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Developmental Biology

Allografts of tumor nuclear transplantation embryos: Differentiation competence

(Lucké renal carcinoma/cloning/specialized cell nuclei/genetic multipotentiality)

JEANNE MARIE LUST*†, DEBRA L. CARLSON*, RICHARD KOWLES‡, LOUISE ROLLINS-SMITH§, JOHN W. WILLIAMS III¶, AND ROBERT G. MCKINNELL*‖

*Department of Genetics and Cell Biology, University of Minnesota, Saint Paul, MN 55108; ‡Biology Department, Saint Mary's College, Winona, MN 55987; §Division of Pediatric Immunology, Vanderbilt University, Nashville, TN 37232; and ¶Department of Biology, Tuskegee University, Tuskegee, AL 36088

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ABSTRACT **The developmental potential of nuclei can be studied by nuclear transplantation. Although amphibian blastula nuclei and other early embryonic nuclei are totipotent, to our knowledge no nucleus from an adult cell has ever been shown to be totipotent by this procedure. Transfer of Lucké renal carcinoma nuclei into enucleated eggs results in prefeeding swimming tadpoles. Inasmuch as these tadpoles die, rescue of this pluripotential tissue was attempted by grafting fragments of triploid tumor nuclear transplant tadpoles to the tails of normal diploid *Rana pipiens* hosts. Grafts of tumor nuclear transplant tadpole tissue were histologically indistinguishable from grafts of normally fertilized embryos and developed normal-appearing structures such as complete eyes, well-differentiated neural tissues, kidney tubules, and gut epithelium. Moreover, histological differentiation in tumor nuclear transplant grafts was comparable to that observed in 50-day-old normal larvae. Grafting enhanced the survival of tumor nuclear transplant tissue from no more than 14 days as part of the donor tadpole to 40 days at which time the grafts were harvested as healthy tissue. Thus, both differentiation and survival of tumor nuclear transplant tissue were augmented with the grafting procedure. Cytophotometric analysis of ploidy was used to confirm the tumor origin of the donor tissue.**

The amphibian nuclear transplantation procedure was designed to characterize the competence of a genome to program for embryonic and adult differentiation (1–3). Totipotency of blastula nuclei was demonstrated by the nuclear transplant frogs, which produced normal progeny (4, 5). Similar studies with mammalian embryos indicate that their early cleavage nuclei give rise to normal adults (6, 7). There is a progressive loss of developmental potency in amphibians associated with increased age of the nuclear donor (8) such that no normal frog has ever ensued from the transplantation of a nucleus derived from an adult donor. Recently it has been shown that differentiation obtained by the conventional transplantation procedure of transfer of a nucleus to an enucleated mature ovum can be enhanced by prior exposure of the donor nucleus to oocyte cytoplasm (9). We report here the enhancement of the developmental potential of nuclear transplant embryos by allografting fragments of these embryos to normal host embryos.

The North American leopard frog, *Rana pipiens*, can be afflicted with a herpesvirus-induced renal carcinoma (10). The tumor is malignant as evidenced by its invasiveness (11, 12) and by its propensity for metastasis (13–16). Frog renal carcinoma nuclei, when transplanted into activated and enucleated eggs, form embryos (17) and swimming tadpoles that fail to feed (18). In the present study, we sought to ascertain if the limited pluripotentiality of the tumor nuclear transplant (TNT) tadpole is due to a genetic limitation imposed on all of the cells of the tadpole, or alternatively, if failure to thrive might be due to one or a few genomic restrictions that lead to improper function in certain tissues, which in turn leads to the death of the tadpole. Our results show that the potential for survival, growth, and differentiation of parts of the tadpole exceeds that of the intact tadpole. Hence, the tumor nuclear transplant cells have not undergone irreversible changes that prevent them from responding to the host environment.

