Journal of Andrology, Vol. 10, No. 5, September/October 1989

Transplantation of Newborn Rat Testis Under the Kidney Capsule of Adult Host as a Model to Study the Structure and Function of Leydig Cells

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Newborn rat testis was transplanted under the kidney capsule of adult castrated and uncastrated male rats to develop and characterize a model system for studies on Leydig cell development. Two weeks after transplantation, the number of Leydig cells and the size of their nuclei in the transplants had increased. Secretion of testosterone was indicated by increased seminal vesicle weights and decreased pituitary LH in the castrated host animals. Pituitary FSH content increased significantly in the uncastrated animals with transplants, which suggested production of an FSH-stimulating factor. Cells with the morphologic features characteristic of fetal- and adult-type Leydig cells were observed in the transplants. The seminiferous tubules with spermatocytes, incipient lumina, and significantly larger average diameters showed more advanced development than those in the normal 2-week-old testis. By the present morphologic and functional criteria, the kidney subcapsular transplantation technique provides a suitable model for studies of fetal and adult Leydig cell development.

Key words: rat, testis, Leydig cell, transplantation, kidney capsule.

J Androl 1989; 10:335–345.

Supported by grants from the Sigrid Jusélius Foundation and the Academy of Finland.

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Received for publication July 11, 1988; accepted for publication November 29, 1988.

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Leydig cells have a biphasic pattern of development in many mammalian species (Christensen, 1975). In the rat, the first period of development begins *in utero* when the so-called fetal Leydig cell population appears (Roosen-Runge and Anderson, 1959; Lording and de Kretser, 1972; Gondos, 1977). During the first 2-3 weeks of postnatal life, the fetal cells are replaced by the adult generation of Leydig cells (Roosen-Runge and Anderson, 1959; Lording and de Kretser, 1972; Zirkin and Ewing, 1987), which presumably differentiate from fibroblast-like precursors (Christensen, 1975). Factors regulating the change in the populations are not fully understood, although pituitary gonadotropins probably play an important role (Sharpe, 1982). To continue studies on Leydig cell development in the rat (Huhtaniemi et al, 1984, 1985a; Tapanainen et al, 1984; Kuopio et al, 1989), a model experimental system was developed, which would maintain the

structural integrity of the testis and its connection to the circulatory system.

Transplantation of newborn testis into an adult mesenteric pouch is possible (Grund, 1987), although there are several technical difficulties. The subcapsular space of the kidney seems to provide favorable nutritional and immunologic conditions for experimental tissue transplantation (Bogden et al, 1979; Kangas and Perilä, 1985). Exogenous hormone administration, hypophysectomy or castration of the adult host animal are technically easy and can be used to study different hormonal effects on the transplanted tissues. Experiments of this kind have been used in studies of ovarian development (Henzl et al, 1971; Arrau and Roblero, 1979; Arrau et al, 1983; Taketo-Hosotani et al, 1984, 1985; Taketo-Hosotani, 1987). However, reports on testicular transplantation are few and have mainly concentrated on the development of germinal rather than Leydig cells (Chan et al, 1969; Mangoushi, 1977; Ozdzenski and Presz, 1984). The purpose of the present study was to adapt the kidney subcapsular transplantation technique for studies on the development of rat Leydig cells.

Materials and Methods

Animals and Testis Transplantation

Twenty-four male rats of the Wistar strain from our own colony were divided in two groups. Twelve rats were castrated, and 12 were sham-operated at the age of 65 days. The testicular transplantations were performed 9–11 days later. Bothkidneys of each animal were exposed via dorsolateral incisions under barbiturate anesthesia (Mebunat™, Orion, Espoo, Finland). A small cut was made in the kidney capsule, the capsule gently lifted, and a testis from a freshly decapitated newborn animal was transplanted under the capsule of each kidney. Both castrated and uncastrated animals had sham-operated controls. The animals were killed for specimen collection 2 weeks after transplantation. The structure of the transplanted testis was compared with the normal development of organs from normal 5-, 11-, 14- and 20-day-old animals.

Morphologic Analysis

One transplanted testis from each animal was dissected out and immersed in 5% glutaraldehyde in 0.16 mol/l 2-, 4-, 6-collidine-HCl buffer, pH 7.4. Three castrated and three uncastrated animals were perfusion-fixed via the thoracic aorta with the same fixative for 20 min before the transplanted organs were dissected out. After fixation, the specimens were washed and postfixed in potassium ferrocyanide-reduced osmium tetroxide for 2 h (Karnovsky, 1971). The tissue pieces were embedded in epoxy resin (Glycidether 100, Merck, Darmstadt, FRG). Sections with thickness of 1 μm were cut on a Porter-Blum ultramicrotome and stained with toluidine blue for light microscopy. Ultrathin sections for electron microscopy were cut on a Huxley-LKB ultramicrotome (LKB, Bromma, Sweden), stained with uranyl acetate and lead citrate, and examined with JEOL (Tokyo, Japan) JEM-100C electron microscope.

The seminiferous tubule diameters of the transplanted and normal 14-day-old testis were measured using a light microscope fitted with a microscale in the eyepiece. When the cord profile was ovoid instead of circular, the short axis perpendicular to the long axis was measured. Three animals from both transplanted groups and five from normal 14-day-old controls were examined. Altogether 50 cords per animal were measured. To determine the mean cross-sectional area of Leydig cell nuclear profiles, 50 nuclear profiles from three animals per transplantation group were drawn on paper using a drawing tubus. The areas of the profiles were measured with a MOP-AMO3 digitizer (Kontron A.G., München, FRG).

