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Pituitary Hormones Are Not Required for Sexual Differentiation of Male Mice: Phenotype of the T/ebp/Nkx2.1 Null Mutant Mice

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We have studied male sexual differentiation of null mutant mice (−/−) for the thyroid-specific enhancer-binding protein (*T/ebp* or *Nkx2.1*) gene, a homeodomain transcription factor that plays a role in organogenesis of the thyroid, lung, ventral forebrain, and pituitary gland. Because the *T/ebp/Nkx2.1* (−/−) mice do not develop the pituitary gland, their sexual differentiation, if any, must occur in the absence of action of gonadotropins and other pituitary hormones. The (−/−) mice survive only until birth (embryonic d 19–19.5 of pregnancy), and when their external and internal genitals were inspected at embryonic d 18.5, they were indistinguishable from the (+/−) and (+/+) control mice. The testis weights of (−/−) mice were 20% lower than in (+/+) and (+/−) mice. The testosterone content of the (−/−) testes (13.5 ± 2.4 pg/gonad, mean ± SEM, n = 11) was dramatically reduced, compared with (+/−) (165 ± 22.5 pg, n = 14) and (+/+) (234 ± 37.3 pg, n = 10) littermates. Light microscopy revealed no difference in seminiferous tubules, interstitial tissue, or relative proportions of the two-cell compartments between the (−/−) and (+/+) testes. However, electron microscopy confirmed that Leydig cells in the (−/−) testes were much smaller, with smaller mitochondria and proportion of smooth endoplasmic reticulum than found in the controls, which was in support of the low androgen content of the knockout testes. In conclusion, this study on *T/ebp/Nkx2.1* knockout mice, devoid of the pituitary gland, demonstrates that pituitary hormone secretion is not needed for stimulation of sufficient fetal testicular androgen synthesis to induce male sexual differentiation. The endogenous testosterone level in the null mutant testes is 5–10% of the control level, which suggests that there is a considerable safety margin in the amount of testosterone that is needed for the male fetal masculinization. (*Endocrinology* 143: 4477–4482, 2002)

THERE ARE CLEAR sex differences in the principal mechanisms of mammalian sex differentiation. The gonadal differentiation in both sexes requires a cascade of well-orchestrated gene activities (1–3). For the differentiation of the extragonadal sex organs, a clear mechanistic difference prevails between the female and male. Although the female differentiation seems to occur autonomously without influences from the fetal ovary, the male differentiation is critically dependent on fetal testicular function. The fetal testis produces three important hormones. The Leydig cell product, testosterone, causes stabilization of the Wolffian ducts, and the Sertoli cell product, anti-Müllerian hormone (AMH), causes involution of Müllerian ducts. A third hormone produced by fetal Leydig cells has been recently discovered: it is insulin-like factor 3 (INSL3), which regulates the first phase of testicular descent, *i.e.* the transabdominal passage of testes from the vicinity of the kidneys to the inguinal region (2). Data concerning the regulation of fetal testicular hormone production have been scanty. Various fragments of information suggest that at least initially, testicular hormone production is not dependent on pituitary gonadotropins but is either autonomous or regulated by paracrine and autocrine mechanisms (4–6). Earlier studies on anencephalic human fetuses (7, 8) and hypophysectomized mammalian fetuses (9, 10) provided evidence that fetal masculinization is possible without the input of pituitary hormones. This information is supported by more recent observations on normal masculinization of fetuses of gonadotropin-deficient *hpg* mice (11, 12) and common α-subunit (13) and LH receptor (6, 14) knockout mice. Because other pituitary hormones are able, either directly or indirectly, to stimulate testicular function (15), clear evidence for or against pituitary involvement in the fetal testicular function is not available.

