Effects of an *Igf1* Gene Null Mutation on Mouse Reproduction

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Both sexes of adult mice homozygous for a targeted mutation of the *Igf1* gene, encoding insulin-like growth factor I, are infertile dwarfs (~30% of normal size). The testes are reduced in size less than expected from the degree of dwarfism but sustain spermatogenesis only at 18% of the normal level. The epididymides are overall nearly allometric to the reduced body weight, but the distal regions of the duct, vas deferens, seminal vesicles, and prostate are vestigial. Despite the mutational impact on the epididymis, capacitated sperm are able to fertilize wild type eggs *in vitro*. It is hypothesized that the infertility of male mutants is caused by failure of androgenization resulting in absence of mating behavior, due to drastically reduced levels of serum testosterone (18% of normal). This hormonal deficiency was correlated with an ultrastructural analysis of mutant Leydig cells revealing a significant developmental delay, while assays in organ culture showed that the basal and LH-stimulated production of testosterone by testicular parenchyma is reduced in comparison with wild type controls. The female mutants fail to ovulate even after administration of gonadotropins, which is apparently the primary cause of their infertility, and possess an infantile uterus that exhibits a dramatic hypoplasia especially in the myometrium. The phenotypic manifestations of the mutation were correlated with the localization of transcripts for insulin-like growth factor I and its cognate receptor in wild type reproductive tissues by *in situ* hybridization. (Molecular Endocrinology 10: 903–918, 1996)

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INTRODUCTION

The insulin-like growth factors, IGF-I and IGF-II, are small polypeptides with structural homology to proinsulin that are produced by many tissues and function as autocrine/paracrine signals (for reviews see Refs. 1–3). They may also act as classical hormones, since they circulate in the plasma associated with cognate binding proteins. The signaling of both of these ligands is mediated by the type-1 IGF receptor (IGF1R), while a second receptor (type-2; IGF2R) serves for IGF-II turnover.

The *in vivo* growth-promoting role of the IGFs has been demonstrated conclusively from the dwarfing phenotypes observed after targeted mutagenesis of the mouse *Igf1*, *Igf2*, and *Igf1r* genes [(4–7); reviewed in Ref. 8]. The phenotypes manifested by these null mutations, alone or in combination, were compared in terms of embryonic growth kinetics, developmental delays in particular tissues, severity of growth deficiency, and survival after birth. The onset of mutational effects and the *in vivo* ligand/receptor interactions were defined from these comparisons, which indicated that IGF-I functions exclusively through IGF1R, while IGF-II utilizes an additional, unknown receptor (XR). At present, XR has been identified only genetically but appears to be distinct from IGF2R (7). Studies with embryonic fibroblasts from the mutants lacking IGF1R showed that the cell cycle is 2.5-fold longer than normal (9), indicating that this signaling system influences the most important growth determinant: the rate of cellular divisions that increase total cell number.

In contrast to IGF-II, which has a predominantly embryonic role in rodents, IGF-I functions both during embryonic and postnatal development (6, 7). Thus, the viable and fertile *Igf2* null mutants exhibit dwarfism at birth but grow postnatally at a normal rate (4), whereas the postnatal growth rate of *Igf1* null mutants, which

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survive variably depending on genetic background, is reduced (7). The latter mutants exhibit a birth weight that is 60% of normal, but their size becomes only 30% that of their wild type littermates at 8 weeks of age and plateaus at that level thereafter. As expected from the well established postnatal role of IGF-I, which acts as a mediator for many of the growth-promoting effects of GH (see, *e.g.* Ref. 10), analysis of long bone development in the mutants has revealed significant delays in ossification, including the closure of the growth plate (Ref. 7 and our unpublished results).

Here we report that the *Igf1* null mutation has also a dramatic impact on the development and physiology of the reproductive systems of both sexes. These results provide the first direct evidence, in support of previous suggestions (see *Discussion*), that the IGF system plays an important role in mammalian reproduction.

RESULTS

Igf1 Null Mutants Are Infertile

Both sexes of *Igf1* nullizygous mutants reaching adulthood lack sex drive and are infertile in general. The males do not exhibit aggressive behavior when caged with other males, while behavioral signs of estrus were never observed in females. Many attempted matings between nullizygous mutants or between such mutants of either sex and wild type partners failed to produce offspring, with one exception. After caging each of nine *Igf1* null males with two wild type females over a 1-yr period, only a single litter of progeny was obtained. Genotyping showed that all nine offspring of this litter were heterozygous for the mutation, demonstrating that they were indeed sired by an *Igf1* nullizygous male (data not shown).

Reproductive Phenotype of Male *Igf1* Null Mutants

Morphological examination of the reproductive organs of male mutants revealed that they were all significantly smaller than in wild type littermates (Fig. 1A), but their reduction in weight was not uniformly proportionate to the reduction in body size (28% of wild type; Table 1). The testicular weight was reduced less than expected (41% of the wild type value); the total epididymal weight was nearly allometric (23% of control); and the vas deferens, seminal vesicles, and prostate were disproportionately small (Table 1).

Histological examination of the testis (Fig. 1, D and E) showed that the seminiferous epithelium contained Sertoli cells and germ cells at all stages of differentiation and in apparently normal cellular associations, including spermatogonia, primary spermatocytes in meiotic prophase, and spermatids undergoing acrosome formation, nuclear condensation, and tail assembly.

The Leydig cells of the mutants appeared to be condensed and clustered into small groups (Fig. 2B). Based on stereological data, the volume of these cells was about 50% of normal, while their number was reduced to a nearly expected level (33% of normal; Table 1). Examination of Leydig cells in the mutants by electron microscopy revealed that they were delayed in their development. Thus, when Leydig cells of 4-month old null mutants were examined, we observed that the numerous cytoplasmic lipid inclusions were not surrounded by abundant concentric membranous whorls of smooth endoplasmic reticulum (Fig. 2D), which are characteristic of wild type cells involved vigorously in steroid synthesis (Fig. 2C). Moreover, the mutant cells exhibited glycogen particles scattered throughout their cytoplasm, which is a typical feature of undifferentiated cells, while the characteristic intramitochondrial granules of mature wild type cells were absent (*cf.* Fig. 2, C and D). These observations suggested that, despite the advanced age of the examined mutant animals, their Leydig cells had only reached a stage of differentiation corresponding to days 10–14 of postnatal development (11–13). Clearly, a study of Leydig cells at several postnatal ages is required, to examine the progress of their development in mutant mice in detail. Nevertheless, our results are probably indicative of a delay in differentiation; additional electron microscopic analysis of mutant Leydig cells at 2 weeks after birth showed that the interstitial space contained predominantly undifferentiated mesenchymal cells, while fetal and immature Leydig cells were present in wild type animals of the same age (data not shown).

Our tentative conclusion about the slow rate of differentiation of the male steroidogenic compartment is consistent with comparative measurements of serum testosterone, which indicated that the concentration of this androgen in the mutants is 18% of the normal level (Table 1). Moreover, measurements of basal and LH-stimulated testosterone production by testicular cells in organ culture indicated that the amount of testosterone secreted over a period of 3 h, and normalized per weight of incubated tissue, was for the mutants 62% and 28%, respectively, of the values detected in wild type controls (Table 2). In contrast, the corresponding values for androstenedione production under the same assay conditions were 8- and 6-fold higher than in the controls (Table 2). This indicates further that the Leydig cells in the mutants had not yet differentiated fully (see *Discussion*).