Hormone Measurements

Biochemical measurements were carried out on 7-10 individuals per treatment group. Testicular testosterone (T) was measured in the homogenates by RIA after diethyl ether extraction as previously described (Huhtaniemi et al, 1985b). The sensitivity of the method was 500 fg/tube, intra-assay variation was below 6% and inter-assay variation below 12%. LH and FSH of pituitary homogenates were determined by a double-antibody RIA as previously described with reagents provided by the National Pituitary Agency and NIADDK (Bethesda, MD) (Huhtaniemi et al, 1986). Samples were diluted appropriately with 0.01 mol/l phosphate-buffered saline (PBS, pH 7.4) containing 0.1% (w/v) BSA. The assay sensitivities were 1.0 ng and 7.0 ng/tube for LH and FSH, respectively, and intra-assay and interassay variations were below 8 and 15%, respectively. These levels were expressed in terms of the standard NIADDK-RP-2.

Preparation of Radioiodinated Human Chorionic Gonadotropin (hCG)

Highly purified hCG (CR-121, 13.500 IU/mg by bioassay) was prepared by Dr. R. Canfield (Columbia Univ., New York, NY) and provided by the Center for Population Research and NICHHD (Bethesda, MD). The hormone was radioiodinated by a solid-phase lactoperoxidase method described by Karonen et al (1975), and purified on an 0.6 × 10-cm column of BioGel P-60 (Catt et al, 1976). The specific activity of labeled hCG was determined by self-displacement analysis in a radioligand-receptor assay employing rat testis homogenate (Catt et al, 1976). A correction was also made for the fraction of radioactivity

associated with biologically active hormone, as measured in the labeled hormone preparation by determining the maximum specific binding of a sample of the tracer to an excess of rat testis receptors (Catt et al, 1976). The fraction was 50%, and the corrected specific activity was 70 Ci/g.

Measurement of Testicular LH Receptors

The testes were thawed and each was homogenized in 0.6 ml of Dulbecco's PBS (pH 7.4), containing 0.1% BSA. Binding of hCG to the homogenates was measured by incubating 150,000 cpm (2 ng) [^{125}I] iodo-hCG with duplicate 100-μl samples of testicular homogenate. Nonspecific binding was measured in matched samples in the presence of a 1000-fold molar excess of Pregnyl (Organon, Oss, The Netherlands). The incubations were performed for 16 h at room temperature (23 C) in a final volume of 250 μl. Bound and free hormones were separated by 15-fold dilution and centrifugation (at 4 C) of the samples. The concentration of hCG used in the incubations was near saturation (Catt et al, 1976; Huhtaniemi et al, 1981), and therefore, the binding assay gives a reliable estimate of testicular LH receptor content.

Results

Morphology

Two weeks after testicular transplantation, 38 of the 48 transplanted newborn organs (79%) were found under the kidney capsules of the host animals. The testes had grown in size and had vascularized, as indicated by clearly visible arteries on the surface and interior of the organ (Figs. 1, 2B, 2C, 4A, and 4B). Microscopic observations showed that testicular development had continued after transplantation. Blood vessels, well-preserved interstitial cells and seminiferous tubules with occasional incipient lumen formation in the transplants indicated that histologic organization of the tissues in the transplanted and normal postnatal testes was comparable (Fig. 2). Some areas in the center of the transplanted organs were degenerated, which was probably due to incomplete revascularization.

The most advanced germ cells in both the transplants and the normal 14-day-old controls were primary spermatocytes, indicating progression of spermatogenesis in the transplants. The pooled mean seminiferous tubule diameter (± SEM) of the transplanted testes (87 ± 3 μm, n = 6; 86 ± 5 μm, n = 3 in the uncastrated group and 89 ± 6 μm, n = 3 in the castrated group) was significantly ($p < 0.05$, student's t-test) higher than that in the normal 14-day-old control animals (76 ± 1 μm, n = 5). This suggested that tubular development in the transplants was slightly more advanced than in the controls of a corresponding age.

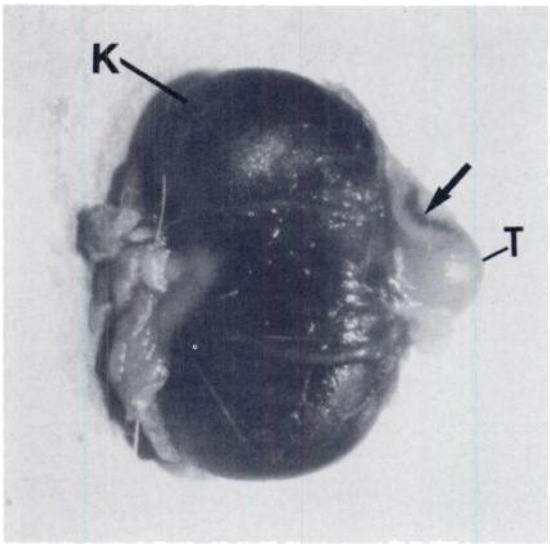

Fig. 1. Photograph of a newborn testis (T) under the kidney (K) capsule of a castrated adult male rat 2 weeks after transplantation. A new large artery grown into the testis is indicated with an arrow.

The numerous interstital Leydig cells of the castrated group formed large confluent areas, which were never found in the uncastrated group or in the 14-day-old normal controls (Fig. 2). The average surface area of the Leydig cell nuclear cross sections was significantly larger ($p < 0.05$, student's t-test) in the castrated group (23 ± 0.1 μm^2, ± SEM, n = 3) than in the uncastrated group (19 ± 0.1 μm^2, n = 3).