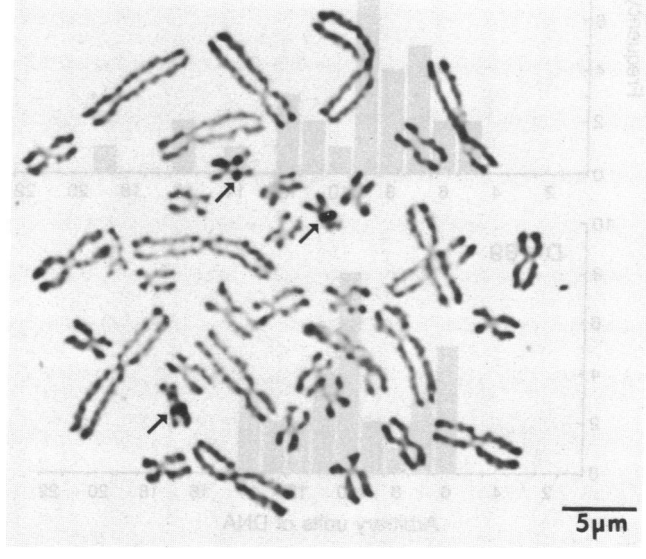

FIG. 1. Silver-stained chromosomes from a triploid, $3n = 39$, renal carcinoma of *R. pipiens*. Arrows indicate the 10q location of three active nucleolar organizer regions as expected in triploid cells.

This study relates not only to nuclear potential as assayed by transplantation but also to the question of whether malignant transformation is due to an irreversible genomic lesion. Many forms of malignancy may be related to genetic defects (19). However, normal differentiation may ensue from cancer cells despite a genetic lesion (20), and many tumor cells can be differentiated to their nonmalignant mature tissue counterpart (21–24). In this study, we report the normal differentiation of a *diversity* of tissue types that are mitotic progeny of a neoplastic genome. Moreover, these differentiated tissues represent all three embryonic germ layers and are not limited to the tissue of origin of this tumor.

MATERIALS AND METHODS

Triploid Renal Carcinomas. Triploid *R. pipiens* embryos were produced by retention of the second polar body shortly

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Abbreviation: TNT, tumor nuclear transplant.

†Present address: College of Saint Benedict, Saint Joseph, MN 56374.
‖To whom reprint requests should be addressed.

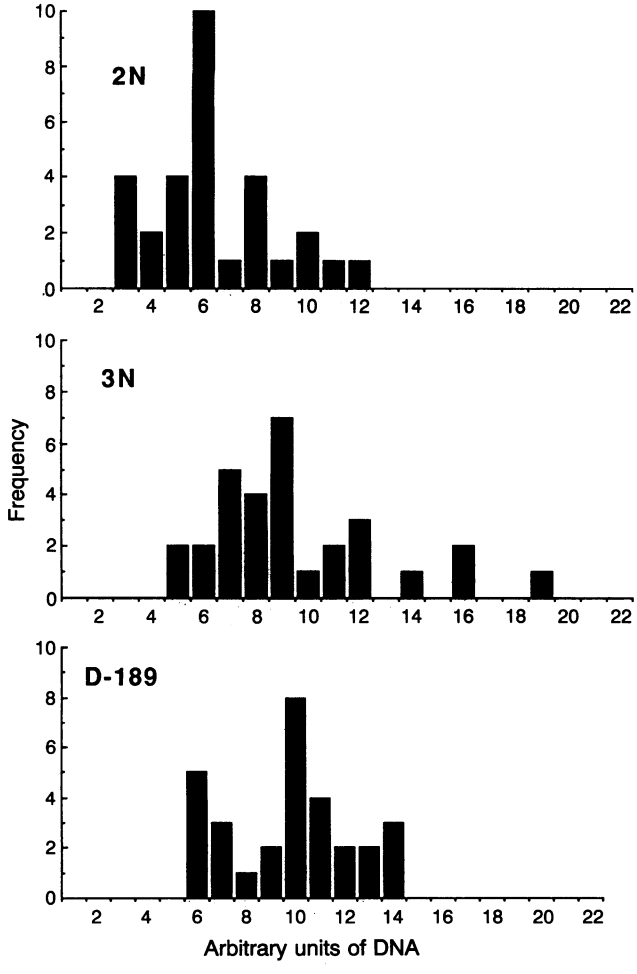

FIG. 2. Representative histograms showing DNA measurements obtained from TNT nuclei compared with nuclei from 2*n* and 3*n* control embryos. Statistical tests for comparing the differences in the means of the TNT and control nuclei confirm the triploidy of the TNT cells and thus their tumor origin.

after insemination of mature eggs with a sperm suspension. The haploid polar body fuses with the diploid zygote nucleus, producing triploid embryos. Polar body retention was induced by hydrostatic pressure of 6000 psi (1 psi = 6.89 kPa) for 6 min, 4 min after insemination (25). The triploid embryos were injected at prefeeding stages with a tumor cell fraction containing Lucké tumor herpesvirus (26, 27). The renal carcinomas, which arose in the triploid animals just prior to metamorphosis, were expected to be triploid (28). Triploidy of the carcinomas was established by chromosome counts of metaphase plates prepared according to Picciano and McKinnell (29) and silver stained (30) to demonstrate active nucleolar organizer regions.