Despite the overall nearly allometric weight of the epididymis in the mutants, its reduction in size was not uniform for the entire duct. Thus, the caput segment (27% of normal) appeared proportionate to body weight, whereas the corpus and the cauda were affected severely by the mutation (each about 15% of normal; Table 1). Moreover, we observed that the cauda lacked the numerous ductal convolutions that are characteristic of this region in wild type mice. Nevertheless, abnormalities in the differentiated cells

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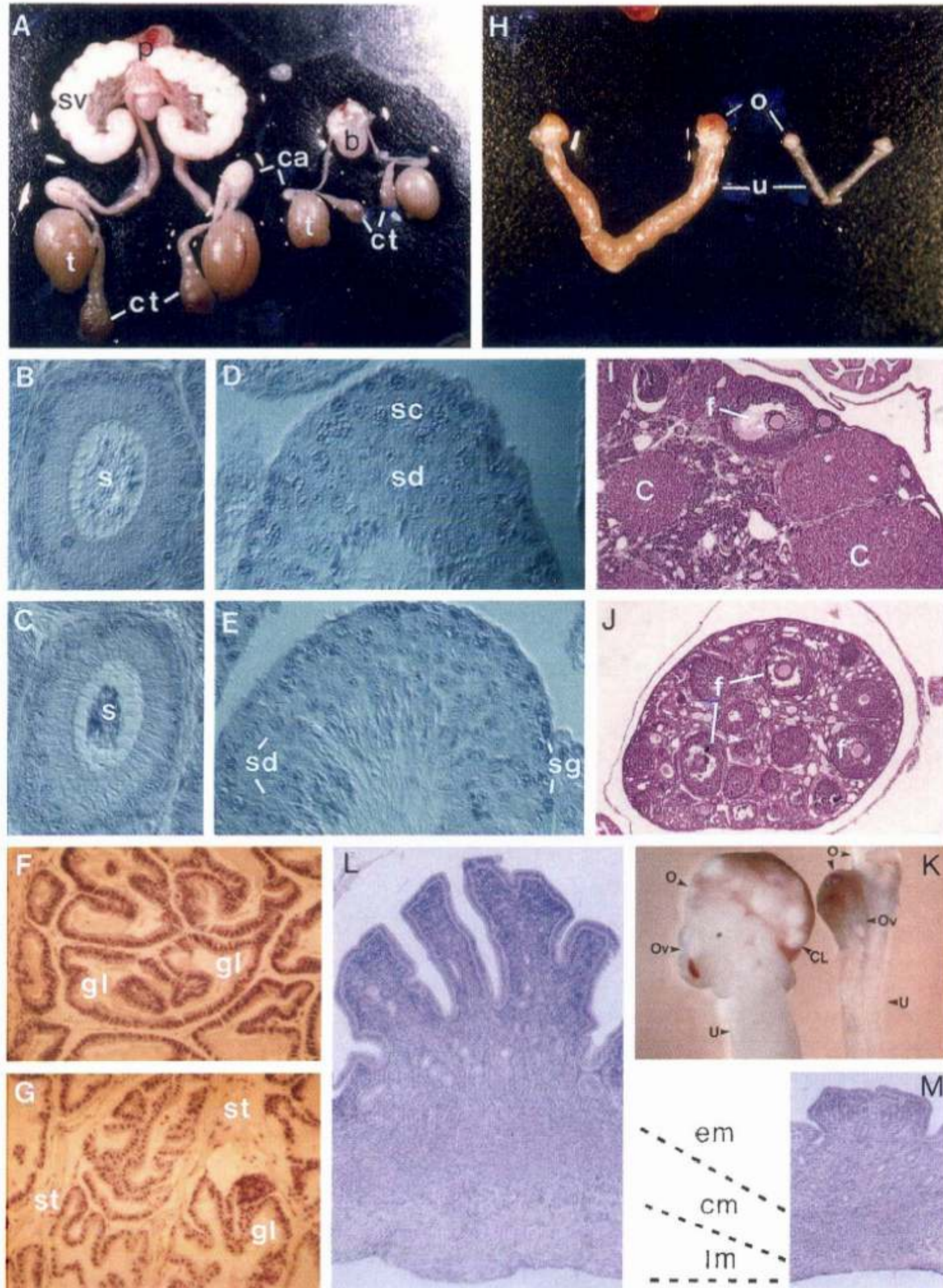

Fig. 1. Comparison of Male and Female Reproductive Systems of Adult Wild Type and *Igf1* Null Mice

A, Male reproductive tracts from wild type (*left*) and mutant (*right*) animals. The testes (t), caput (ct), and cauda (ca) of the epididymis, prostate (p), bladder (b), and seminal vesicles (sv) are indicated. B and C, Transverse sections through the proximal epididymis of wild type (panel B) and mutant (panel C) mice. The lumen of the duct is filled with sperm (s) in both cases. D and E, Transverse sections of the tests of wild type (panel D) and mutant (panel E) mice. Spermatogonia (sg), spermatids (sd), and spermatocytes (sc) are indicated. F and G, Sections of seminal vesicles from wild type (panel F) and mutant (panel G) animals. The glands (gl) and fibromuscular stroma (st) are underdeveloped in the mutant. H, Female reproductive systems from wild type (*left*) and mutant (*right*) animals. The ovary (o) and uterus (u) are indicated. The histological section of a wild type ovary (panel I) shows the presence of antral follicles (f) and corpora lutea (c), while only the former are present in a mutant ovary (panel J). K, Comparison of wild type (*left*) and mutant (*right*) ovaries from 3-month old littermates after administration of PMSG and hCG to induce superovulation. The organs were dissected 12.5 h after injection of hCG. Corpora lutea (CL) indicating the occurrence of ovulation are evident only on the surface of the wild type ovary. The oviduct is indicated as Ov. L and M, Uterine sections of wild type (panel L) and mutant (panel M) females. In the mutant, the glandular elements of the endometrium (em) are less complex, while the myometrial hypoplasia is more severe in the longitudinal (outer) layer of smooth muscle (lm) than in the circular (inner) layer (cm).

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Table 1. Weights of Reproductive Organs and Testicular Parameters in Male Wild Type and *Igf1* Null Mutant Mice

	Mutant	n_1	Wild type	n_2	Mutant/wild type (% of normal)
Body weight (g)	9.9 ± 0.8	8	35.6 ± 2.0	11	27.8
Testis (mg)	46.7 ± 3.3	12	114.9 ± 6.5	16	40.6
Epididymis (mg)	10.6 ± 1.0	10	46.6 ± 3.5	14	22.7
Caput	4.4 ± 0.3	4	16.5 ± 1.6	5	26.7
Corpus	1.4 ± 0.2	4	8.9 ± 1.0	5	15.7
Cauda	1.7 ± 0.6	4	11.3 ± 0.5	5	15.0
Vas deferens (mg)	1.2 ± 0.1	4	11.8 ± 0.6	5	10.2
Seminal vesicle (mg)	13.5 ± 2.7	13	135.3 ± 10.4	17	10.0
Prostate (mg)	3.8 ± 0.7	6	28.8 ± 3.5	11	13.2
Testicular sperm ($\times 10^{-6}$)	4.6 ± 0.5	6	26.0 ± 3.1	10	17.7
Epididymal sperm ($\times 10^{-6}$)	4.0 ± 0.9	6	23.3 ± 1.9	10	17.2
Caput	1.3 ± 0.5	4	2.4 ± 0.3	5	54.2
Corpus	1.4 ± 0.4	4	6.0 ± 1.0	5	23.3
Cauda	1.4 ± 0.5	4	16 ± 1.8	5	8.8
Vas	0.3 ± 0.07	4	5.7 ± 1.2	5	5.3
Sperm motility (%)	45.8 ± 6.9	4	41.8 ± 5.4	4	[a]
Leydig cells:					
Total cell number ($\times 10^{-6}$)	0.65 ± 0.22	4	2.0 ± 0.43	4	32.5
Volume per cell ($\mu m^3 \times 10^3$)	0.92 ± 0.10	4	1.86 ± 0.58	4	49.5
Serum testosterone (ng/ml)	0.60 ± 0.17	11	3.3 ± 0.60	13	18.2

The numbers of mutant and wild type animals examined are indicated in columns n_1 and n_2, respectively. For each animal, the wet weights of paired organs were averaged, and this single value was used to calculate means ± SE. To avoid loss of sperm for measurements from the same specimens, the vas deferens was dissected together with the epididymis, and its weight is included in the value of total epididymal weight. The age of male animals ranged between 3 and 14 months. Thus, the various values were averaged, since the growth of all reproductive organs ceases at about 2 months of age, and their weights do not change significantly thereafter (100–103).

[a] Statistical analysis using Student's t test ($P < 0.05$) did not show a significant difference for sperm motility (using sperm recovered from the cauda epididymis) between the means of the wild type and mutant samples.

comprising the epididymal epithelium were not evident by light microscopic examination (*cf.* Fig. 1, B and C). The size of the vas deferens was also dramatically reduced (~10% of normal; Table 1).

In the mutants, the total number of sperm in the testis and epididymis were significantly lower than in wild type controls (only ~17%-18% on average; Table 1). However, the sperm content, expressed as a percentage of the corresponding normal values, exhibited a gradient in the four segments of the excurrent ducts, being highest in the caput epididymis and lowest in the vas deferens. Thus, there was an apparent correlation between sperm content and the sizes of the epididymal segments that were differentially compromised. Nevertheless, sperm recovered from the cauda epididymis of the mutants were normal in morphology and motility (Table 1) and apparently functional; after capacitation, they were able to fertilize wild type eggs *in vitro*, producing apparently normal zygotes that developed successfully into two-cell embryos (see Table 4A).