Electron microscopic analysis of the castrates revealed two kinds of Leydig cells: those in distinct clusters surrounded by envelope cells (Fig. 3), and those organized as irregularly outlined confluent areas without conspicuous clustering (Figs. 4A and 4B). The clustered Leydig cells had large spherical nuclei, numerous lipid inclusions, and abundant smooth endoplasmic reticulum (Figs. 3A and 3B). All the cells seemed to be at the same stage of differentiation. No capillaries or cell types other than Leydig cells were found inside the clusters. Occasionally the clusters were surrounded by a continuous basement membrane (Figs. 3A and 3B).

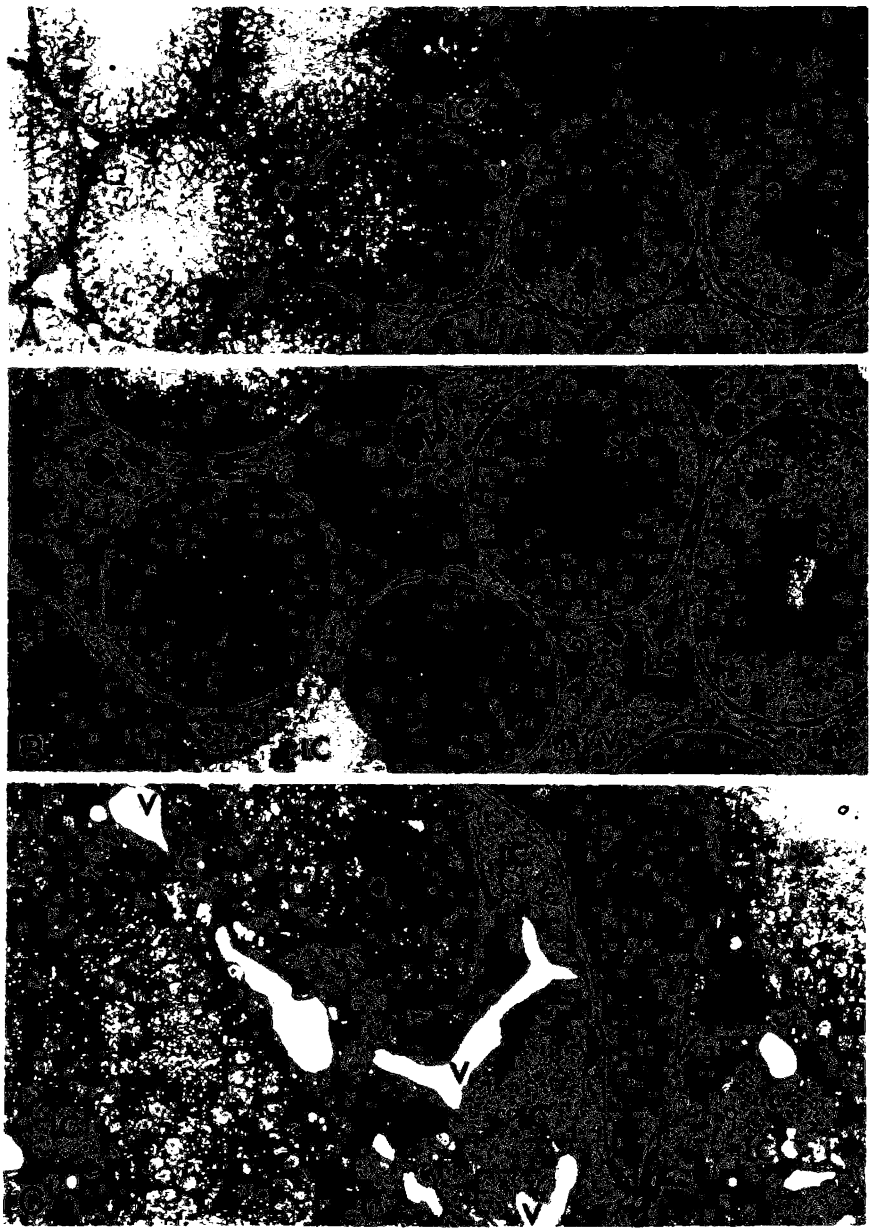

Fig. 2. Light micrographs of a testis from a normal 2-week-old control rat (A) and of newborn testes transplanted under the kidney capsules of uncastrated (B) and castrated (C) adult male rats 2 weeks after transplantation. Leydig cells (LC) are most numerous in the castrated group (C). Incipient lumen formation (asterisks) occurs in the transplanted organs. V = blood vessel. Toluidine blue stained sections, × 330.

In contrast to the clustered Leydig cells, the Leydig cells forming irregularly outlined confluent areas were not distinctly separated from the surrounding interstitium by enveloping cells. They were often in close association with interstitial blood vessels (Figs. 4A and 4B), and the size and shape of their nuclei and the amount of their cytoplasm varied considerably (Figs. 4A and 4B). With increasing cell size, the shape of the nuclei changed first from irregular to oval and then to spherical (Fig. 4A), which was thought to represent advancing cell development. The cytoplasm of all these Leydig cells contained abundant, smooth endoplasmic reticulum (Figs. 4 and 5), but the amount of lipid varied from one cell to another. There were unclustered well-developed Leydig cells with both few (Fig. 4A) and numerous (Figs. 4B and 5) lipid inclusions simultaneously present in the transplants. Several mitoses of both Leydig cells and undifferentiated cells in peritubular location were noticed (Fig. 6).

