the rat fetus. A–C, Schematic drawings of the gonadal position on E16, the indifferent stage, and on E19 in the male and female rat fetus. A, During the indifferent stage, the developing gonads of both male and female fetuses are positioned on the ventrolateral aspect of the kidney (k). Gonads and ducts (d) are attached to the abdominal body wall by two ligaments, the cranial mesentery (Cm) and the gubernaculum. B, In the male, the testis gains a position lateral to the bladder (bl), connected to the inguinal region via a developing gubernaculum. CSL development is lacking. C, In the female, the ovary is positioned lateral to the kidney, attached to the last rib via a well developed CSL. The gubernaculum does not further differentiate.

bernaculum development, it remains to be determined whether the targeted factors are directly involved in regulation of gubernaculum development. In the present study, development of the rat gubernaculum during the first phase of testis descent was studied *in vivo* with respect to cell differentiation and proliferation. Furthermore, an organ culture technique for *in vitro* culture of the gubernaculum was established to study the effects of different testicular hormones/factors on rat gubernaculum development.

Materials and Methods

Animals and tissue collection

Adult rats (Wistar) and mice were housed under standard animal housing conditions in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Female animals were placed in individual cages with males, and the morning a vaginal plug was found was designated day 0 [embryonic day 0 (E0)] of pregnancy.

For immunohistochemical analysis of the rat gubernaculum, fetuses were removed from the uterus on E17 and E19 and fixed in 10% neutral buffered formalin for 24 h. After fixation, fetuses were embedded in paraffin and sectioned at 7 μm, and sections were selected for immunohistochemistry.

Cell proliferation in the fetal rat gubernaculum *in vivo* was assessed using 5′-bromodeoxyuridine (BrdU), a thymidine analog that is incorporated into the DNA during the S phase of the cell cycle. Pregnant rats were injected ip with a single pulse of BrdU (Roche Molecular Biochemicals, Mannheim, Germany) dissolved in saline (100 mg/kg BW) on E17. Two hours after injection, fetuses were removed from the uterus, fixed in 10% neutral buffered formalin, and further processed for immunohistochemical detection of incorporated BrdU.

Organ cultures

For each culture, fetal rat gubernacula and mouse gonads were isolated. Gubernacula were obtained from E17 male rat fetuses. After the mothers were killed, the rat fetuses were quickly removed and placed on ice until dissection. Fetuses were examined under a dissecting microscope, and gubernacula were obtained from male fetuses and placed in PBS under sterile conditions. Urogenital ridges were also removed.

Gonads were obtained from neonatal mice (FVB strain) on postnatal day 6. Additionally, two different mutant mouse models were used: AMH mutant mice (B6 strain) (9) and Insl3 mutant mice (129/SV strain) (15). Homozygous $AMH^{-/-}$ or $Insl3^{-/-}$ female mice were bred with heterozygous $AMH^{+/-}$ or $Insl3^{+/-}$ males, respectively, to obtain homozygous and heterozygous neonates. The macroscopic anatomy of the neonates was determined with a dissection microscope, and homozygous AMH and Insl3 mutant neonates could be reliably distinguished from heterozygous ones based on their phenotypes. Nevertheless, tails were collected for genotyping by PCR. After dissection, testes were decapsulated.

The culture technique used was similar to that used by Cooke *et al.* (17) for mouse neonatal bulbo-urethral gland. Briefly, tissues were transferred onto CM filters (Millipore Corp., Bedford, MA) and floated on 1 ml medium in four-well plates (Nunc, Roskilde, Denmark). The culture medium used was DMEM/Ham's F-12 (Life Technologies, Inc., Breda, The Netherlands) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), fungizone (0.6 μg/ml), insulin (10 μg/ml), and transferrin (10 μg/ml; all from Sigma, St. Louis, MO). The medium was supplemented with 2% stripped FCS (Greiner Labortechnik, Kremsmuenster, Austria). In some of the cultures, the synthetic androgen methyltrienolone (R1881; 10^{-8} M) was added to the medium. The culture dishes were placed in a humidified incubator at 37 C in a 5% CO_2 atmosphere, and the medium was changed daily.

Gubernacula were cultured for 5 days with or without added gonads. The gonads were placed in close proximity to the gubernacula on a filter (Millipore Corp.) at the ratio of one gonad to one gubernaculum. After 5 days of culture, some explants were chosen randomly for immunohistochemical analysis. Tissues were fixed in neutral-buffered formalin and embedded in paraffin, and serial sections of 5 μm were cut.