In addition to the vas deferens, the most dramatic reduction in the size of accessory reproductive organs was observed in the seminal vesicles and the prostate (~10% and 13% of the normal sizes, respectively; Table 1). In most mutant animals, the seminal vesicles were vestigial buds, and their identification at autopsy was occasionally difficult. Histologically, these infantile glands consisted of very small and simple glandular saccules, which were lined with apparently normal epithelium, but exhibited poorly developed fibromuscular lamina propria and had only a limited deposition of secretory protein in the lumen (*cf.* Fig. 1, F and G). Similar observations were made for the prostate of the mutants, which, if visible, was generally very hypoplastic and exhibited a marked reduction in the size and complexity of the tubuloalveolar glands, although the epithelium appeared to be cytologically normal.

Reproductive Phenotype of Female *Igf1* Null Mutants

The ovaries of female mutants, ranging in age between 3 weeks and 4.5 months, were approximately 25% of the normal weight (Table 3), and, thus, almost commensurate to the reduced body size. On histological examination, these ovaries contained primordial, primary, and secondary antral follicles, but only rare and small preovulatory Graafian follicles (Fig. 1J). Extensive comparative measurements of oocyte and follicular diameters in histological sections of mutant and control ovaries indicated that these sizes were some-

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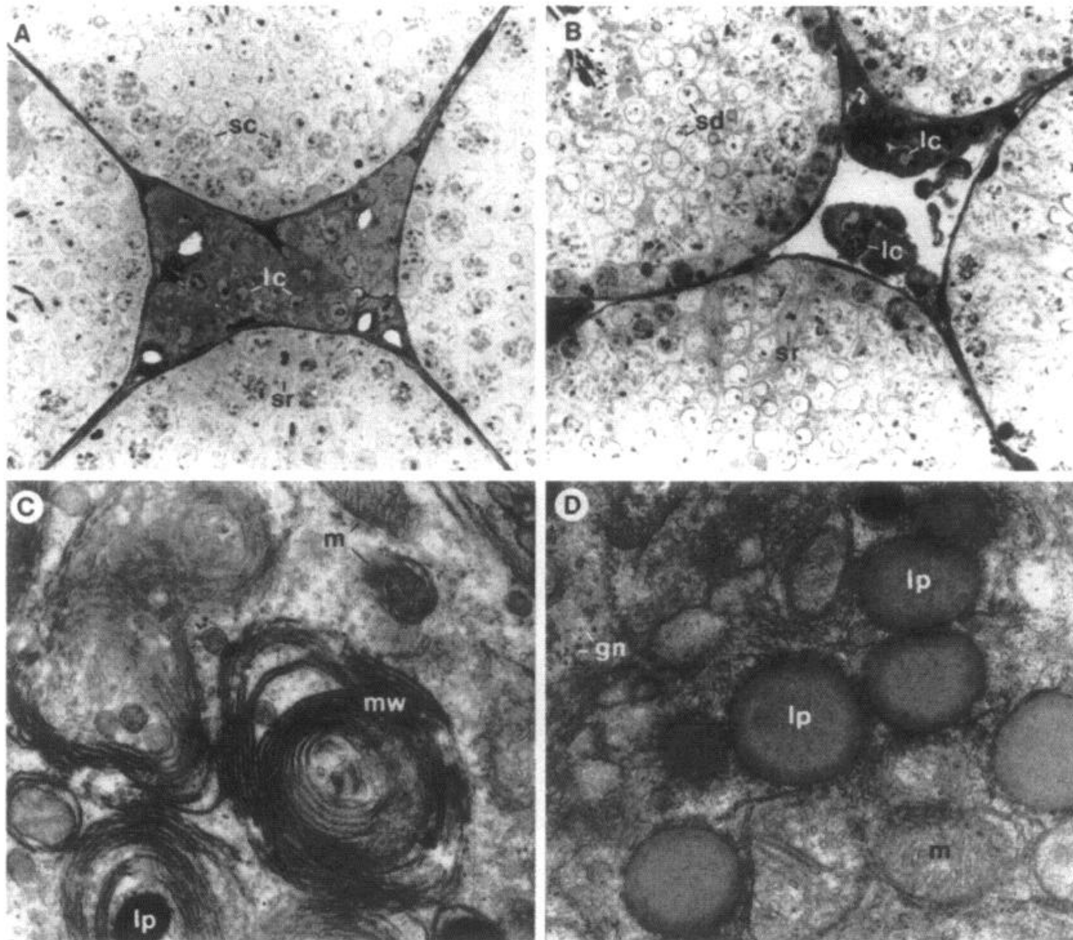

Fig. 2. Light (Panels A and B) and Electron (Panels C and D) Micrographs of Leydig Cells from Wild Type (Panels A and C) and Mutant (Panels B and D) 4-Month Old Littermates

In A and B, the Leydig cells (lc) of the interstitium are surrounded by seminiferous epithelium. Sertoli cells (sr), spermatids (sd), and spermatocytes (sc) are indicated. In the mutant (panel B), the Leydig cells are smaller and clustered, while a lymphatic duct occupies part of the interstitial space. Comparison of the Leydig cell ultrastructure in panels C and D shows that the membranous whorls (mw) of smooth endoplasmic reticulum surrounding lipid droplets (lp) in wild type animals, which are indicative of active steroidogenesis, are absent from the mutants. In contrast, the mutant cells contain glycogen granules (gn) that are characteristic of an undifferentiated state. Mitochondria (m) are also shown.

what smaller for the primordial follicles of the mutants (~78% of normal; Table 3). Although this difference is statistically significant, its biological importance, if any, is obscure because at later stages of folliculogenesis the sizes of primary and secondary oocytes and follicles of the mutants were similar or indistinguishable from wild type (Table 3). Interestingly, the number of follicles per mm^2 was 2.4-fold higher in the mutants. This crowding of follicles is consistent with the interpretation that their total number is not reduced as a consequence of the mutation. Under the assumption that the ovary is a spherical organ, the surface area of any section would be 40% of normal in the mutants, given that the corresponding ovarian weight (and, therefore, volume) is 25% of normal (see legend to Table 3 for details of this calculation). Thus, the mutant to wild type ratio of the number of follicles should not differ from unity (2.4 ratio of follicles/mm^2 $\times$ 0.4 ratio of surface areas = 0.96).

In some cases, the granulosa cells of antral follicles exhibited highly condensed, basophilic chromatin, nuclear blebbing, and dehiscence, typifying early atresia. However, a similar level of atresia was also noted in wild type animals (Table 3). Currently, the morphological observations on mutant ovaries cannot be correlated with function. Nevertheless, it appears that ovarian steroidogenesis was affected by the absence of IGF-I, since the serum concentration of estradiol was reduced to about 53% of the normal value in female mutants (Table 3). This level seems to be still quite high, but the potential contribution of extraovarian sources to serum estradiol is unknown. The thecal cells surrounding antral follicles appeared to be cytologically normal, but distinct external and internal layers were not discernible.

The mutant ovaries did not contain corpora lutea or corpora albicantia, indicating that ovulation never occurred in mutant females. Attempts to induce ovula-

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Table 2. Androgen Production by Testicular Parenchyma *in Vitro*

	Mutant	Wild type	Mutant/wild type (% of normal)
	(ng/mg parenchyma · 3 h)		
A. Testosterone			
Basal	0.92 ± 0.55 (4)	1.48 ± 0.63 (5)	62.2
LH-stimulated	1.56 ± 0.55 (4)	5.60 ± 0.93 (5)	27.9
Fold stimulation	1.7	3.8	44.7
B. Androstenedione			
Basal	0.56 ± 0.18 (8)	0.07 ± 0.02 (10)	800.0
LH-stimulated	1.73 ± 0.63 (8)	0.29 ± 0.10 (10)	596.6
Fold stimulation	3.0	4.1	73.2

For details see *Materials and Methods*.

tion in the mutants by standard injections of gonadotropins (PMSG and human CG) were unsuccessful. While 33 eggs were recovered on average from each of the wild type littermates after hormonal stimulation, no eggs were found in the oviducts of the mutant females in six independent experiments (Table 4B). Moreover, when the ovaries of the injected mutants were examined macroscopically (Fig. 1K) or histologically, no corpora lutea were detected, although a significant number of large Graafian follicles were observed in the sections, in contrast with unstimulated mutant animals (not shown). When nine mutant females received a 10-fold higher dose of human CG (hCG), 12 ova were recovered, but they all had abnormal morphology and were incompetent for fertilization (only one of them was able to bind capacitated wild type sperm).