The thyroid-specific enhancer-binding protein (T/ebp) transcription factor, also called NKX2.1 or thyroid transcription factor 1, is known to control thyroid- and lung-specific gene transcription. It is also essential for organogenesis of the thyroid gland, lung, ventral forebrain, and pituitary gland, as has been demonstrated with a *T/ebp/Nkx2.1* null mouse model (16). Heterozygous mice developed normally, whereas those homozygous for the disrupted gene were born dead and lacked the lung parenchyma. Furthermore, the homozygous mice had no thyroid gland but had a normal parathyroid. Extensive defects were found in the brain, especially in the ventral region of the forebrain. The entire pituitary, including the anterior, intermediate, and posterior lobes, was also missing. *In situ* hybridization showed that the T/ebp gene is expressed in the normal thyroid, lung bronchial epithelium, and specific areas of the forebrain during early embryo-

Abbreviations: AMH, Anti-Müllerian hormone; E, embryonic day; INSL3, insulin-like factor 3; T/ebp, thyroid-specific enhancer-binding protein.

genesis. The knockout fetal mice survive throughout a normal-length gestation but die at birth mainly because of severely hypoplastic lungs. We therefore surmised that the *T/ebp/Nkx2.1* null mouse would be a perfect model for deciphering the role of the pituitary gland in fetal gonadal function and sexual differentiation.

Materials and Methods

Animals

T/ebp/Nkx2.1 null mice were produced by homologous recombination as previously described (16). The *T/ebp/Nkx2.1* heterozygous mice were maintained and mated to obtain homozygous embryos and wild-type littermates. Embryos were collected by dissection of pregnant mice on d 18.5 of pregnancy, snapped frozen in liquid nitrogen, and kept frozen until use. Embryonic day (E) was designated as 0.5 at noon of the day that the copulatory plug was detected. Genotyping of the embryos was carried out by PCR using the yolk sac as a source of DNA. Appropriate permissions from the local ethical committee on animal experimentation were obtained for the experiments.

Testosterone measurements

Testicular testosterone was measured from diethyl ether extracts of testicular homogenates by a RIA as described earlier (17). A pair of testes was used for the homogenate.

Electron microscopy

For electron microscopy the gonads were prepared as previously described (18). Briefly, the testes were fixed by immersion in 5% glutaraldehyde (Merck, Darmstadt, Germany) in 0.16 mol/liter s-collidine-HCl buffer (pH 7.4) and postfixed with potassium ferrocyanide-osmium fixative (19). The tissues were embedded in epoxy resin (Glycidether 100, Merck) and sectioned for light and electron microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate (Reichert Ultrostainer, Leica Corp., Vienna, Austria) and examined in a Jeol JEM-100XS electron microscope (JEOL, Tokyo, Japan). For light microscopy, 1-μm-thick sections were cut and stained with 0.5% toluidine blue.

Morphometry

Sections stained with toluidine blue (see above) were viewed and photographed using a DM RB E research microscope with a digital camera (DC 200, Leica Corp.) and printed on paper. The tubular and interstitial compartments were cut from the paper images and weighed. The volume density of the interstitial tissue was calculated. One arbitrary section of each testis studied was used for calculations.

Stereomicroscopy

The fetuses were halved at the level of diaphragm, and the caudal parts were fixed in glutaraldehyde [0.2% glutaraldehyde, 0.1 mol/liter sodium phosphate buffer (pH 7.3), 5 mmol/liter EGTA, 2 mmol/liter $MgCl_2$, 2% formalin] for 30 min, rinsed with water, and stained with Mayer's hematoxylin for 1 min. Tissues submerged in buffer were placed under an SZX9 stereomicroscope (Olympus Corp., Hamburg, Germany) and photographed using a DC 100 camera (Leica Corp.).

Statistics

Two-sided unpaired *t* tests were used for statistical comparison of two experimental groups. To compare three groups, one-way ANOVA was used, followed by Tukey's test. A *P* value less than 0.05 was taken as the limit of statistical significance.