The testis in normal postnatal rats also contained clustered Leydig cells surrounded by slender

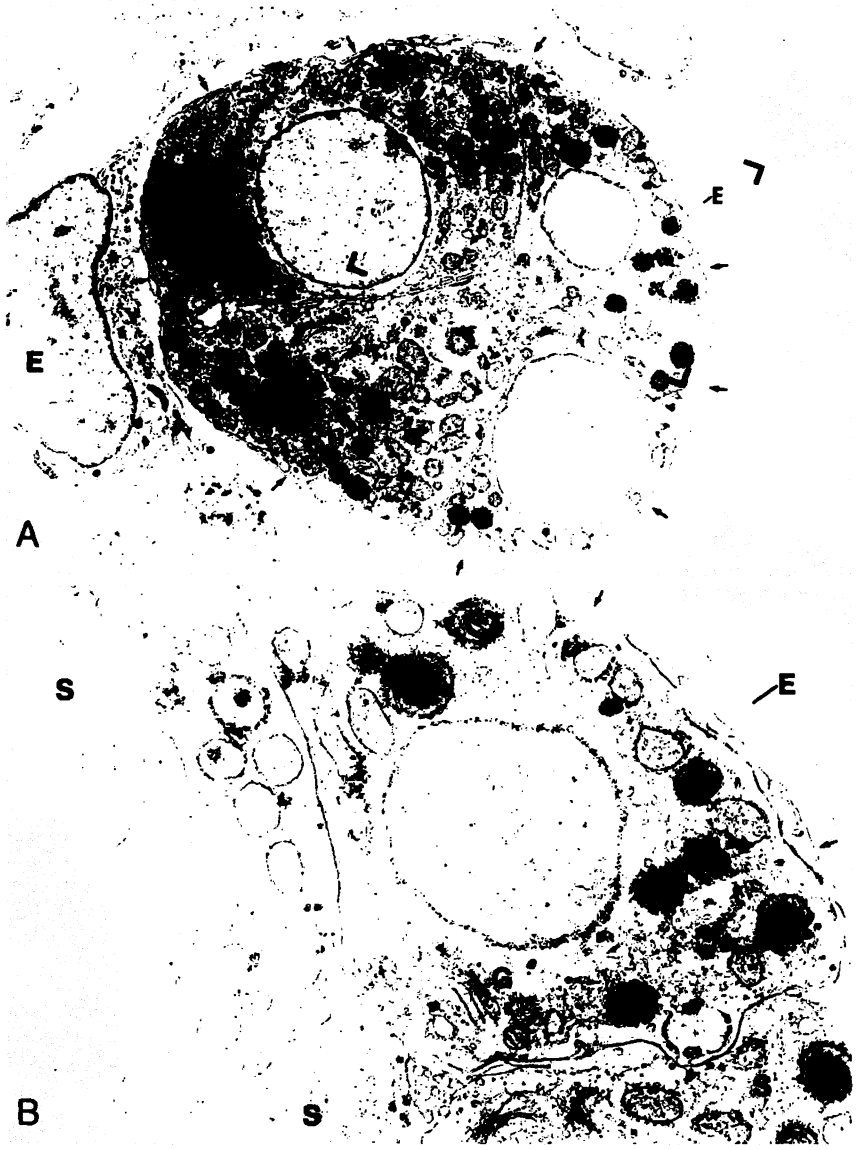

Fig 3. Electron micrographs of Leydig cells in testis transplant from a castrated adult host 2 weeks after the transplantation. The Leydig cells have characteristic features of postnatal rat fetal-type Leydig cells: they are in a cluster surrounded by an enveloping cell (E) and have numerous cytoplasmic lipid inclusions (L). The rectangle indicated by corner marks in A represents the area seen at higher magnification in B. The cluster is surrounded by a continuous basement membrane (arrows). S = smooth endoplasmic reticulum, G = golgi complex. Scales (A) × 3,400; (B) × 8,500.

enveloping cells. These characteristic fetal-type Leydig cells with spherical nuclei, rich supply of lipid droplets, and abundant smooth endoplasmic reticulum were very similar to the clustered cells of the transplants, except that no continuous basement membrane was found around them. During and after the second postnatal week, cells with characteristically adult-type Leydig cell features appeared. These cells were not aggregated into distinct clusters. The shape of their nuclei changed first from irregular to oval and then to spherical, while their cytoplasm contained abundant smooth endoplasmic reticulum but only few lipid inclusions. Mitoses of these adult-type Leydig cells were not found in our present material, but several dividing undifferentiated cells in peritubular location were observed.

Serum Testosterone and Pituitary Gonadotropins

Serum T concentrations in the castrated animals with transplants were significantly ($p < 0.05$) higher than those in the untransplanted controls (Fig. 7). No significant difference was found between transplanted and control animals in the uncastrated groups. The Leydig cells of the transplanted testis produced testosterone in quantities that were sufficient to enlarge the size of the regressed seminal vesicles of the castrates (Table 1). In uncastrated animals, the effect did not reach statistical signif-