No attempt to weigh the oviducts of female mutants was made because their extremely small size precluded accurate measurements. However, the cytological features of the ducts did not differ from wild type (not shown). The infantile uterus (Fig. 1, H and K), which did not exceed 13% of the normal weight in the mutants (Table 3), was thin and flaccid. Although the endometrium was lined with differentiated columnar epithelial cells, the abundance and complexity of the secretory glandular elements were significantly reduced, while the myometrium exhibited a dramatic hypoplasia; the outer longitudinal layer in particular was limited to only a few layers of smooth muscle cells (Fig. 1M; compare with Fig. 1L).

Igf1 and *Igf1r* Gene Expression in Reproductive Tissues

Details about the pattern of expression of genes encoding members of the family of IGF ligands and receptors in the cellular constituents of mouse reproductive tissues have not been reported previously. Thus, to correlate the phenotypic manifestations of the *Igf1* null mutation with the localization of transcripts, we performed *in situ* hybridization analyses, to assess the expression of the *Igf1* and *Igf1r* genes in the testis, epididymis, ovary, and uterus of wild type mice.

In the testis of prepubertal mice (postnatal day 14; p14), moderately abundant *Igf1* transcripts were localized in the interstitial compartment, but no hybridization signal was detected in the seminiferous epithelium (Fig. 3, A-C). The diffuseness of the signal in the interstitium did not allow us to conclude whether immature Leydig cells were included among the positive components, although this is likely. Interestingly, we observed a significant change in the cellular pattern of *Igf1* gene expression, occurring sometime after p14. Thus, in older animals (p35), *Igf1* transcripts were detected in the seminiferous epithelium but not in interstitial cells (Fig. 3, D–F). However, a strong hybridization signal was evident only in spermatids and not in spermatogonia or primary spermatocytes (Fig. 1F). Whether a positive signal was also present in Sertoli cells is uncertain. In contrast to the results obtained for *Igf1* transcripts, the sites of *Igf1r* gene expression did not appear to change with developmental age. Thus, at both examined ages (p14 and p35), strong *Igf1r* expression was evident in Leydig and peritubular interstitial cells, and occasionally in spermatogonia, while the hybridization signal in other cells of the seminiferous epithelium did not exceed background levels (Fig. 3, G–I).

At p14, *Igf1* transcripts were absent from the epithelial elements of the epididymis, but a moderately intense hybridization signal was detected in myofibroblastic cells surrounding the duct (Fig. 3A). At p35, however, expression was diminished to barely detectable levels, and only a few scattered mesenchymal cells that were positive for hybridization could be observed (not shown). Regardless of age, *Igf1r* mRNA was detected in the epithelium of the duct, with highest levels concentrated in a region of the caput, as compared with the corpus and the cauda (Fig. 3, J–L).

In the ovary, the patterns of *Igf1* and *Igf1r* gene expression occurring in granulosa cells did not differ from those previously described in the rat (14, 15). Thus, *Igf1* transcripts were observed exclusively in a subset of follicles, while all follicles exhibited a positive signal for *Igf1r* expression (Fig. 4, A–C). The hybridization signal for *Igf1r* transcripts in the thecal-interstitial cells was extremely weak. Parallel *in situ* hybridization

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Table 3. Weights of Reproductive Organs and Ovarian Parameters in Female Wild Type and *Igf1* Null Mutant Mice

		Mutant	n_1	Wild type	n_2	Mutant/wild type (% of normal)
Uterus (mg)						
Age (months):	0.75.8	1	14.3	1	12.6	
	2.0	3.0	1	35.1	1	8.6
	2.5	8.7	1	56.3	2	15.5
	4.0	8.3	1	57.3	1	14.5
	4.5	12.2	1	78.5	1	15.5
						Average 13.3 ± 1.3
Ovary (mg)						
Age (months):	0.75	1.7	1	5.7	1	29.8
	2.0	1.8	1	7.2	1	25.0
	2.5	2.1	1	9.4	2	22.3
	4.0	2.4	1	11.0	1	21.8
	4.5	3.4	1	12.1	1	28.1
						Average: 25.4 ± 1.6
Follicles (diameter; μm)						
Primordial		20.06 ± 0.22	379	25.85 ± 0.30	340	77.6
Primary		52.17 ± 2.07	145	49.44 ± 1.15	195	105.5
Secondary		147.26 ± 6.42	98	154.27 ± 4.80	178	95.5
Graafian		282.00	1	345.00 ± 17.4	20	
Follicles/mm^2		3.35 ± 0.25		1.38 ± 0.06		2.4
Atretic follicles (%)		22.1%	54	17.8%	70	
Oocytes (diameter; μm)						
in primordial follicles		14.74 ± 0.12	379	18.77 ± 0.23	340	78.5
in primary follicles		31.18 ± 1.31	145	31.67 ± 0.76	195	98.5
in secondary follicles		60.70 ± 0.97	98	71.06 ± 1.00	178	85.4
in Graafian follicles		75.00	1	87.67 ± 1.84	20	
Serum estradiol (ng/ml)		0.03 ± 0.006	3	0.057 ± 0.016	3	52.6

The growth of the ovary reaches an apparent plateau between 3 and 6 months of age (104, 105), whereas the uterus continues to grow for at least 7 months (106) or considerably longer in some mouse strains (107, 108). Thus, the corresponding measurements are presented individually, and then the means ± SE of mutant/wild type ratios are shown. The uterine weight corresponds to the entire organ (both horns). The numbers of mutant and wild type animals examined are indicated in columns n_1 and n_2. The numbers of follicles and oocytes examined are shown in the same columns. The latter data represent averages from three animals of each category. Statistical analysis using Student's *t* test ($P < 0.05$) showed a significant difference in the means of follicular and oocyte diameters between control and mutant samples only for primordial follicles. The number of follicles per mm^2 was calculated after counting of oocytes and measuring the surface area of 166 and 255 ovarian sections from mutant and wild type animals, respectively. Assuming that the ovary is a sphere with radius R and volume $4.189R^3$, it can be calculated from the known mutant (m) to wild type (w) ratio of ovarian weights (volumes) of 0.254 that $R_m = 0.633R_w$ (0.633 is the cubic root of 0.254). Therefore, the ratio of the surface area ($3.14R^2$) of a circular section between the mutant and the wild type will be 0.4 (0.633^2). Accordingly, the number of follicles in such a section should be the same in mutants and controls (2.4 ratio of mutant/wild type follicles/mm^2 × 0.4 ratio of areas = 0.96).

analysis of mutant ovaries indicated that the pattern of *Igf1r* gene expression was indistinguishable from wild type (data not shown).

In the uterus of cycling mice, *Igf1* transcripts were detected in endometrial stromal cells but not in the glandular epithelium (Fig. 4, F and G). Positive hybridization signal was also observed in the myometrium, especially in the outer longitudinal layer (Fig. 4E). The distribution of *Igf1r* transcripts was more extensive, and their abundance was significantly higher in the glandular elements and also in the epithelium lining the uterine lumen (Fig. 4I). Similarly, in the oviducts, widely expressed *Igf1r* transcripts were detected, more abundantly expressed in the lining epithelium, while *Igf1* mRNAs were concentrated in the muscular layers (data not shown).

DISCUSSION

A large number of studies have previously suggested that the IGF system is involved in mammalian reproductive functions (for reviews see Refs. 16–20). This general conclusion is now firmly supported by the direct evidence derived from our genetic analysis, which can establish causal relationships based on mutational consequences in the context of the whole experimental animal.