Results

The (−/−) embryos survived until d 19–19.5 of gestation, *i.e.* the day they were born. When inspected at E18.5, their internal and external genitals, in both sexes, were indistinguishable from those of the (+/+) and (+/−) littermates. There is ample evidence that fetal ovaries do not play a role in the extragonadal female sex differentiation (2). The gonadotropin receptors appear in rodent ovaries only after birth (20). We therefore found it more intriguing to do a careful analysis of sexual differentiation of the male fetuses that are known to produce three hormones, AMH, testos-

TABLE 1. Testis weights and volume density of interstitial tissue of total testicular volume (mean ± SEM) of control (+/+) and *T/ebp/Nkx2.1* heterozygous (+/−) and homozygous (−/−) knockout mice

Genotype	Testis weight (mg)	n	Interstitial tissue (% of testicular volume)	n
+/+	0.64 ± 0.07	8	53.4 ± 1.0	2
+/−	0.65 ± 0.03	30	52.0 ± 1.4	3
−/−	0.52 ± 0.02[a]	22	55.1 ± 4.1	3

[a] $P < 0.05$ vs. (+/−) mice; ANOVA/Tukey's test.

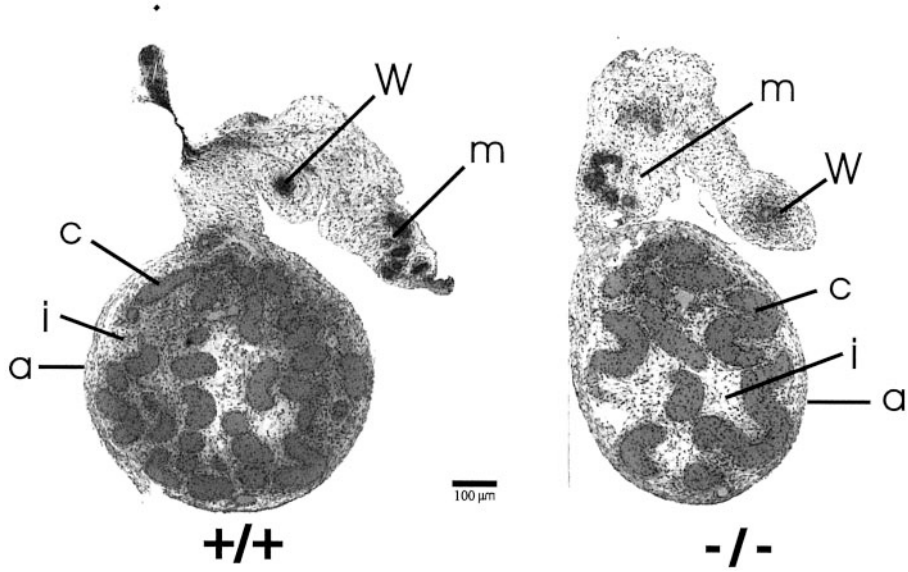

FIG. 1. Light micrographs of mouse fetal testis on E18.5 showing convoluted solid cylindrical testicular cords (c) and interstitium (i), which consists of loose connective tissue and Leydig cells. The tunica albuginea (a) is also well developed. The Wolffian duct (W) is well preserved, and no Müllerian duct is seen in either section; m, convoluted mesonephric remnants. Transverse equatorial epoxy resin section stained with toluidine blue, thickness 1 μm. *Left panel,* Testis of wild-type mouse; *right panel,* testis of a homozygous knockout mouse. *Scale bar,* 100 μm.

terone, and INSL3, all potentially under the regulation of pituitary gonadotropins.

Weights of the [(+/−) and (−/−)] testes were monitored, and they were 0.64 ± 0.07 mg in (+/+) mice (n = 8), 0.65 ± 0.03 mg in (+/−) mice (n = 30), and 0.52 ± 0.02 mg in (−/−) mice (n = 22). Hence, the weights of the (−/−) testes were about 20% smaller than in the other groups (Table 1). Except for the slightly smaller overall size, consistent with the about 15% lower body weight (16), the testes in the knockout and normal animals were histologically similar. The testicular cords were convoluted cylindrical structures embedded in interstitial tissue, which contained stromal and Leydig cells (Fig. 1). The number of Leydig cells per unit volume of testis tissue appeared on light microscopy similar in the (+/+) and (−/−) testes (Table 1). The tunica albuginea was well developed in both cases. Electron microscopic examination confirmed the presence of ultrastructurally well-differentiated Leydig cells with similar cytoplasmic organelles both in transgenic and normal testes, although the Leydig cells in the (−/−) animals were consistently smaller in size (Figs. 2 and 3). From the ultrastructural point of view, the Leydig cells in the transgenic testes despite their smaller size are fully capable of synthesizing and secreting steroid hormones.