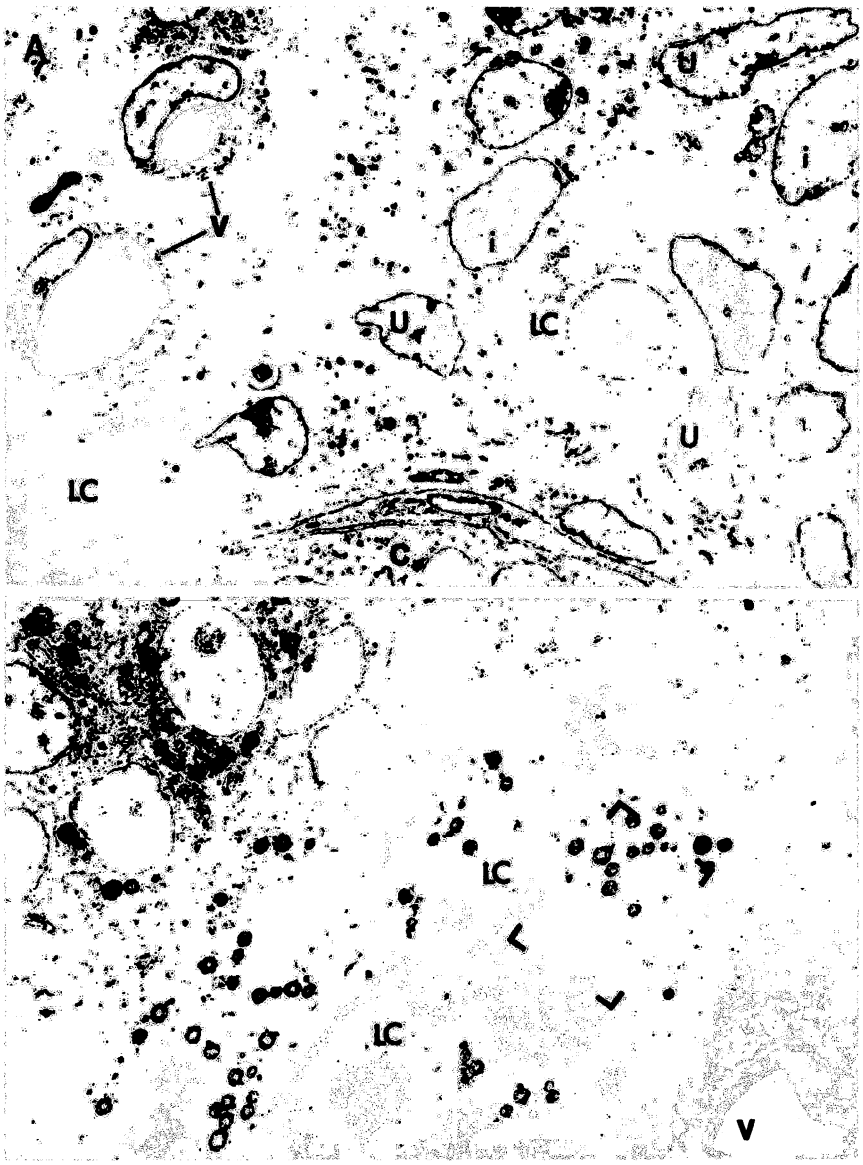

Fig. 4. Electron micrographs of Leydig cells of the same testis transplant as in Fig. 3. Well-developed Leydig cells (LC) with few (A) and numerous (B) lipid inclusions are not aggregated into distinct clusters. The Leydig cells form large confluent areas where undifferentiated interstitial cells (U) and immature Leydig cells (i) with large oval or irregular nucleus and scanty cytoplasm are also seen. Rectangle indicated by corner marks in B represents the area seen at higher magnification in Fig. 5. C = seminiferous tubule, V = blood vessel. (A) × 1,700; (B) × 1,800.

icance. Pituitary LH content was lower in the castrated group with transplants, showing that T production of the transplanted Leydig cells had a negative feed-back effect on LH production (Fig. 8). In contrast, the pituitary FSH content was significantly ($p < 0.05$) elevated by the transplanted testis in the uncastrated animals (Fig. 9).

Tissue Testosterone and LH Receptors

Although transplant tissue T and LH receptor levels varied greatly between individual measurements, a signficant ($p < 0.01$) positive correlation ($r = 0.65$) was observed between the hormone and receptor concentrations (Fig. 10).

Discussion

The present survival rate of the newborn testis after transplantation was similar to that of an earlier transplantation study with fetal hamster ovaries (Arrau and Roblero, 1979). Revascularization in our transplants also resembled the findings of Arrau and Roblero (1979), who described an extensive vascular supply to the transplants beneath the kidney capsule.

Seminiferous tubules developed in both the castrated and uncastrated groups during the 2 weeks

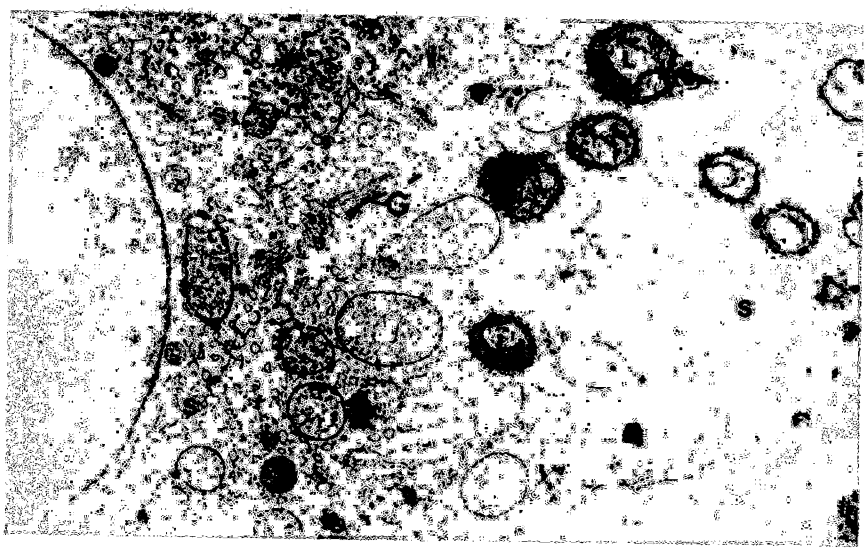

Fig. 5. Electron micrograph of a portion of Leydig cell inside the marked area in Fig. 4(B). S = smooth endoplasmic reticulum, L = lipid inclusions, G = golgi complex. × 9,400.