Currently, our data are consistent with the working hypothesis that the absence of IGF-I results primarily in impairment of gonadal steroidogenesis. However, mechanistic aspects of the role(s) of this ligand in reproduction have not yet been addressed experimentally. Moreover, it is unwarranted at present to extrap-

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Table 4. Comparisons of Gamete Physiology between Wild Type and *Igf1* Null Mutant Mice

A. *In vitro* fertilization	Wild type ova	Two-cell embryos	Embryos/ova (%)
Wild type sperm	13	10	76.9
Sperm of mutants			
Exp 1a	24	19	79.2
1b	10	6	60.0
2	45	35	77.8
3	18	8	44.4

B. Superovulation	Wild type females	Mutant females
Average number of ova recovered per animal	33 ± 4 (6)	0 (6)

Each *in vitro* fertilization experiment was performed with sperm from a single animal, except for Exp 1a and 1b (sperm of the same mutant was used). The numbers of female animals injected with gonadotropins are shown in *parentheses*.

olate observations with mice indiscriminantly to other mammals, since the mechanistic details of the IGF-I action in developmental or physiological aspects of reproduction may differ between species. This possibility has been suggested by differences in the expression patterns of *Igf1* and *Igf1r* mRNAs, detected by *in situ* hybridization, which reflect variations at the sites of local synthesis of the cognate proteins, under the reasonable assumption that these messages are translatable. For example, in contrast to rodents, the *Igf1* gene is not expressed in the human testis (21). In addition, there are differences in the localization of *Igf1r* transcripts. Thus, in rodents, expression at high levels is confined to Leydig and peritubular cells and some spermatogonia, whereas in humans the Leydig cell expression is low, and *Igf1r* transcripts are detected predominantly in Sertoli cells and spermatocytes (21). Unexpectedly, some differences also exist between rats and mice (this paper and C. Bondy, and J. Zhou, unpublished results). Thus, in contrast to mice, the *Igf1* gene is not expressed in rat spermatids, although it is expressed in Leydig cells in both rodent species. Moreover, in mice, but not in rats, expression of the *Igf1* gene in Leydig cells depends on postnatal age, and is apparently extinct in older animals. In regard to the female reproductive system, differences between rodents have not been detected. It is also notable that the ovarian *Igf1r* gene expression is essentially the same in rodents, monkeys, and humans (Refs. 14, 15, 22, and 23 and C. Bondy and J. Zhou, unpublished data). On the other hand, the ovarian expression of *Igf1* differs between species. Thus, *Igf1* mRNA is not detected in human granulosa cells, whereas the *Igf2* gene is transcribed in these cells, but only in mature follicles (22).

These differences in mRNA expression patterns among species imply that some temporal and/or spatial variations in the IGF ligand/receptor relationships have apparently emerged during mammalian evolution, although the extent of their potential differential effects on reproductive physiology remains to be seen. Nevertheless, to avoid a misleading discussion, we have decided to restrict its content to rodents, focusing on mice and using information pertaining to rats with caution. Moreover, of previously reported experiments, we chose to discuss primarily *in vivo* results and refer to *in vitro* data only selectively. Despite the unquestionable significance of many observations with primary cultures of Leydig, Sertoli, granulosa, and thecal cells, direct comparisons with the data derived from our mutational analyses are difficult and may not lead to reliable interpretations. For example, while effects of IGF-I on cultured rat Sertoli cells have been described (reviewed in Ref. 24), the mouse *Igf1* null mutation has no apparent impact on Sertoli cells, which is consistent with the lack of *Igf1r* expression in this testicular compartment. Similarly, there are several reports on the consequences of addition of IGF-I to cultures of rat thecal cells (reviewed in Ref. 25), which cannot be easily interpreted without invoking some influence of the *in vitro* conditions, since the corresponding *Igf1r* expression in the ovarian interstitial compartment is barely detectable in intact rats and mice and may not really exceed background levels. Finally, it is notable that when *in vivo* and *in vitro* experiments were performed, to examine the role of IGF-I in granulosa cell physiology, conflicting results were obtained that could not be interpreted in a straightforward manner (26, 27).

IGF-I and Male Reproductive Functions

Formal evidence that points unequivocally to the cause of the general infertility of male *Igf1* null mutants is currently lacking, since sperm recovered from such animals are functional *in vitro*, and an exceptional case of productive natural mating has been recorded. In principle, the drastic reduction of sperm number in the cauda epididymis and the vas deferens of the mutants could be a contributing factor, but its significance cannot be easily assessed, since the vast majority of males that were caged with wild type females did not exhibit mating behavior. Thus, we speculate that a likely explanation for the infertility of *Igf1* null males is predominantly the absence of sex drive, which is apparently due to inadequate serum testosterone levels, according to the following considerations.

It has been firmly established that, during a crucial period spanning the first 5 postnatal days, the presence of testosterone is absolutely necessary for masculinization (for reviews see Refs. 28–30). This hormone is aromatized and converted into estrogen in the brain, where the latter steroid promotes the sexual differentiation of hypothalamic nuclei that are involved in mating and aggressive behavior. In this regard, the hypogonadal (*hpg*) mouse mutant (31) provides an interesting paradigm. The mutational defect in *hpg* mice is a 33.5-kb deletion encompassing the 3′-portion (two of four exons) of the gene encoding the

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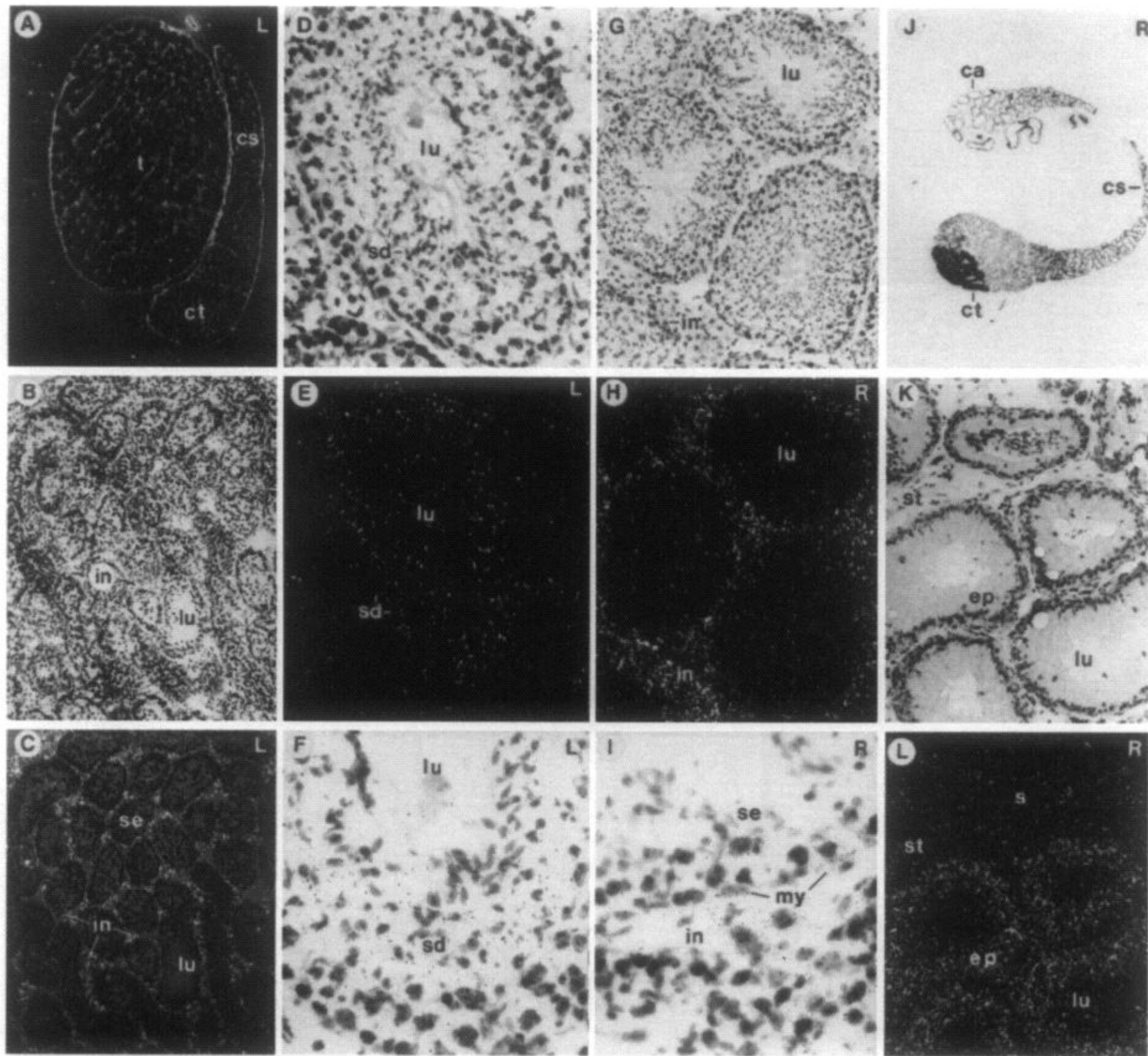