The testicular testosterone contents of the (−/−) mice were 90–95% reduced, compared with the (+/−) and (+/+) mice. However, they were 2- to 3-fold higher than in all the female groups ($P < 0.05$) (Table 2).

On stereomicroscopic examination, no differences were seen in the external genitals of the male (+/+), (+/−), and (−/−) mice, and, respectively, among the female animals of the different genotypes, including the sex differences observed in anogenital distances (Fig. 4). Likewise, the internal genitals of the different genotypic groups of male and female fetuses did not show any structural alterations, indicating

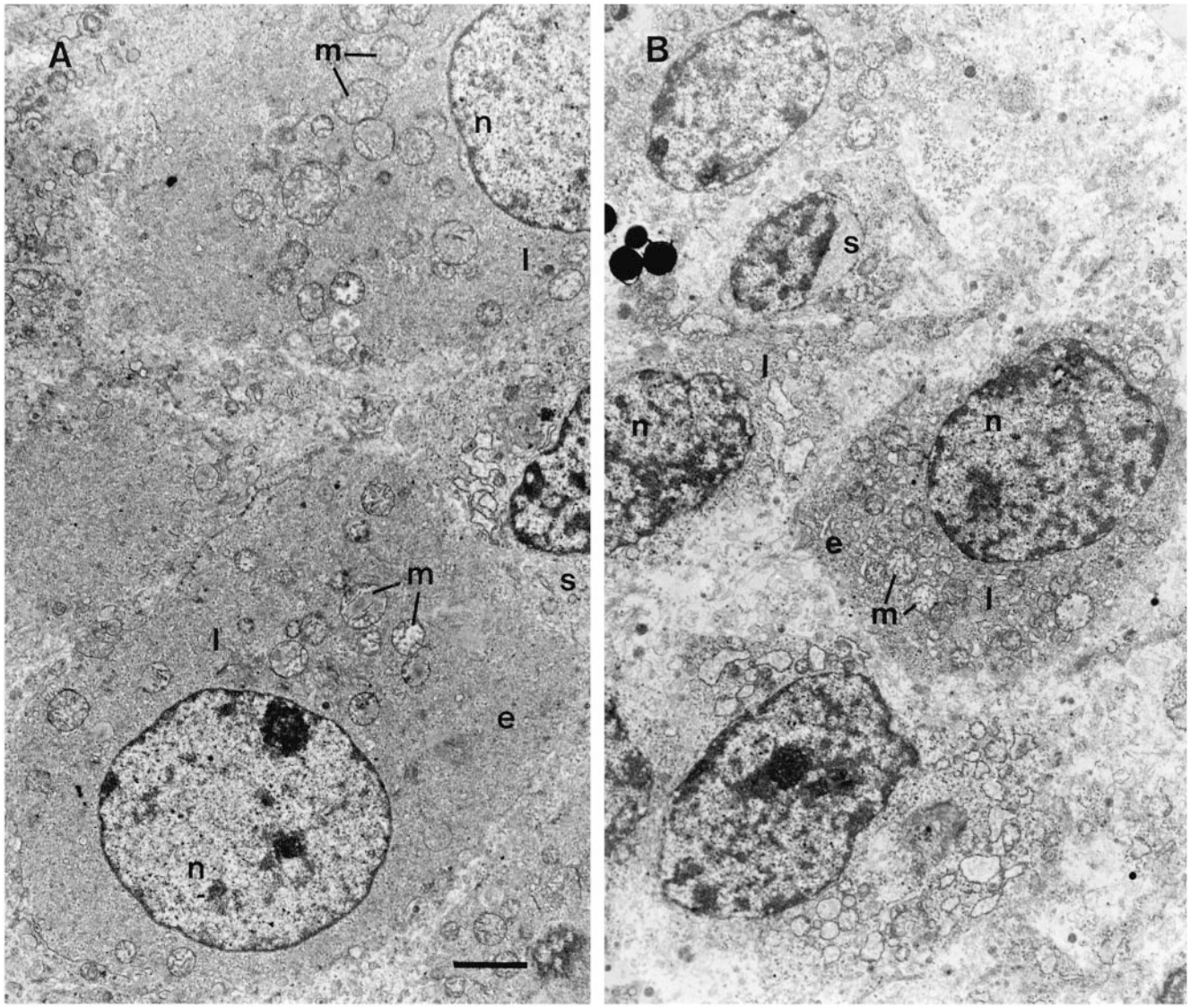

FIG. 2. Survey electron micrographs of testis from a wild-type (A) and a knockout (B) mouse fetus on d E18.5. Leydig cells (l) in the interstitium are surrounded by stromal cells (s). The Leydig cells are large with a spherical nucleus (n), wide cytoplasm filled with smooth endoplasmic reticulum (e), and spherical mitochondria (m). *Scale bar,* 2 μm.