Thymidine incorporation

The proliferation of [^{3}H]thymidine into DNA was measured on culture day 5. At the end of day 4, 5 μCi/ml [*methyl*-^{3}H]thymidine (SA, 48 Ci/mmol; Amersham Pharmacia Biotech, Little Chalfort, UK) was added to the culture medium. After incubation overnight, the cultured tissues were removed, rinsed with 10% ice-cold trichloroacidic acid, and centrifuged. The precipitate was washed twice with cold 5% trichloroacidic acid and hydrolyzed in 25 μl 1 M NaOH (68 C, 30 min). The radioactivity of the supernatant was measured using a liquid scintillation counter, and the incorporation of radioactive thymidine was calculated as disintegrations per min/gubernaculum.

Immunohistochemistry

Selected sections were mounted on silane-coated slides. After deparaffinization, sections were treated with 3% H_2O_2/methanol solution to block endogenous peroxidase activity.

To study gubernaculum development *in vivo* and *in vitro*, polyclonal antibodies against the rat androgen receptor (SP197) (18) were used as marker for the mesenchymal core, and monoclonal antibodies against sarcomeric myosin (MF20, obtained as hybridoma from the Developmental Studies Hybridoma Bank) (19) were used as a marker for the skeletal muscle outer layer. The procedure used was based upon that described by Janssen *et al.* (20). Briefly, sections were placed in 10 mM citrate buffer and microwaved three times for 5 min each time at 700 watts. Sections were preincubated with normal goat serum, followed by incubation with the primary polyclonal antibodies SP197 (diluted 1:7000) or monoclonal antibody MF20 (diluted 1:100) overnight at 4 C.

The antibodies were detected using biotinylated goat antirabbit antibody (dilution 1:400) or biotinylated goat antimouse antibody (diluted 1:50), respectively, by incubation for 30 min at room temperature, followed by treatment with streptavidin-biotin-peroxidase complex. The peroxidase activity was visualized with 3,3′-diaminobenzidine tetrahydrochloride. Negative controls were included by replacement of the primary antibody by PBS. Cells with nuclear [androgen receptor (AR)] or cytoplasmic (myosin) immunostaining were interpreted as positive. AR expression in the gubernaculum was assessed in qualitative terms by visually determining the average intensity of the positive nuclear staining in this structure.

The BrdU staining procedure has been described previously (21). Briefly, labeling was detected in 7-μm sections, pretreated with 0.1% pronase for 30 min and 2 N HCl for 30 min, followed by 1-h incubation with mouse monoclonal anti-BrdU antibodies (diluted 1:25; Sigma), all at 37 C. Sections were then incubated with peroxidase-conjugated goat antimouse antiserum (diluted 1:100) for 30 min at room temperature. BrdU-labeled nuclei were visualized with 3,3′-diaminobenzidine tetrahydrochloride. Slides were counterstained with hematoxylin.

Controls and statistical analysis

The proliferation index of the rat gubernaculum was estimated by determining the proportion of BrdU-labeled nuclei per total number of cells ($\times$100) per fetus. Statistical significance was assessed using Student's two-tailed *t* test.

All culture experiments were performed in triplicate, and the data are expressed relative to [^{3}H]thymidine incorporation induced in gubernaculum cultured with R1881 (10^{-8} M) in the same experiment. All of the data shown were collected in at least two independent experiments. Statistical analysis was performed using one-way ANOVA supplemented with Hochberg's GT2 test. All data are presented as the mean ± SEM.

Results

Sexually dimorphic development of the gubernaculum

Histological analysis of rat fetuses on E17 and E19 showed sexually dimorphic development of the gubernacular bulb (Fig. 2). The sexually dimorphic development of the gubernaculum was further examined by determination of the proliferation index and by studying the organization of the gubernacular bulb into mesenchymal cells and myoblasts using two different cell differentiation markers. On E17, the bulb in both sexes is organized into a mesenchymal core and an outer muscular layer, but the male bulb is enlarged compared with the female gubernacular bulb. The proliferation indexes for the male and female gubernacula on E17 were estimated using the BrdU-labeling technique. BrdU incorporation was significantly higher in the male gubernacular bulb (15.8 ± 0.5%; n = 4) than in the female bulb (7.2 ± 1.8%; n = 4; $P < 0.005$). The enlargement or swelling of the male gubernacular bulb progressed to E19, whereas in the female fetus no further development occurred (Fig. 2, A and B).