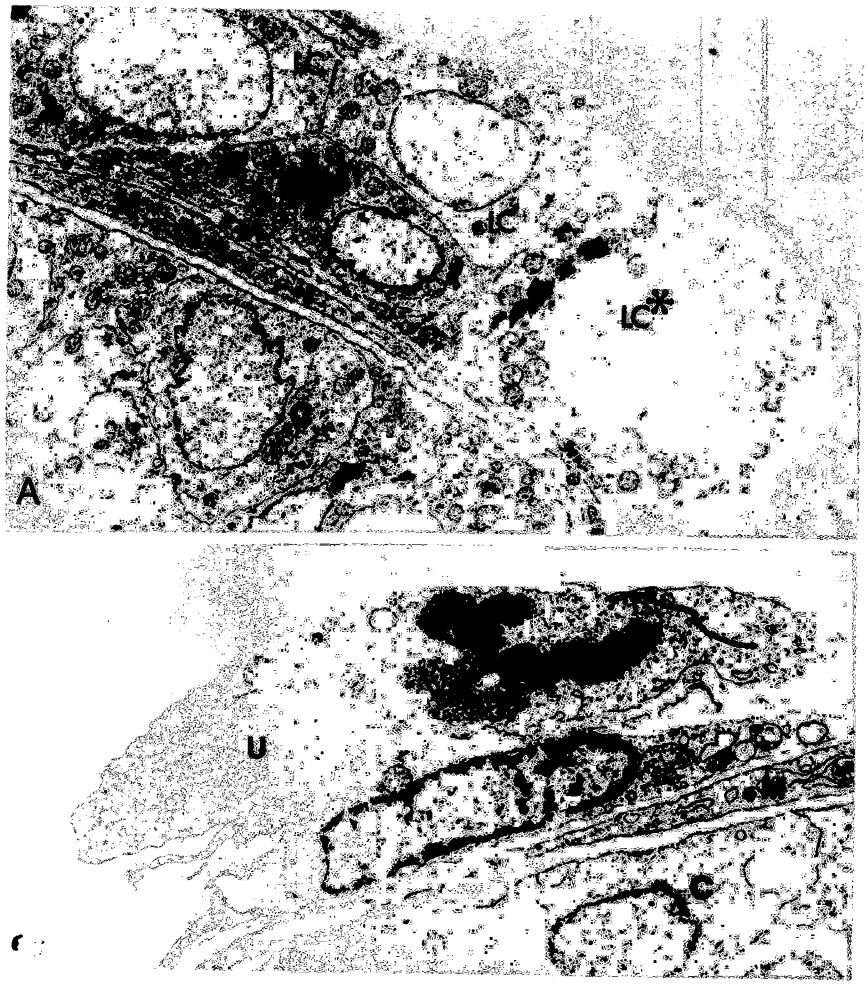

Fig. 6. Electron micrographs of a tubule-associated Leydig cell (*) and an undifferentiated peritubular interstitial cell (U) in mitosis. LC = Leydig cell, M = myoid cell, L = lipid inclusions, C = seminiferous tubule. (A) × 2,700; (B) × 4,800.

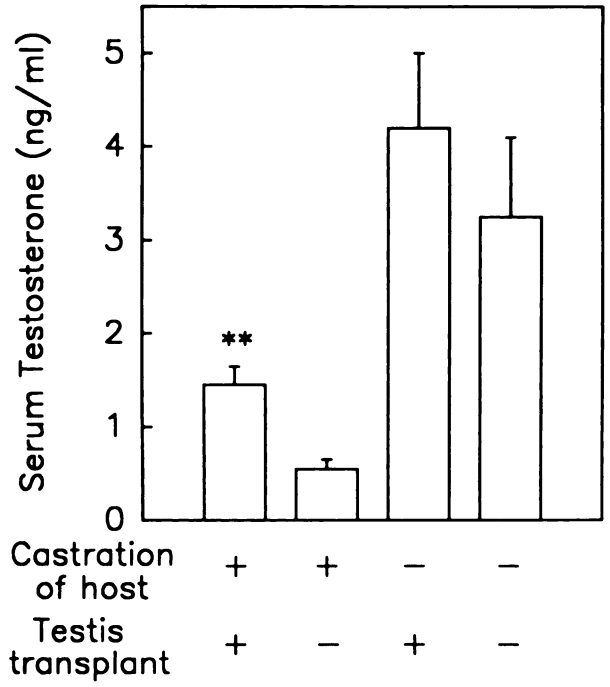

Fig. 7. Serum T concentrations of adult castrated (+) and uncastrated (−) male rats with (+) and without (−) newborn testis transplants 2 weeks after transplantation. Each bar represents mean ± SEM. Asterisks indicate a statistically significant difference from the respective controls, **p < 0.01, student's t-test.