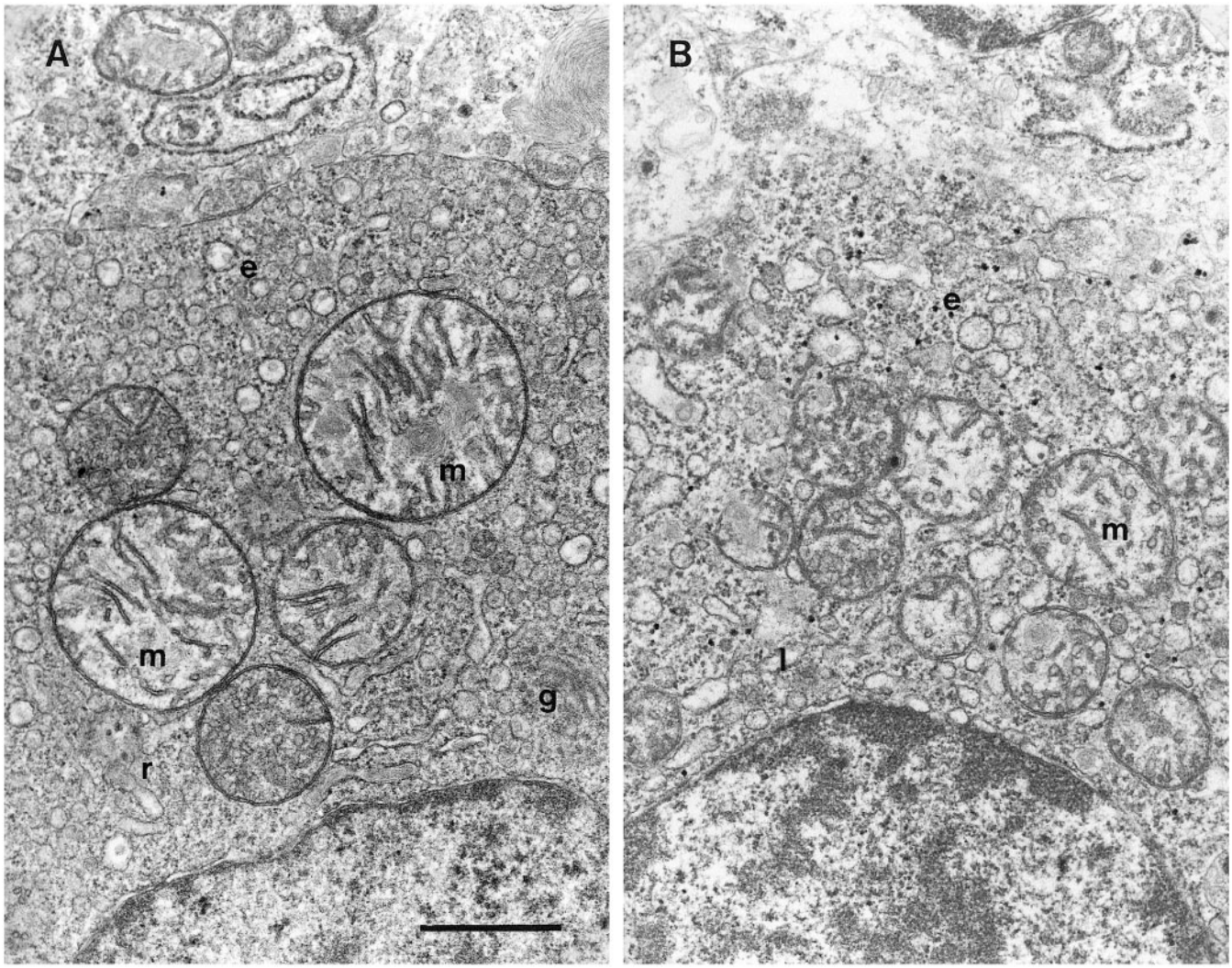

FIG. 3. High-power electron micrographs of testis from a wild-type (A) and a knockout (B) mouse fetus on d E18.5. The Leydig cell cytoplasm is composed of the typical organelles of a steroid-secreting cell: abundant smooth endoplasmic reticulum as small vesicles (e) and large spherical mitochondria (m) with tubular cristae, Golgi complex (g), cisternae of granular endoplasmic reticulum (r), and many free polysomes. *Scale bar,* 1 μm.

TABLE 2. Testosterone concentration of gonads of control (+/+) and *T/ebp/Nkx2.1* heterozygous (+/−) and homozygous (−/−) knockout mice (mean ± SEM)

Genotype	Sex	n	Testosterone (pg/gonad)
+/+	Male	10	234 ± 37.3
	Female	2	2.5 ± 0.5
+/−	Male	14	165 ± 22.5
	Female	9	5.0 ± 2.5
−/−	Male	11	13.5 ± 2.4[a]
	Female	5	4.6 ± 2.7

[a] $P < 0.05$ *vs.* (−/−) females.

that lacking pituitary gland development does not affect genital differentiation in either sex during fetal life. In particular, the location of the testes was verified in the inguinal region on both sides of the urinary bladder, indicating that the transabdominal descent of the testes, regulated by INSL3, had occurred normally. A view of male (+/−) and (−/−) internal genitals is shown in Fig. 5. Likewise, the involution of the Müllerian ducts was similar in all males, irrespective of their genotype (results not shown).

Previous studies of the *T/ebp/Nkx2.1* knockout mice showed no phenotypic effects in (+/−) mice. We therefore did not observe systematically the (+/+) and (+/−) groups for each parameter but collected representative data on either of them and the (−/−) animals. All findings supported the earlier findings on absence of effect of heterozygosity on the phenotype.

Discussion

The finding on intrauterine development of testes and genitals of the *T/ebp/Nkx2.1* (+/+), (+/−), and (−/−) fetal mice allow the following conclusions: 1) The lack of pituitary development documented in the (−/−) mice does not affect either male or female fetal genital differentiation up to the age of E18.5; 2) the production of the three fetal testicular hormones, needed for male-type genital differentiation, AMH, testosterone, and INSL3, is sufficient in the absence of

pituitary gonadotropin stimulation; 3) fetal Leydig cell steroidogenic capacity of the (−/−) animals is decreased in the absence of pituitary hormones, most likely of gonadotropins, because Leydig cell steroid content is suppressed by 90–95% and the Leydig cell size is clearly reduced when measured at the age of E18.5; 4) despite missing tropic stimulation, the fetal Leydig cells show all ultrastructural features of steroidogenic cells; and 5) there appears to be a large safety margin in the need of fetal Leydig cell testosterone production for masculinization because the clearly suppressed levels in the (−/−) males are able to induce normal fetal masculinization. Although we did not monitor the production of the other two fetal testicular hormones, AMH and INSL3, their production in (−/−) mice appeared sufficient because of normal transabdominal descent of the testes and regression of the Müllerian ducts.