Immunohistochemical analysis of the gubernaculum demonstrated expression of sarcomeric myosin in the periphery of the gubernacular bulb of both sexes on E17 and E19 (Fig. 2). However, in the male fetus, the muscular layer expanded between E17 and E19, and the myoblasts started to differentiate (Fig. 2, C–E). AR expression was restricted to the mesenchymal cells of the gubernacular bulb (Fig. 2F). AR expression in the male gubernaculum was consistently higher than that in the female gubernaculum at both time points (not shown).

An in vitro model of gubernaculum development

To analyze hormonal control of gubernaculum development, the possibility of using a culture system was studied. As the *in vivo* studies demonstrated that gubernaculum development is characterized by rapid cell proliferation, it was decided to follow gubernaculum development *in vitro* by measurement of the incorporation of [^{3}H]thymidine into DNA. Development of the Wolffian duct *in vitro* is also marked by rapid proliferation when cultured in presence of a testis or androgen (22). Therefore, the Wolffian duct was used in each culture experiment as a control androgen-responsive tissue.

Initial studies indicated that E17 rat gubernacular explants responded to the presence of testicular tissue in culture by increased incorporation of [^{3}H]thymidine. To determine the effects of gonads from AMH$^{-/-}$ and Insl3$^{-/-}$ mice on gubernaculum development *in vitro*, a series of culture experiments with mouse gubernacula and gonads was carried out. However, in contrast to fetal rat gubernacular explants, fetal mouse gubernacular explants did not show any indication of a proliferative response to testicular tissue. It was therefore decided to perform a coculture of rat gubernaculum with mouse gonads, outlined in Fig. 3A, which was successful. Maximal differences in [^{3}H]thymidine uptake per rat gubernaculum cultured in the presence or absence of mouse testis were observed after 5 days of culture. [^{3}H]Thymidine uptake by the gubernacular explants was enhanced after 5 days when cultured in the presence of testis or R1881, the effect of testis being greater than that of R1881 (Fig. 3B). Ovarian tissue had no effect on gubernacular growth. As a prenatal mouse testis is much smaller then a prenatal rat testis, three prenatal mouse testes per rat gubernaculum were necessary to produce a growth response similar to that produced by one prenatal rat testis. However, replacing these three fetal mouse testes by one neonatal mouse testes, dissected on postnatal day 6, induced a growth response in a E17 rat gubernaculum that compared well with that of an E17 rat testis.

At the light microscopic level, the explants were studied after 5 days of culture to determine the viability of the tissues (Fig. 2, G and H). Addition of 2% FCS and insulin (10 μg/ml) to the medium enhanced the viability of gubernaculum and gonadal tissue during culture. On day 0, the gubernacular bulb was organized into a muscular outer layer and an inner mesenchymal core (Fig. 2G). By day 5, the entire gubernaculum had enlarged. The organization into muscular outer layer and mesenchymal core was preserved in the gubernacular bulb cultured in the presence of a testis (Fig. 2H).

Immunohistochemistry was performed to study the expression of both differentiation markers, AR and myosin, in cultured gubernacula (Fig. 2, I–L). Myosin was expressed in the gubernaculum under all culture conditions, including in gubernacula cultured without hormone or in the presence of ovarian tissue (Fig. 2, I and J). AR expression was detected in gubernacular explants cultured in the presence of either R1881 or testis (Fig. 2, L). The organization of the gubernacular bulb into an outer muscular layer and an inner AR-positive mesenchymal core, as seen *in vivo*, was also observed in gubernacular explants *in vitro*, but only when cultured in