Fig. 3. Localization of *Igf1* Ligand (L) and *Igf1r* Receptor (R) Transcripts in the Testis (Panels A-I) and Epididymis (Panels J-L) of Wild Type Mice by *in Situ* Hybridization Using ^{35}S-Labeled Antisense cRNA Probes

The specimens in panels A-C and panels D-L are from 14- and 35-day-old animals, respectively. The hybridization signal in panel J was detected by x-ray film autoradiography, while emulsion autoradiography was used for the other panels. Panels B and C, D and E, G and H, and K and L are paired bright and dark field illuminations of the same microscopic field. In the dark field view, the hybridization signal has the appearance of white grains. Panel A is a low power dark field micrograph, while panels F and I are high power bright field micrographs in which the hybridization signal appears as *black grains*. A-C, In the testis (t) of younger animals, the *Igf1* hybridization signal is localized in the interstitium (in) but not in the seminiferous epithelium (se). In the epididymis (panel A), only myofibroblastic cells are labeled. D-F, In older animals, the hybridization profile changes, and *Igf1* hybridization signal is detected in the seminiferous epithelium, predominantly in spermatids (sd) as shown in panel F at high magnification (grains), while the interstitium remains unlabeled. G-I, *Igf1r* transcripts are detected predominantly in Leydig and peritubular myoid (my) cells as shown in panel I at high magnification (grains). This pattern does not change with developmental age. The lumen is indicated as lu. J-L, In the epididymis, *Igf1r* transcripts are localized in the epithelium (ep) but not in the stroma (st) of the duct. As shown in panel J, the strongest signal is concentrated in a region of the caput (ct), as compared with the corpus (cs) and the cauda (ca). In all cases, hybridization with a sense (control) probe did not exceed background levels (not shown).

common hypothalamic precursor polypeptide of GnRH and GnRH-associated peptide (32). Although the deletion leaves the GnRH-coding sequence intact, and the gene is transcribed, GnRH peptide is undetectable. As a result gonadotropins are not produced, and, consequently, neither spermatogenesis nor steroidogenesis can occur in *hpg* males. In these mutants, the body weight is not affected, but the weights of the testes and seminal vesicles are 3% and 0.65% of normal, respectively, while testosterone secretion is reduced to 0.3% of normal, and no sperm are formed (33). Interestingly, transplantation of tissue from the preoptic area (containing GnRH-producing cells) into the third ventricle of *hpg* males resulted in increased

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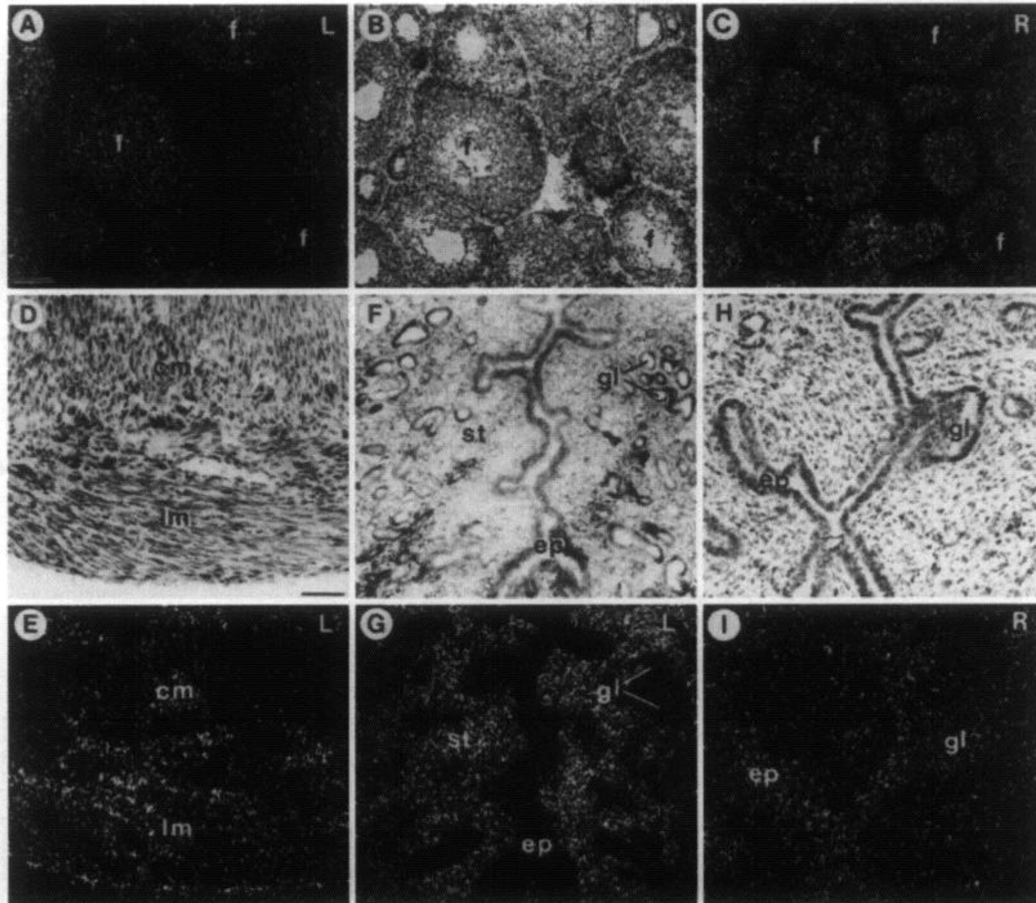

Fig. 4. Localization of *Igf1* Ligand (L) and *Igf1r* Receptor (R) Transcripts in the Ovary (Panels A-C) and Uterus (Panels D-I) of Wild Type Mice by *in Situ* Hybridization Using ^{35}S-Labeled Antisense cRNA Probes

Panels A and B are paired dark and bright field micrographs of the same ovary section, while panel C is a dark field view of an adjacent section for comparison of ligand and receptor gene expression in the same follicles. Panels D and E, F and G, and H and I are paired bright and dark field micrographs. A-C, In the ovary, *Igf1* and *Igf1r* transcripts are localized in granulosa cells, but only a subset of follicles (f) are labeled with the *Igf1* probe. D-G, In the uterus, the circular (cm) and longitudinal (lm) muscle layers of the myometrium (especially the latter), and also the endometrial stromal cells (st), exhibit a positive hybridization signal with an *Igf1* probe, while the glandular epithelium (gl) remains unlabeled. H and I, *Igf1r* transcripts are localized in the glandular epithelium (gl) and in the epithelium (ep) lining the uterine lumen.

production of gonadotropins, steroidogenesis, and gametogenesis (34), but, despite this improvement, the treated animals failed to display masculine sexual behavior or to impregnate females. In contrast, normal sexual behavior and fertility was observed in *hpg* mutants that were injected perinatally with testosterone and then received a testosterone implant, whereas control mutants receiving the same implant without prior androgenization failed to impregnate females (35). Extrapolating from these and other observations, we propose that, although testosterone is not completely absent from *Igf1* null males, its reduced level in serum is inadequate for perinatal androgenization.

Initially, the deficiency of *Igf1* null mutants in androgens was evidenced indirectly by the dramatic reduction in the size of male accessory glands, particularly exemplified by the vestigial appearance of the seminal vesicles and prostate, which are known to be extremely sensitive to serum testosterone levels (36–38). Our direct measurements of serum testosterone have confirmed this conclusion. In fact, the hormonal deficiency of the mutants is very significant, since we are not measuring the absolute amount of testosterone, but, rather, its concentration in serum, which, regardless of the reduction in body size, should have been normal if the steroidogenic capacity of Leydig cells was not drastically impaired. Moreover, our *in vitro* data have provided an indication that the reduction in serum testosterone levels in the mutants may be attributed, at least in part, to reduced testosterone synthesis and/or secretion in the testis.