Nuclear Transplantation. Dissected renal carcinomas were dissociated in modified (calcium- and magnesium-free) Steinberg's solution (2). Dissociated cells were drawn into a micropipette and subsequently inserted into a previously activated and enucleated ovum. The trauma of drawing a tumor cell into the small bore of the micropipette ruptures the plasma membrane, which liberates the nucleus to interact with the cytoplasm of the egg (2, 31). Triploid nuclear transplant embryos developing from recipient ova were cultured in 10% Steinberg's medium until grafted to diploid normal hosts of the same age.

Grafting Procedure. Jelly and vitelline membranes were removed from both donor and recipient embryos (Shumway stage 17; ref. 32) in 100% Steinberg's solution fortified with antibiotics (penicillin at 100 units/ml, streptomycin at 100 μg/ml, and amphotericin B at 0.25 μg/ml). Fragments of donor tissue, in the form of small cubes, were taken from eye, gill, pronephric, heart, brain, and gut forming areas. Each fragment was placed in the tail mesenchyme after an incision was made in a recipient embryo. The fragment was held in position by a small piece of coverglass positioned over the operated area. Healing generally took place in several hours, after which time the animals were transferred to 10% Steinberg's solution containing antibiotics (same concentrations as given above). When host animals had a completed operculum (Shumway stage 25; ref. 32), they were reared in dechlorinated water until the grafts were harvested. Evidence of rejection (lymphocytic infiltration of tissue) was observed in normal diploid and triploid control and experimental grafts harvested after 40 days. Accordingly, the data presented in this study are from grafts maintained for 40 days or less. Harvested grafts were fixed in 10% neutral buffered formalin, sectioned, and stained with hemotoxylin/eosin.

Table 1. Tissues differentiating in allografts of TNT embryos

	Donor embryos					
	D-11	D-157	D-189	167	171	D-288
Ectoderm						
Normal eye with lens		+	+	+		
Abnormal eye	+			+		
Olfactory epith.	+	+	+	+		
Auditory vesicle	+	+		+		
CNS cells	+	+	+	+		+
Ganglion cells			+			
Ependymal cells	+	+	+	+		+
Mesoderm						
Kidney tubules	+	+	+	+	+	
Kidney blastema				+	+	
Hematopoietic tissue				+		
Erythrocytes	+			+		
Cardiac cells	+	+	+	+	+	+
Cartilage	+	+	+	+	+	+
Smooth muscle			+	+		+
Endoderm						
Goblet cells			+	+		
Cuboidal epith.	+	+	+	+		+
Ciliated cuboidal epith.			+			
Columnar epith.		+		+		+
Ciliated columnar epith.			+	+		
Cuboidal/columnar ducts			+	+		+
Pancreas			+	+		+
Liver		+	+			
Stomach		+	+			
Pharyngeal epith.	+	+	+	+	+	
Gill epith.	+	+	+			+
Thymus		+	+	+	+	

CNS, central nervous system; epith., epithelium. A plus sign indicates detection of the given tissue type.

Confirmation of Ploidy of Donor Embryo. Small fragments of donor embryos, retained after grafting, were fixed in 10% buffered formalin, sectioned, and stained according to the Feulgen procedure. Diploid and triploid control tissues were fixed and stained at the same time for comparison. The relative quantity of DNA in the graft and control tissues was ascertained by microdensitometry using the two-wavelength method of Mendelsohn (33). Random nuclei were measured for percent transmission at 495 and 570 nm with a Zeiss photomicroscope III equipped with a MPM 01K photometer. The measurements were converted to arbitrary units of DNA according to the formula of Patau (34). The mean values of the DNA content were calculated, and the grafted donor embryo DNA was compared with the DNA values of diploid and