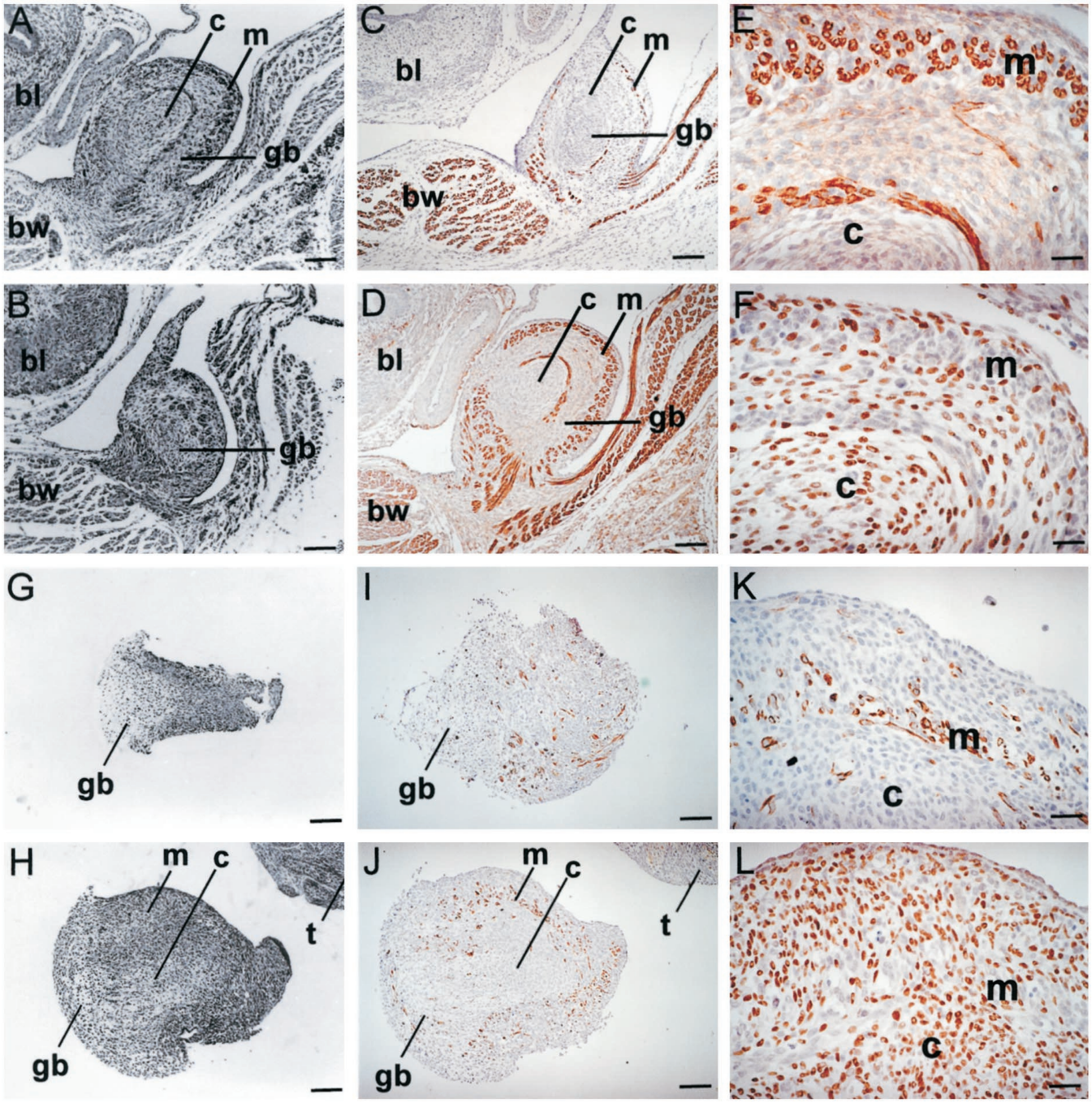

FIG. 2. Histology and immunohistochemistry of rat gubernaculum development *in vivo* and *in vitro*. A and B, Histological analysis of the gubernaculum on E19 in the male (A) and female (B) rat fetus *in vivo*. In the male gubernacular bulb (gb), the muscular layer (m) and mesenchymal core (c) can be discriminated. Although the gubernacular bulb of the female fetus also consists of myoblasts and mesenchymal cells, it is smaller and less well organized compared with the male bulb. bl, Bladder; bw, body wall. C–F, Immunohistochemical localization of myosin (C–E) and AR (F) in the developing male gubernacular bulb of the rat *in vivo*. C, On E17, the muscular layer of the gubernacular bulb can be clearly distinguished by myosin immunostaining. D, On E19, the muscular layer is more pronounced compared with that on E17, as demonstrated by a strong antin-myosin immunoreaction. E, Detail of the muscular layer from section D, showing differentiating myoblasts that are myosin positive. F, A section from the same male rat fetus (D and E), but stained with antibodies against the AR, showing that the mesenchymal cells are AR positive (F). G and H, Morphology of gubernacular explant before culture (G) and after 5 days of culture in the presence of mouse testicular tissue (t) (H). Note the pronounced increase in size of the gubernacular bulb after culture. I–L, Immunolocalization of myosin (I–K) and AR (L) after culture in the presence of R1881 (10^{-8} M; I) or testis (J–L). Both gubernacular explants (I and J) demonstrate immunostaining with myosin. In the gubernacular explant cultured in the presence of R1881 (I), myosin-positive cells can be seen across the whole explant. In contrast, the gubernacular explant cultured in the presence of testicular tissue (J) shows a myosin-positive outer layer. Detail (K) of the muscular layer from section J, showing myoblasts that are myosin positive. L, A section from the same explant (J and K), but stained with antibodies against the AR, showing that the mesenchymal cells are AR positive. *Scale bar*: A, B, C, D, G, H, I, and J, 100 μm; E, F, K, and L, 25 μm.