Interestingly, androgen deficiency in the mutants can be correlated with an apparently retarded differentiation of Leydig cells, demonstrated by the absence of membranous whorls from the cytoplasm of adult mutant Leydig cells and the low production of testosterone, which are also characteristic features of the immature form of these cells in normal animals before

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puberty. During mammalian development, there are distinct phases in the growth and differentiation of Leydig cells. Initially, androgens are produced by a population of fetal cells, the fate of which is a matter of controversy (they either degenerate or continue to be present as a minor component within the population of Leydig cells that appear later; see Refs. 39–41). It is undisputed, however, that during the prepubertal period, mesenchymal progenitor cells in the testicular interstitium differentiate into immature Leydig cells, which, in turn, give rise to their adult counterparts. Although an exact analysis of the *Igf1* mutational impact on this steroidogenic compartment is pending, our current data suggest that lack of IGF-I impairs the second phase of Leydig cell differentiation. Our testable hypothesis pertaining to perinatal androgenization predicts that the fetal Leydig cell population should also be affected. A relevant correlation is that the steady state level of intratesticular *Igf1* mRNA is highest perinatally and then declines progressively to approximately 55% of its perinatal value by p20, remaining subsequently constant up to p60 (42).

Previously, *in vitro* studies suggested that there is a relationship between IGF-I and androgen production in rodents. Although ineffective by itself, IGF-I apparently stimulated LH-dependent androgen synthesis in primary cultures of Leydig cells from immature rats (see Ref. 43 and other references therein). Our results are consistent with these observations, as they showed that, in comparison with wild type controls, the LH stimulation of testosterone production over the basal level by testicular parenchyma in culture was 55% lower in *Igf1* null mice. Interestingly, both basal and LH-stimulated androstenedione production was much higher in the *Igf1* null mutants than in wild type controls. This may reflect the developmental immaturity of Leydig cells in the mutants (*cf.* Ref. 44), while the activity of steroidogenic enzymes, and particularly the action of 17β-hydroxysteroid dehydrogenase, which converts androstenedione to testosterone, may also be affected differentially by the mutation. Reportedly, LH also up-regulated the binding capacity of IGF1R in Leydig cells (45), but its overall effects on the IGF system may be complex. It was shown, for example, that in highly purified cultured Leydig cells from mature rats, hCG decreased significantly the rate of transcription of *Igf1* mRNA without affecting its stability (46). Whether an indirect relationship between FSH and IGF-I could also exist is unclear. FSH can affect the physiology of Leydig cells only indirectly through its effects on Sertoli cells, which, in contrast to Leydig cells, do express FSH receptors, but not IGF1R.

It is notable that the role of GH, if any, on the regulation of intratesticular IGF-I cannot be significant, in contrast to its important hepatic action that controls the level of circulating IGF-I. First, the GH receptor gene does not appear to be expressed in the testis (Refs. 47, 48; but see Ref. 49). Second, in contrast with the *Igf1* null mutants, male *little* (*lit*) mice (50), carrying a missense mutation of the gene encoding the receptor for the GH-releasing hormone (51, 52), are fertile (53). These mutants maintain only about 4–8% of the normal level of GH in the pituitary, and their serum levels of GH and IGF-I are 1.3% and 10% of normal, respectively (54–57). However, all reproductive organs are allometric to the reduced body size (~50% of normal), and steroidogenesis and spermatogenesis are normal (58). Similarly, fertility is not impaired in homozygous *dr/dr* (spontaneous dwarf) rats (59), in which a base substitution in the GH gene results in abnormal splicing deleting a base from the GH mRNA that leads to premature translational termination, due to a frameshift (60). As a consequence, the amount of pituitary GH does not exceed 0.04% of normal, while the serum IGF-I concentration is about 13% of normal (61). Homozygous mutant rats, which carry a different dwarfing mutation (*dw/dw*) potentially affecting the GH-releasing hormone signal transduction pathway (62), are also fertile, and despite GH deficiency in the pituitary (<5% of normal) and low serum IGF-I concentration, they exhibit normal levels of testicular IGF-I (63). Thus, in addition to being apparently GH-independent, the testicular functions of IGF-I seem to be served by its local production (autocrine/paracrine action) without an endocrine contribution by the circulating form of this factor. This conclusion is seemingly inconsistent with the observation that the reproductive phenotype of *Igf1* null mice bears a striking resemblance to that of mouse Snell (*dw/dw*) dwarfs (reviewed in Ref. 64), which carry a missense mutation of the gene encoding the pituitary-specific transcription factor Pit-1 that activates the expression of the GH and PRL genes (65, 66). In *dw/dw* mice, the reduction in testis size (~30% of normal) is proportional to body size, while the seminal vesicles are disproportionately small (only 4% of normal; Ref. 58). Moreover, after stimulation by LH, the testis of these mutants secretes only 9% of the normal level of testosterone (58). However, despite these similarities in mutational manifestations and the fact that the IGF-I concentration in serum is exceedingly low in *dw/dw* mice (67, 68), a direct comparison with the *Igf1* null mutants is unwarranted, since the Snell dwarfs lack both GH and PRL and have reduced levels of thyroid hormones. Moreover, their spermatogenesis is not affected (69), in contrast with the males lacking IGF-I. This indicates that neither GH nor the circulating IGF-I is involved in the spermatogenic process.

IGF-I and Female Reproductive Functions

A novel conclusion that can be reached from our results is that IGF-I is an indispensable component of the ovulatory pathway. Thus, even if they exhibited mating behavior, the female *Igf1* null mutants would still be infertile due to the lack of ovulation, a phenotypic feature also observed in *dw/dw* female dwarfs (reviewed in Ref. 64). On the other hand, our data have indicated that IGF-I is not an obligatory participant in follicular development, at least up to the formation of

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antral follicles. It is apparent, therefore, at least on the basis of histological criteria, that the gonadotropins, which were shown (by hypophysectomy and hormone replacement experiments) to regulate mouse follicular development and differentiation from the early preantral to preovulatory stages (70–73), are essentially able to perform these functions in the absence of IGF-I. However, even after a surge of exogenously administered gonadotropins, the pathway of follicular responses culminating in rupture does not function in the mutants. We believe that, whatever the unknown mechanism of the ovulation block may be, our observations reveal an important interdependence of the gonadotropin and IGF-I signaling pathways, which is perhaps related to events necessary for the generation of Graafian follicles and subsequent ovulation. Such a transition was observed in mutant ovaries only after administration of gonadotropins at a very high pharmacological dose, which also induced rupture of a few follicles, but yielded abnormal oocytes. Thus, if our hypothesis is correct, follicular development cannot reach a stage responsive to physiological ovulatory stimuli, unless IGF-I is present. In this regard, a correlation with the expression pattern of *Igf1* during follicular development may be eventually attained, considering for example the following observation. During oocyte maturation in rats, the *Igf1* transcripts, initially found in all granulosa cells, become confined to their layer closer to the antrum, including the cumulus surrounding the oocyte, while in atretic follicles, *Igf1* expression ceases (15). At least in rats, the ovarian *Igf1* gene expression is apparently not subject to gonadotropin regulation, since the level of *Igf1* transcripts detected by *in situ* hybridization in granulosa cells remains unchanged after hypophysectomy with or without gonadotropin replacement (15). In contrast, the level of *Igf1r* mRNA is diminished upon hypophysectomy and restored or up-regulated by administration of gonadotropins (15, 26). However, both IGF-I and its cognate receptor are either insensitive to GH control (15), despite the presence of GH receptors in the ovary (74), or GH may be playing some minor (nonessential) role. This view is consistent with the fact that female *lit* mutants (75) and all of the aforementioned GH-deficient female rat dwarfs (59, 62) are fertile. However, even if the ovarian IGF-I has some degree of autonomy, it may be regulated by other hormonal stimuli. Estrogen, for example, is apparently able to regulate the level of *Igf1* mRNA (76) and also of *Igf1r* mRNA (26). Reciprocally, IGF-I seems to be involved in ovarian steroidogenesis, as suggested by *in vitro* results. Thus, IGF-I enhances LH binding (77) and FSH-induced aromatase activity (78) in cultured granulosa cells from immature hypophysectomized rats. It is believed that in rodents androgen synthesized in theca cells is converted to estrogen by the aromatase enzymatic complex in granulosa cells (for a review, see Ref. 79). The strong positive correlation between granulosa cell DNA synthesis, measured by bromodeoxyuridine incorporation, and *Igf1* gene expression, detected by *in situ* hybridization in the same cells of a subset of rat ovarian follicles, suggests an involvement of IGF-I in the proliferation of this steroidogenic compartment (80), although an additional differentiative role cannot be excluded. Our preliminary measurements of serum estradiol in *Igf1* null mice showed that this estrogen is reduced to about 53% of the normal level, but the exact link between estrogen and ovarian IGF functions is still unclear. Nevertheless, targeted ablation of the estrogen receptor (ER) gene (81) showed for the first time that estrogen is directly involved in the growth and differentiation of follicles. Thus, only primary and secondary follicles are present in the ovaries of ER null mice, while administration of exogenous gonadotropins results in the formation of a few antral or Graafian follicles, but predominantly in the appearance of atretic cysts (81, 82).