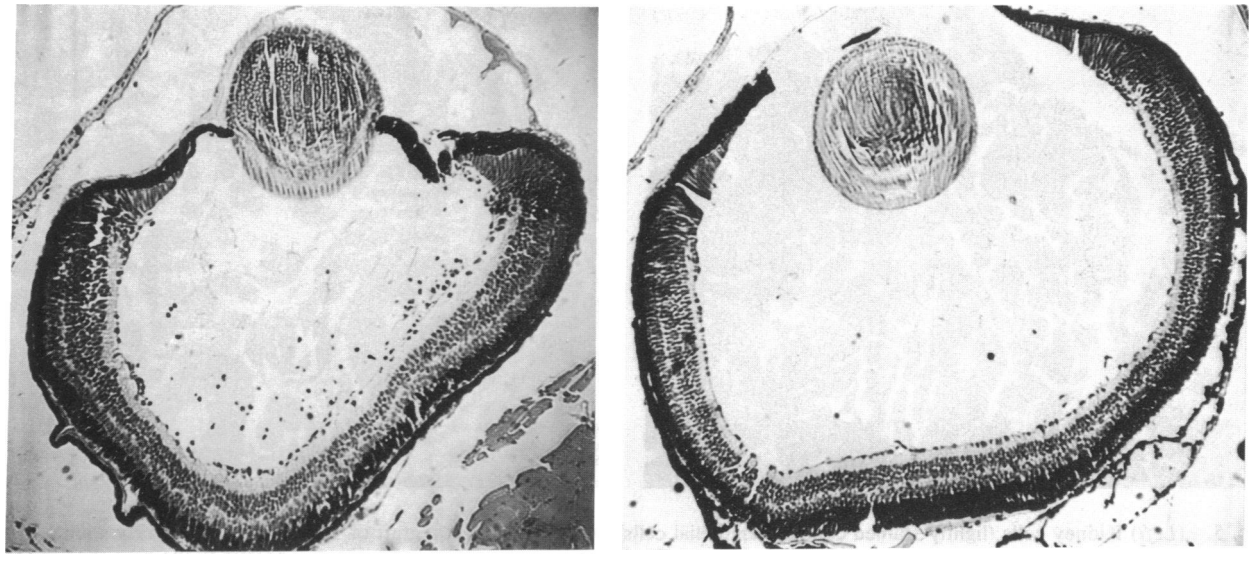

FIG. 3. Lens and optic cup that developed in a graft of TNT tissue (*Left*) and in a graft of normal tissue (*Right*). (×65.)

triploid embryos of the same age by using two statistical tests (Student's *t* test and Kolmogorov–Smirnov test).

RESULTS

Induced Lucké renal carcinomas were ascertained to be triploid ($3n = 39$) by chromosome count and by the presence of three active nucleolar organizer regions (Fig. 1). TNT embryos, produced by transplantation of triploid carcinoma nuclei, were confirmed to be triploid (as was expected) by Feulgen cytophotometry (Fig. 2). Six triploid TNT embryos and several normal control triploid embryos provided the tissue for the grafting of this study.

Among the 82 surviving hosts that retained grafts, 70 animals had persisting TNT donor tissue that differentiated to organs and tissues of all three embryonic germ layers. Table 1 lists the diverse tissues and organs that differentiated in the grafts as revealed by histological examination. All of the tissue types previously observed in ungrafted TNT tadpoles were detected in grafted tissue, with the exception of epidermis and skeletal muscle. More importantly, numerous tissues and organs such as kidney, gut, eyes with lenses, gills, olfactory epithelium, and auditory epithelium, which differentiate poorly or are not found at all in the ungrafted TNT tadpole, differentiated normally in the grafts. In addition, the rhythmic contractions of cardiac tissue and slow peristalsis of the gut were clearly visible in some grafts. These contractions witnessed to the functional capacity of these tissues prior to histological examination of the graft.

Grafting of tissues from the experimental tadpoles not only resulted in differentiation of a greater number of tissues but also resulted in increased survival time for the tissue. The maximum survival time exhibited by a TNT tadpole thus far is 14 days. Tissues survived up to 40 days after grafting and still appeared normal and healthy when they were taken for fixation.

Grafts of TNT embryos, made at the same stage as the grafts of control triploid embryos, were judged to be normal and indistinguishable by histological analysis. For example, the lens and optic cup of a TNT embryo (Fig. 3 *Left*) were morphologically normal and similar to control lens and optic cup (Fig. 3 *Right*). Further, examination at higher power showed that the choroid, pigmented epithelium, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, and ganglion cells of the TNT optic cup (Fig. 4 *Left*) were not discernibly different from the structure of the optic cup of a control graft (Fig. 4 *Right*). Kidney tubules in TNT grafts (Fig. 5 *Left*) were of simple cuboidal epithelium and showed no evidence of a neoplastic transformation and resembled kidney tubules of control grafts (Fig. 5 *Right*).