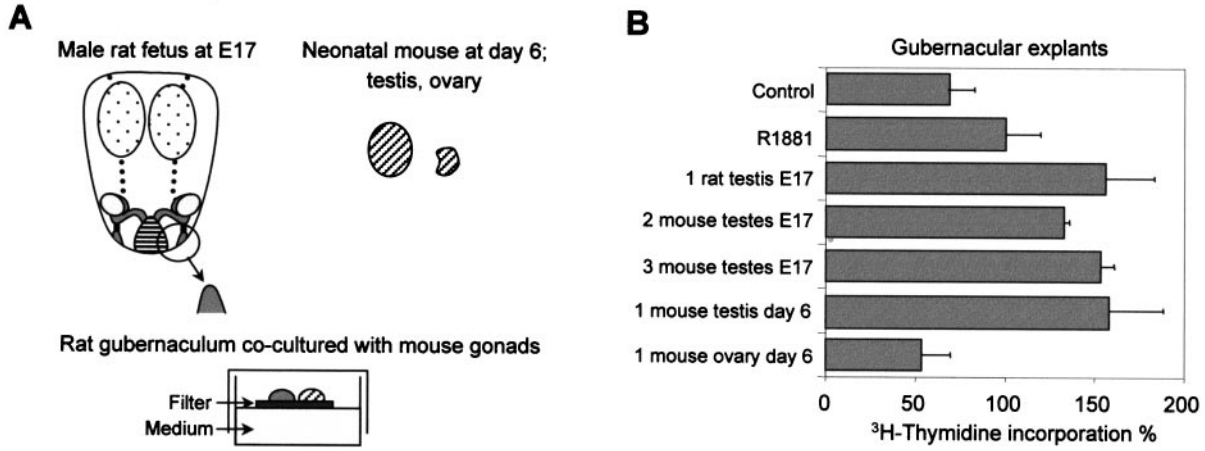

FIG. 3. *In vitro* culture of gubernacular explants. A, Schematic outline of the organ culture system used for coculture of rat gubernacular explants and mouse gonads, as described in *Materials and Methods*. B, A representative experiment of [^{3}H]thymidine incorporation by rat gubernacular explants cultured without hormone (control) or in the presence of R1881 (10^{-8} M), testicular tissue or ovarian tissue. Testis was derived from E17 rat fetuses, E17 mouse fetuses, or postnatal day 6 mice. Ovarian tissue was only derived from postnatal day 6 mice. Values are expressed relative to the effect of R1881 (set at 100%).

presence of a testis (compare Fig. 2, K and L, with Fig. 2, E and F). However, the muscular layer was thinner and less differentiated compared with *in vivo* development (compare Fig. 2K with Fig. 2E). In all other cultures, myosin-positive cells were found all across the gubernaculum (Fig. 2I).

Effects of testes from different mutant mice

The culture system described above was used to study the stimulatory effects of gonads from different mutant mice on gubernacular explants. Two different mutant mouse models were available, AMH$^{-/-}$ and Insl3$^{-/-}$ mice, as well as the heterozygous animals. Although *in vivo* analysis of heterozygous and homozygous AMH and Insl3 mutant male mice did not indicate any defect in androgen production by the testis, R1881 was added to the culture medium to compensate for a possible partial deficiency in androgen production by the testis *in vitro*. In Fig. 4, the effect of testicular tissue from the different mutants on [^{3}H]thymidine uptake by gubernacular explants, cultured in R1881-containing medium, is shown. AMH$^{-/-}$, AMH$^{+/-}$, and Insl3$^{+/-}$ testicular tissue stimulated [^{3}H]thymidine incorporation by the gubernacular explants to the same extent as control testis. In contrast, in the presence of a Insl3$^{-/-}$ testis, [^{3}H]thymidine incorporation was significantly reduced compared with the effect of the control testis ($P < 0.05$), to a level similar to that seen with R1881 alone. Heterozygous and homozygous mutant ovarian tissue did not induce any increase in the proliferative activity of gubernacular explants compared with the proliferative activity of gubernacular explants cultured in basal medium (results not shown).