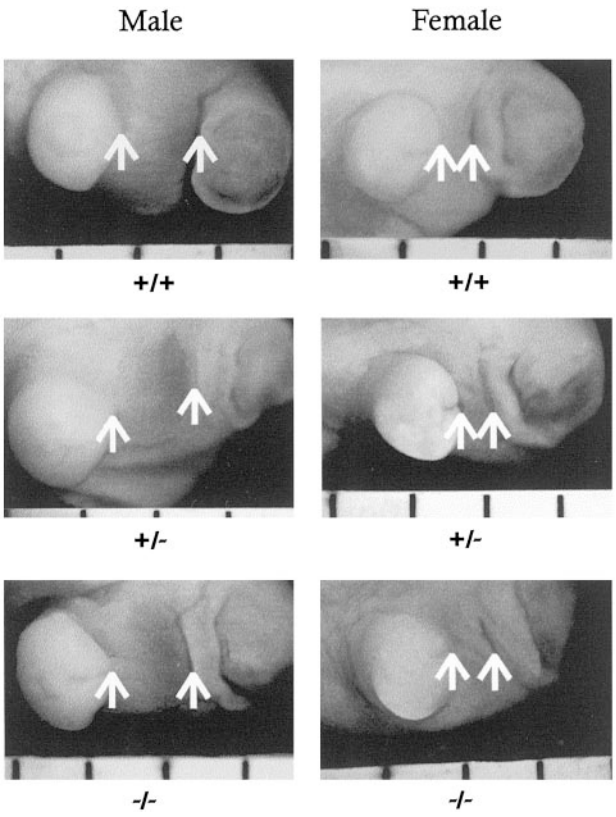

FIG. 4. Stereomicroscopic views demonstrating the anogenital distances of (+/+), (+/−) and (−/−) male *(left)* and female *(right)* on d E18.5. The *left-hand arrows* show the urethral orifices and *right-hand arrows* the anal orifices. The tail *(on the right)* has been removed to improve the view.

The absent phenotype in the female (−/−) mice was expected in light of the documented endocrinological quiescence of the fetal ovary, compared with the testis. Because of this anticipated finding, we concentrated our attention on characterization of phenotype of the male *T/ebp/Nkx2.1* (−/−) mice. The finding that fetal masculinization in the absence of pituitary hormones is normal is supported by a number of other recent findings. Male fetal sexual differentiation is normal in *hpg* mice that are nearly totally devoid of gonadotropins because of inactivating deletion mutation of the gonadotropin-releasing hormone gene (11, 21). However, because these mice have very low but measurable gonadotropin levels (12), they are not a fully convincing model to demonstrate gonadotropin independence of fetal masculinization. Likewise, mice with targeted disruption of the common α-subunit gene, lacking both gonadotropins and TSH, and having reduced levels of growth hormone and PRL, masculinize normally *in utero* (13). Also, mice with disrupted FSHβ or FSH receptor genes show normal masculinization (22–24). The latter finding shows only that the fetal Sertoli cell product AMH is not physiologically dependent on FSH secretion, although both stimulatory and inhibitory effects have been demonstrated (25).