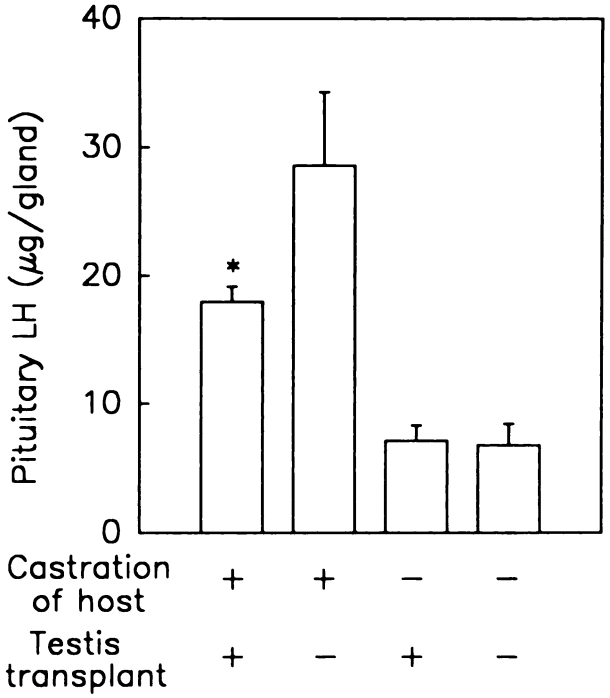

Fig. 8. Pituitary LH content of the host animals 2 weeks after the transplantation. Each bar represents mean ± SEM. Asterisks indicate statistically significant difference from the respective controls, *p < 0.05, student's t-test.

after transplantation, as indicated by the presence of primary spermatocytes, which are the most advanced germ cell type in normal 2-week-old rats (Hodgen and Sherins, 1973). The difference between the average diameter of the seminiferous tubules in the transplanted organs and the normal controls can be explained by the tropic action of the host's gonadotropins, which are at a higher level than those measured in normal male rats during the first 2 weeks of life (Huhtaniemi et al, 1985a).

TABLE 1. Seminal Vesicle Weights of Castrated and Uncastrated Host Animals and Their Controls 2 Weeks after Transplantation of Newborn Testes under the Kidney Capsule

Group		Weight (μg)*	n
Castrated	Controls	70 ± 7	5
	With transplants	90 ± 4†	9
Uncastrated	Controls	410 ± 20	6
	With transplants	590 ± 60	9

*Mean ± SEM.

†Statistically significant difference from controls ($p < 0.05$, student's t-test)

The distinct increase in the Leydig cell number and the significant hypertrophy of their nuclei in the castrated group show that responses of transplanted Leydig cells to changed gonadotropin levels of the host animal are similar to those found following exogenous gonadotropin stimulation (Chemes et al, 1976; Christensen and Peacock, 1980; Nussdorfer et al, 1980; Huhtaniemi et al, 1981; Kuopio et al, 1989). The present observations of peritubular mitotic cells and Leydig cells at varied developmental stages also agree with earlier studies in which mitoses and differentiation of Leydig cells and their precursors have been observed in immature rats stimulated with gonadotropins (Chemes et al, 1976; Kerr and Sharpe, 1985). The close peritubular location of the dividing cells suggests that the Sertoli cells in the transplants are stimulated by the host gonadotropins to produce local mitogenic factors. This suggestion agrees with earlier studies, where seminiferous tubules have been reported to regulate Leydig cells by secreting paracrine factors (Sharpe, 1986).

Postnatal rat fetal-type Leydig cells in distinct clusters surrounded by delicate enveloping cells are

generally known to have spherical nuclei, abundant smooth endoplasmic reticulum and numerous cytoplasmic lipid inclusions (Roosen-Runge and Anderson, 1959; Lording and de Kretser, 1972; Tapanainen et al, 1984; Mendis-Handagama et al, 1987; de Kretser and Kerr, 1988; Kerr and Knell, 1988; Kuopio et al, 1989). Developing adult-type rat Leydig cells have been distinguished from the fetal-type cells principally by their lower number of lipid inclusions (Lording and de Kretser, 1972; de Kretser and Kerr, 1988; Kerr and Knell, 1988). Additionally, in contrast to the fetal-type cells, the adult-type Leydig cells are often closely associated with blood vessels and do not appear in the clusters characteristic of postnatal fetal-type Leydig cells (Roosen-Runge and Anderson, 1959; Clark, 1976; Mendis-Handagama et al, 1987; Kerr and Knell, 1988). According to these established differences between the two types of cells, it is evident that the clustered Leydig cells in the present testicular transplants represent the fetal Leydig cell population. It also seems probable that the unclustered Leydig cells with few lipid droplets in the transplants are developing adult-type cells. The simultaneous presence of the fetal- and adult-type Leydig cells in the transplants is consistent with our own and several earlier observations on the normal developing rat testis (Tsai-Morris et al, 1985; Risbridger and de Kretser, 1986; Mendis-Handagama et al, 1987; Kerr and Knell, 1988). There is general agreement that interstitial fibroblasts or mesenchymal cells are stimulated by LH to differentiate into the adult-type generation of Leydig cells (de Kretser and Kerr, 1988). Furthermore, there is still the possibility that some fetal-type Leydig cells also transform to adult-type Leydig cells (Gondos, 1977; Mendis-Handagama et al. 1987), even though a very recent report of Kerr and Knell (1988) does not support this view. Because continuous basement membranes were not found around the fetal-type Leydig cell clusters in normal postnatal rats and have not been reported previously (e.g. Lording and de Kretser, 1972; Kerr and Knell, 1988), the continuous basement membrane observed occasionally in the transplants around the Leydig cell clusters suggests that exposure to chronically elevated gonadotropins stimulates basement membrane formation around fetal-type cells.