As mentioned previously, Wolffian duct was used in each culture experiment as a control androgen-responsive tissue. After 5 days of culture, [^{3}H]thymidine uptake by rat Wolffian duct explants was enhanced when cultured in the presence of testis or R1881; the effect of the testis was similar to that of R1881 (Fig. 5). Ovarian tissue did not stimulate the growth of Wolffian duct explants. In contrast to the results obtained with gubernacular explants, the stimulatory effect of Insl3$^{-/-}$ testis on Wolffian duct growth *in vitro* was not different from that of control or Insl3$^{+/-}$ testis.

Discussion

In the present study an organ culture system has been established that provides a unique opportunity to study hormonal regulation of the fetal rat gubernaculum under precise and defined conditions. The organ culture system used was similar to the one used by Cooke *et al.* (17) for the mouse neonatal bulbo-urethral gland. Coculture of a rat gubernaculum with a mouse testis induces a growth response, illustrating that the factors controlling gubernaculum outgrowth in these rodents are most likely similar, to be recognized across species barriers. This is also observed for other structures of the male urogenital tract such as the prostate and seminal vesicles (23, 24). Establishment of an organ culture with mouse gubernaculum failed, as no growth stimulatory effect could be induced by addition of mouse testis. Gubernaculum outgrowth, characterized by rapid cell proliferation, occurs relatively fast in mice, between E15.5 and E17.5. It might be possible that during the culture period (a minimum of 2 days), gubernaculum outgrowth has already ended, and cell proliferation in the mouse gubernaculum has reached a plateau. Thus, by the time the growth response assay was performed, at the end of the culture period, no increase in [^{3}H]thymidine incorporation could be determined anymore. In contrast, the period of gubernaculum outgrowth in the fetal rat is longer, as gubernaculum outgrowth occurs between E16 and E20. This might explain why a growth response could be determined using rat gubernacula.

Immunohistochemical studies presented herein showed that both male and female rat gubernaculum express myosin, a marker for myoblasts, *in vivo*. Furthermore, myosin was also expressed *in vitro*, under all different culture conditions. However, the muscular layer of the gubernaculum *in vitro*, even after coculture with a testis, was thinner compared with the *in vivo* situation. Similarly, Radhakrishnan and Donahoe

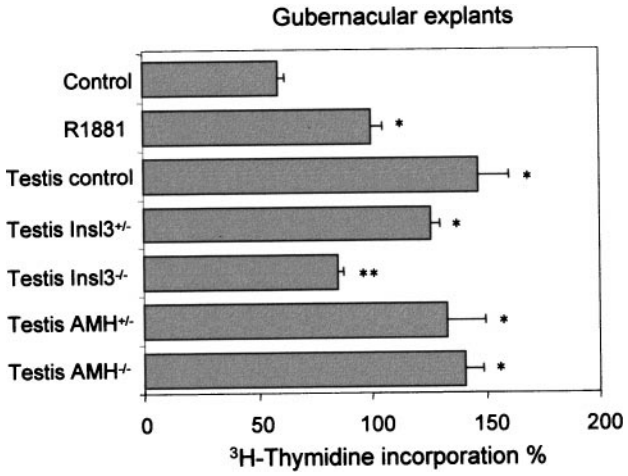

FIG. 4. Effects of testes from Insl3- and Amh-deficient mice on gubernaculum growth *in vitro*. [^{3}H]Thymidine incorporation by gubernacular explants cultured without hormone (control), in the presence of R1881 (10^{-8} M), or in the presence of both R1881 (10^{-8} M) and a testis from control, Insl3$^{+/-}$, Insl3$^{-/-}$, Amh$^{+/-}$, or Amh$^{-/-}$ mice. Values are expressed relative to the effect of R1881 (set at 100%) and are the mean ± SEM of at least two independent experiments performed in triplicate. *, $P < 0.05$ *vs.* control; **, $P < 0.05$ *vs.* control testis.