Very recent studies have shown that mice devoid of LH receptors masculinize normally, again proving that fetal testicular testosterone production is sufficient without gonadotropic stimulation (6, 10). The novel contribution of the present study is to show that the pituitary gland altogether is not needed for sufficient fetal Leydig cell stimulation to induce genital masculinization. However, the very low level of testosterone and the small Leydig cell size show that the Leydig cells in the absence of pituitary gland are not as active as in wild-type animals. Because fetal testicular testosterone levels do not differ from those of controls in gonadotropin-deficient *hpg* mice (8), the lack of other pituitary hormones in the *T/ebp/Nkx2.1* null mice may be responsible for the low steroidogenesis. It is known that at least GH, thyroid hormones, PRL, and posterior pituitary hormones have stimulatory effects on Leydig cell steroidogenesis (15, 26, 27), which may provide explanation why Leydig cell function in mice devoid of all pituitary hormones is more severely reduced than in mice only devoid of gonadotropins. The fact that Leydig cells of the *T/ebp/Nkx2.1* (−/−) mice are able to

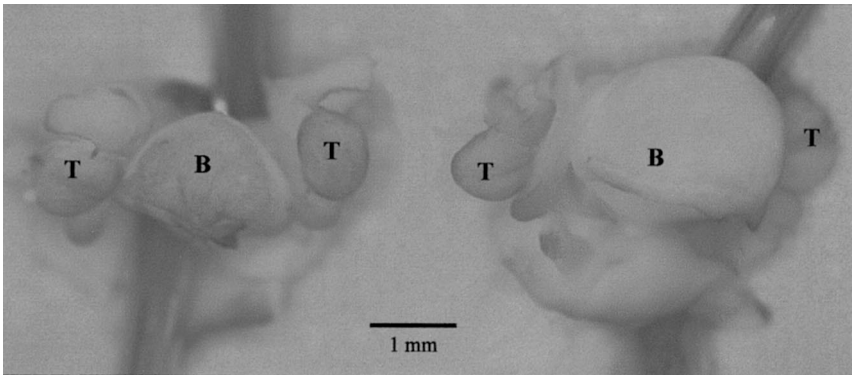

FIG. 5. Internal genitals of a (−/−, *left*) and (+/−, *right*) male mouse at the age of E18.5, showing equal size and position laterally of the testes on each side of the bladder. T, Testis; B, urinary bladder.

produce some testosterone in the total absence of LH was also supported by findings in the LH receptor knockout mice in which low but clearly detectable testosterone concentrations could be found in the testes at all ages studied (Zhang, F. P., M. Poutanen, and I. Huhtaniemi, unpublished data). The residual steroidogenesis may be either constitutive in nature or caused by stimulation by some paracrine or autocrine factors or by nonpituitary-derived humoral factors (15, 28–30).

Finally, it is interesting to compare the current findings in the mouse with the regulation of sexual differentiation in human. Fetal masculinization is clearly dependent in both species on fetal Leydig cell testosterone production. In the mouse, there seems to be a large safety margin in the dose needed because clearly reduced levels are sufficient to cause genital masculinization. Lack of pituitary LH stimulation is not sufficient to block masculinization in the rodent, as shown by the numerous natural and experimental models of suppressed gonadotropin secretion and/or action (see above). In the human, in contrast, complete inactivation of the LH receptor totally blocks male-type sexual differentiation (31). This indicates that LH action is crucial for sufficient testosterone production. If pituitary LH is deficient, as has been demonstrated by one male with inactivating mutation in the LHβ gene (32), the intrauterine phase of sexual differentiation and development are normal. This can be explained by placental human chorionic gonadotropin that can provide the LH-like stimulus for fetal Leydig cells. Admittedly, only one LH-deficient male has so far been identified, and the finding will have to be validated with additional cases. However, if the LH receptor is defective, masculinization does not take place, as has been shown by pseudohermaphroditism of individuals with completely inactivating LH receptor mutations (31). Hence, the safety mechanism in the human (in the form of human chorionic gonadotropin) assures only the supply of gonadotropin stimulation, but LH receptor activation, unlike in rodents, is absolutely necessary for masculinization. It is curious that such a fundamental difference prevails in hormonal regulation of the masculinization process of these two mammalian species. Whether the high physiological concentration of fetal testosterone, which does not seem to be needed for intrauterine masculinization, has some other effects, not identified in the present study, remains an intriguing question.

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