In the transplants, specific characterization of the unclustered Leydig cells with numerous lipid inclusions is complicated because these cells have features of both Leydig cell types. We have

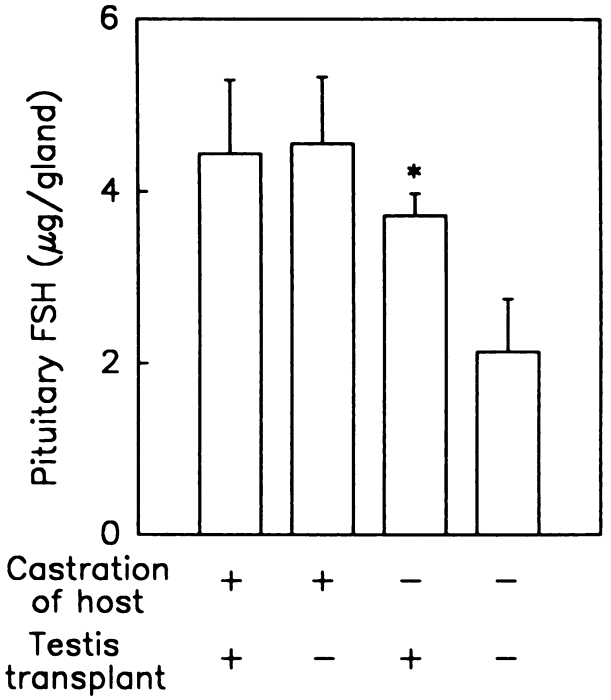

Fig. 9. Pituitary FSH content of the host animals 2 weeks after the transplantation. Each bar represents mean ± SEM. Asterisk indicates statistically significant difference from the respective controls, *$p < 0.05$, student's t-test.

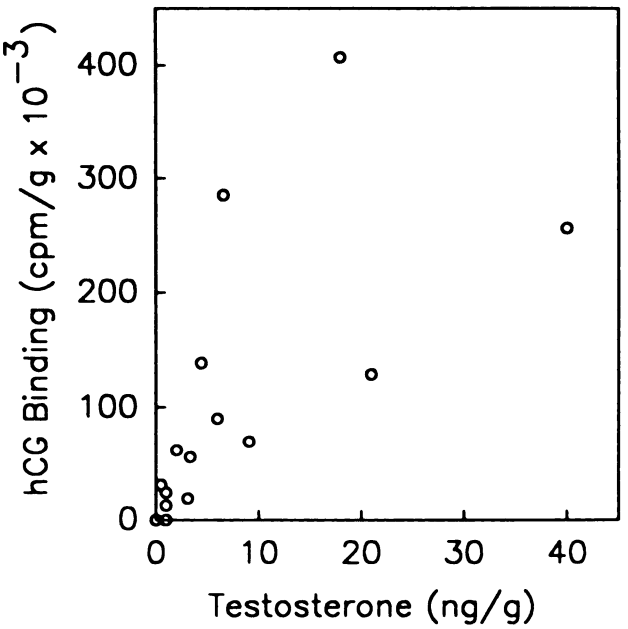

Fig. 10. Tissue T and LH receptor concentrations (reported as hCG binding) of testis transplants. A significant ($p < 0.01$) positive correlation ($r = 0.65$) was observed between testosterone and LH receptor levels.

previously shown that a single injection of hCG increases the number of the fetal-type cells in newborn rat testis (Kuopio et al, 1989). It is possible that some of the fetal-type Leydig cells in the transplanted testis lose their typical clustered organization when they increase in number during chronic gonadotropin stimulation. Another possibility is that a chronic gonadotropin stimulus accumulates lipid in the developing adult-type cells. The latter assumption is consistent with the observations of Nussdorfer et al (1980), who reported a significantly increased amount of lipid in adult rat Leydig cells after chronic hCG treatment. Definite characterization of the unclustered Leydig cells with large quantities of lipid inclusion remains for later studies, where their role as a possible transition phase from fetal- to adult-type Leydig cells should be clarified.

The testis transplants proved to be steroidogenically active and responsive to gonadotropin stimulation. Testosterone was secreted by the transplants in the castrated animals to the extent of increasing seminal vesicle weights and decreasing pituitary LH content. Obviously, such effects were masked by the testicular activity of the intact animals transplants.

The increase of pituitary FSH content in the uncastrated animals bearing transplants was a surprising finding. In fact, a reverse effect of the transplant could have been expected, since the 10 to 20-day-old testis is an especially rich source of inhibin (Ultee-van Gessel and de Jong, 1987). Because inhibin can appear in two forms, one inhibiting and the other (activin) stimulating pituitary FSH secretion (Ling et al, 1986; Vale et al, 1986), the release of an FSH-stimulating principle from the transplanted testis is not impossible. By and large, however, the physiology of FSH regulation is still poorly known, and our findings on a transplant-associated FSH increase remains to be substantiated.

In conclusion, the present kidney subcapsular transplantation technique meets the following criteria set for a successful transplantation: (1) continuation of development of the newborn rat testis after transplantation, (2) production of testosterone by the transplant Leydig cells, (3) participation of the transplant in pituitary-gonadal interactions, and (4) maintenance of host gonadotropin responses in the transplant. These results show that the transplantation technique can be used as an experimental model to study the structure and function of the neonatal testis, including fetal and adult Leydig cells, under different endocrinologic conditions.

Acknowledgments

The authors wish to thank Mrs. R. Andersén, Mrs. S. From, Mrs. M. Huhtinen, Mrs. L. Salminen, Mr. U. Reunanen and Mr. L. H. Wikgren for technical assistance.

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