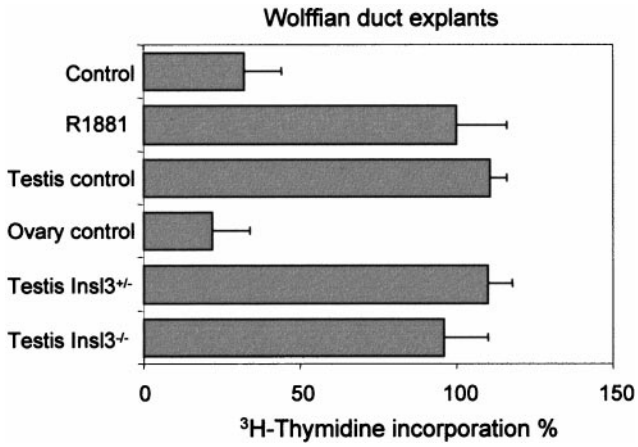

FIG. 5. *In vitro* culture of Wolffian duct explants. A representative experiment of [^{3}H]thymidine incorporation by rat Wolffian duct explants cultured without hormone (control) or in the presence of R1881 (10^{-8} M), testicular tissue, or ovarian tissue. Testis tissue was derived from control, Insl3$^{+/-}$, or Insl3$^{-/-}$ postnatal day 6 mice. Ovarian tissue was derived from postnatal day 6 control mice. Values are expressed relative to the effect of R1881 (set at 100%).

(25) limited their *in vitro* studies to the muscular layer and did not observe induction of muscle differentiation in cultured rat gubernacular tissue by using whole testis. Studies on *in vitro* skeletal muscle development showed that only a limited degree of differentiation can take place *in vitro*, probably due to the lack of innervation, growth factors, and extracellular matrix (26). Although myoblasts can differentiate in the absence of nerves, continuing growth and maturation definitively require innervation (26). Upon isolation, the gubernaculum is separated from the body wall musculature and nerves, which could explain the retarded muscular development *in vitro*.

Immunostaining of the AR *in vivo* demonstrated AR-positive cells in the mesenchymal layer of the gubernaculum of both sexes, which is in agreement with published data (18, 27). The level of AR expression increases in the male, but decreases in the female gubernaculum, indicating an initial androgen-independent expression followed by a hormone-dependent expression (28). In agreement with the *in vivo* situation, the AR was only highly expressed in explants that were cultured in the presence of androgen, either as R1881 added to the medium or in cocultures with testicular tissue.

Generally, gubernacular tissue cultured *in vitro* showed a more condensed structure than gubernacular tissue *in vivo*. This could be due to the flattening of the gubernacular explants when cultured on the two-dimensional filter layer. Furthermore, the organization of the gubernaculum into the mesenchymal core and muscular outer layer, as seen in the male gubernaculum development *in vivo*, was only observed in the gubernacular explants cultured in the presence of a testis. Apparently, testicular factors other then androgens are necessary to maintain the male-specific organization of the gubernaculum during culture.

On E17, the male gubernaculum *in vivo* was enlarged substantially compared with that in the female, which is in agreement with histological observations reported previously (5, 29, 30). This difference can be explained by a difference in cell proliferation, as determined by BrdU labeling indexes. This is in agreement with observations in the pig fetus, in which gubernaculum outgrowth during the transabdominal phase of testis descent is also characterized by rapid cell proliferation (31–33). The regulation of proliferation was used as parameter for further analysis of hormonal control of gubernaculum development.

Surprisingly, R1881 added to the culture medium clearly increased [^{3}H]thymidine uptake by gubernacular explants. In two earlier *in vitro* studies using isolated porcine gubernaculum cells, androgens did not stimulate proliferation (10, 12). As the major difference between rat and pig gubernaculum proper is the presence of a muscular outer layer in the rat (5), the increased proliferation might reflect an increase in the muscular component of the rat gubernaculum. Given the fact that expression of the AR, which is a prerequisite for androgen activity, is detected in the mesenchymal core of both rodent and porcine gubernaculum (27, 34, 35), it is suggested that androgens may act directly on the mesenchymal cells, which, in turn, elicit a growth response on the myogenic cells through paracrine mechanisms. In several androgen-responsive tissues, including prostate, seminal vesicles, and bulbo-urethral glands, testicular androgens act via the AR in the mesenchymal cells (36). In addition, influences of the mesenchyme on the muscular layer and *vice versa* might be necessary for complete gubernaculum development. Such a mechanism of action might also hold true for the process of gubernaculum outgrowth in the pig. Although the porcine gubernaculum proper only consists of mesenchymal cells, it is in close contact with the cremaster muscle, which is the equivalent of the muscular outer layer of the rat gubernaculum (5). The present *in vitro* data are in apparent contradiction with the currently available data from *in vivo* studies that more strongly support a role of androgens in gubernaculum regression then outgrowth (4). However, both Spencer *et al.* (37) and Cain *et al.* (38) demonstrated a decrease in gubernacular bulb development upon antian-

drogen exposure of male rat fetuses. This indicates the complexity of gubernaculum development *in vivo* and underlines the usefulness of organ culture for further studies on the role of androgen.