Our observations have provided direct evidence demonstrating that IGF-I plays a significant role in the development of the uterus, as previous data had indicated (reviewed in Refs. 83–85). In this organ, the *Igf1* mRNA content is regulated predominantly by estrogen. Thus, its level is dramatically reduced as a consequence of ovariectomy and restored by injection of estrogen in animals that are either ovariectomized or both ovariectomized and hypophysectomized. On the other hand, administration of GH is either ineffective or has a minimal effect (86–90).

Our *in situ* hybridization analysis (see *Results*) demonstrated that the pattern of *Igf1r* gene expression, regardless of mouse age, is widespread in both the myometrium and the endometrium, and, in the latter, includes both stromal and glandular elements. In contrast, the hybridization pattern of *Igf1* mRNA is more restricted and depends on developmental age. Thus, a positive hybridization signal, predominantly in endometrial stromal cells, was detected only in adult, cycling mice, while the myometrium expressed *Igf1* at all ages. It was previously shown by *in situ* hybridization and immunohistochemistry that, in adult mice, estrogen administration increases the abundance of *Igf1* mRNA and IGF-I peptide, which were colocalized in the uterus, although expression was apparently observed predominantly in the luminal and glandular epithelia (91). When the uterus of immature rats was examined by *in situ* hybridization, *Igf-1* mRNA was detected in all cell layers, including the luminal epithelium, but it was more abundant in the longitudinal and circular myometrial cell layers and in the outer stromal cells (92). Administration of estrogen increased the intensity of hybridization, while the overall pattern remained unchanged in estrogen-treated, ovariectomized, and hypophysectomized rats (92). Immunochemical experiments able to detect precursor IGF-I polypeptide suggested that this locally produced *Igf-1* mRNA is translated (83).

Estrogen administration also increases the number of IGF1R-binding sites (93, 94). In immature rats, these receptors, identified by cross-linking with labeled IGF-I, were localized to the smooth muscle cells of

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both myometrial layers but were absent from the stroma and the epithelial cells (93). Thus, the colocalization of receptor and ligand in the myometrium, suggesting the operation of an autocrine loop, provides a basis for the interpretation of the marked myometrial hypoplasia in *Igf1* null mutants, which is significantly more severe than that observed in the endometrial layer. In this regard, our observations are consistent with the idea that IGF-I may be a specific mediator of estrogen function in the uterus (estromedin; Ref. 95). It is notable, however, that the weight of the uterus in ER null mice, measured in animals 3–4 months old, is about 25–30% of normal (Ref. 81; and K. C. Korach, personal communication). Thus, the significantly more severe underdevelopment of the uterus in *Igf1* null females (13% of normal) suggests that the uterine IGF system is controlled by other hormonal signals, in addition to estrogen, and may also exhibit some degree of autonomy.

MATERIALS AND METHODS

Anatomical, Histological, and Ultrastructural Analyses

Nullizygous *Igf1* mutants survive to adulthood at variable frequencies depending on genetic background (6). Thus, in this study we used predominantly MF1×129/Sv hybrids (up to 68% survival). Progeny were genotyped by Southern analysis as described (96). For determination of wet weights, reproductive organs were excised from mutants and wild type controls, patted dry with absorbent paper, and weighed on an analytical balance. Tissues were fixed overnight in 4% paraformaldehyde or Bouin's fluid. For histological analysis, the tissues were washed with phosphate buffer, dehydrated through a graded series of alcohols, cleared in toluene, and embedded in paraffin. Paraffin blocks were sectioned at 5–6 μm, and the sections were stained with modified Harris' hematoxylin with or without eosin.

For Leydig cell analyses, the males were perfused with 2.5% glutaraldehyde, 1% acrolein in sym-collidine buffer, pH 7.4, and the testes were cut into 2- to 4-mm^3 segments, postfixed in 1% osmium tetroxide, embedded in Epon-araldite resin, sectioned at 1 μm, and stained with 1% toluidine blue in borax. The total number of Leydig cells per testis and their average cell volume were measured by point counting, as described (97). Ultrathin testis sections were poststained with 1% uranyl acetate and lead citrate, cut in silver/gold sections, and examined with a Philips 300 electron microscope.

Measurements of oocyte and follicle sizes were made using a calibrated ocular grid. To avoid multiple scoring, only oocytes with an identifiable nucleolus were counted in each section.

In situ hybridization analyses with ^{35}S-labeled antisense and sense (control) rat *Igf1* and *Igf1r* cRNA probes were performed as described (15, 80).

Sperm Counts

The numbers of sperm present in the testis, the epididymal segments (caput, corpus, and cauda), and the vas deferens of age-matched mutant and wild type animals were counted by hemacytometry. Tissues of known weight, previously frozen at −70 C, were thawed at 4 C, cut into 2-mm pieces, suspended in 0.5 ml water, and homogenized in a low-clearance homogenizer (10 strokes) to dissociate somatic cells and sever sperm heads and tails. Only sperm nuclei remaining as a monodisperse suspension were counted from several aliquots, and their number in the original sample was determined by correcting for sample volume and tissue weight.

Measurements of Sex Steroid Hormones

For measurement of serum steroids, blood was collected by cardiac puncture from anesthetized animals and allowed to stand at room temperature for 15 min. Serum was obtained by centrifugation of clotted blood and stored at −20 C until measurement of testosterone or estradiol by RIA using specific antibodies (ICN Biomedical, Costa Mesa, CA).

To measure androgen production, the testes were removed from animals killed by CO_2 asphyxiation, and, after removal of the tunica albuginea, the parenchyma was divided into five, approximately equal, portions. These pieces were weighed and then placed into 1.5-ml microcentrifuge tubes and incubated in DMEM/Ham's F-12 culture medium (1:1; Sigma Chemical Co., St. Louis, MO), buffered with 15 mM HEPES, 14 mM $NaHCO_3$ and containing 1% BSA, for 3 h at 34 C with shaking. Duplicate samples were incubated either alone, for determination of basal levels, or in medium supplemented with a maximally stimulating dose of ovine LH (100 ng/ml). At 30-min intervals, aliquots were removed and replaced with fresh medium to assess linearity in androgen production during the period of incubation. The incubations were terminated at 3 h, and the samples were centrifuged at 500 × *g* and stored at −20 C. Testosterone or androstenedione release in the media was measured by RIA (the interassay variation was 7.8% and 15%, respectively). The data were normalized per milligram of tissue.

Superovulation

Wild type females were superovulated by injection of 5 IU PMSG at approximately 1200 h, followed by injection of 5 IU hCG 46–47 h later. Mutant female littermates were superovulated in parallel, but with 1.7 IU of each of the gonadotropins, to adjust for their small size. In addition, nine mutant females were injected with 1.7 IU PMSG, but with a 10-fold higher dose of hCG (17 IU).

In Vitro Fertilization

The cauda regions of both epididymides were dissected from wild type and mutant males and placed in 500 μl of Whittingham's-Tyrode fertilization medium (98) under light mineral oil. Sperm were squeezed out of the cauda by using the blunt end of forceps under a dissecting microscope, after which the tissue was shredded to ensure high yield. After incubation at 37 C in 5% CO_2 for 30 min, the sperm were counted by hemacytometry and their motility assessed. For capacitation, the incubation was extended for an additional 1.5 h.

Superovulated wild type and mutant females were killed 12.5 h after hCG administration, and pairs of dissected oviducts were placed in 500 μl Whittingham's-Tyrode fertilization medium under light mineral oil. To obtain a cumulus mass from wild type animals, ampullae were torn open under a dissecting microscope. This manipulation, however, did not result in release of a cumulus mass from mutant animals, although small ampullae were observed. For this reason, to obtain ova, the oviducts were shredded with forceps.

Capacitated sperm (30–40% of that recovered from each animal) were mixed with each droplet containing ova and incubated at 37 C for 7 h. The ova, no longer attached to cumulus cells, were then washed with KSOM medium (99) and placed under mineral oil (100 ova/100 μl). Fertilized ova were allowed to develop in culture and monitored for progression to two-cell-stage embryos.

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