The presence of testicular tissue during the culture period exceeded the stimulatory effect of androgen on [^{3}H]thymidine uptake by gubernacular explants, whereas ovarian tissue did not stimulate [^{3}H]thymidine uptake. This implies that a testicular factor, different from androgens, is also involved in gubernaculum growth. This is in contrast to the Wolffian ducts, which are stimulated to the same extent by testicular tissue as by androgens alone. Two factors that are secreted by the fetal testis at the time of transabdominal testis descent are AMH and Insl3 (14, 39). As neither factor was available for direct studies, coculture experiments were designed in which gonads of AMH and Insl3 mutant mice were tested. R1881 was added to the culture medium to compensate for a possible partial deficiency in androgen production by the testes of AMH and Insl3 mutant testes *in vitro*. Culturing gubernacula in the presence of Amh$^{-/-}$ testes did not decrease the uptake of [^{3}H]thymidine, compared with the effect of control or Amh$^{+/-}$ testes. This is consistent with the observed lack of a stimulatory effect of AMH on cultured porcine gubernaculum cells (10, 12). It cannot completely be excluded that a small amount of AMH might have been present in the culture medium, as 2% stripped (mixed sex) FCS was used. However, this amount did not cause Müllerian duct regression in a Müllerian duct organ culture assay performed under the same culture conditions as those used for gubernaculum (personal observations).

The effect of an Insl3$^{-/-}$ testes on [^{3}H]thymidine incorporation by the gubernacular explants, cultured in medium containing R1881, was similar to that by explants cultured in medium containing R1881 only. Insl3$^{-/-}$ testes were not competent to further increase the stimulatory effect of androgens present in the culture medium. This result consolidates the putative role of testicular Insl3 in gubernaculum development *in vivo* and indicates that the primary defect of impaired gubernaculum development observed in Insl3-deficient mice appears to be intrinsic to the testis. In contrast, Insl3 appeared not to be essential for growth of the Wolffian ducts.

Whether Insl3 is secreted by the testis and acts directly on gubernaculum cells remains to be determined. Recently, however, Insl3 has been demonstrated in the human circulation using antibodies (40), and specific, high affinity Insl3 receptors have been identified (41), indicating a possible endocrine role of Insl3. According to the predicted structure, the biologically active Insl3 protein consists of a β-chain (3.3 kDa) and an α-chain (13). Fentener van Vlissingen *et al.* (10) and Visser *et al.* (12) previously demonstrated that low mol wt factors ($<$30K and $<$3.5K, respectively, isolated from fetal pig testis stimulated growth in cultured gubernaculum cells. As the Insl3 gene has also been identified in the pig, and porcine Leydig cells express Insl3 messenger RNA (42), the growth stimulatory effect observed by these investigators might have been related to Insl3 activity.

In the pig fetus, gubernaculum growth is determined by cell proliferation as well as by changes in the extracellular matrix, resulting in true swelling of the gubernaculum due to water uptake (43). Whether changes in the extracellular matrix are important during transabdominal testis descent in rodents remains to be determined. Interestingly, however, primary structure analysis revealed that Insl3 is closely related to relaxin (44). Furthermore, synthetic human INSL3 peptide augmented the activity of relaxin in the mouse pubic symphysis bioassay, indicating relaxin-like properties of Insl3 (44). Relaxin is a hormone that plays a significant role in promoting the growth and softening of the cervix and loosening the pubic symphysis before parturition by remodeling connective tissue (45, 46). A similar mechanism of Insl3 action in the male fetus during gubernaculum development and testis descent would be an attractive working hypothesis for further research.

Further analysis of gubernaculum development *in vivo* and *in vitro* in relation to regulation of Insl3 gene expression might lead to a better understanding of the process of testis descent. A major goal of future studies will be to define downstream genes through which the action of Insl3 on gubernaculum development is mediated.

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