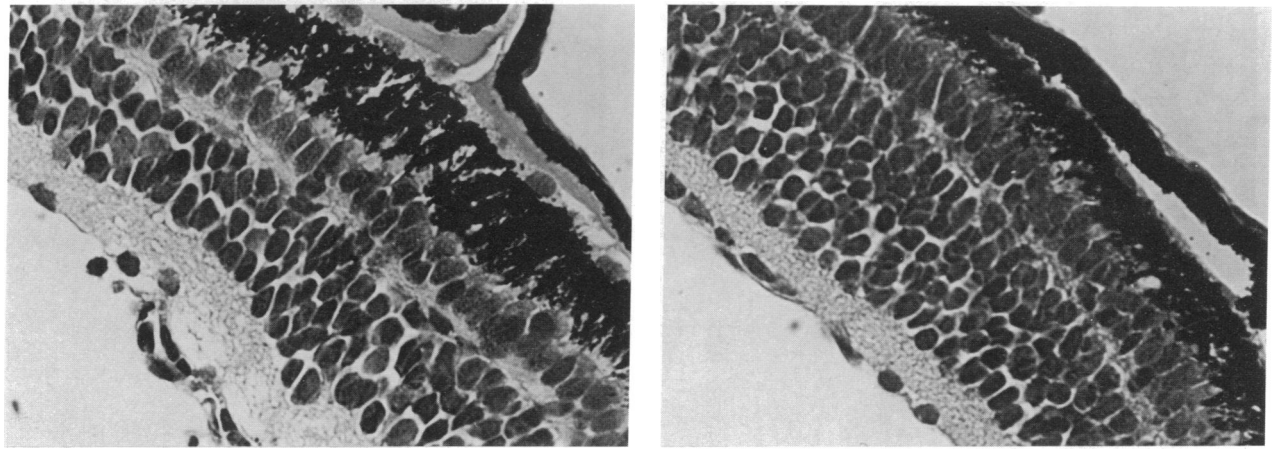

FIG. 4. Retina from a TNT tadpole allograft (*Left*) and a control allograft (*Right*). Choroid, pigmented epithelium, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, and ganglion cells can be identified from the upper right to the lower left. (×400.)

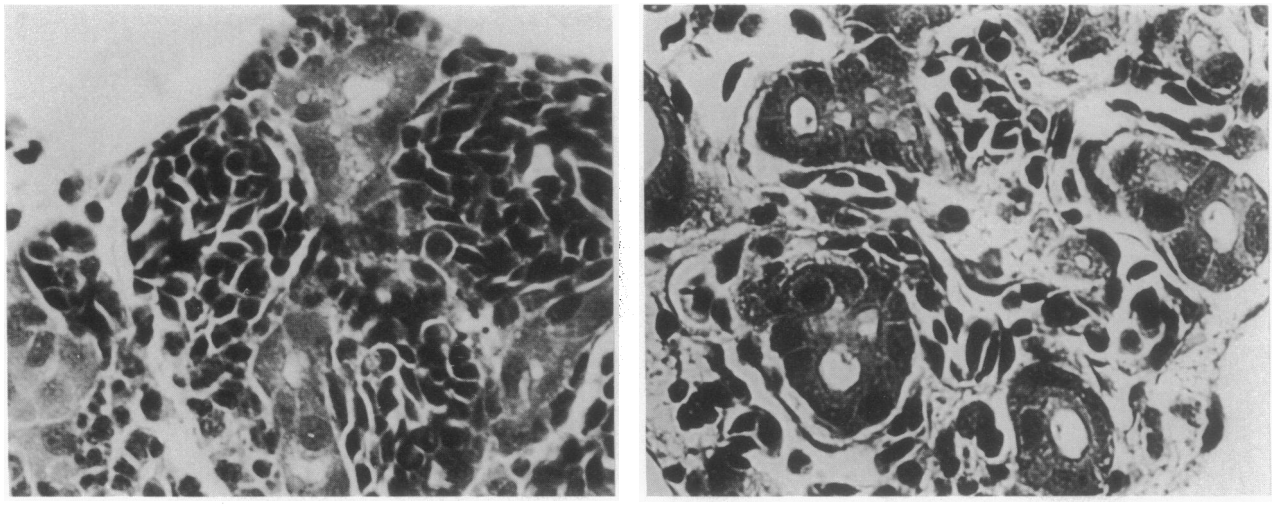

FIG. 5. (*Left*) Kidney cells (lightly stained cuboidal epithelial cells) that developed in a graft of TNT tissue. The darkly stained cells are hematopoietic tissue. (*Right*) Kidney cells that developed in a graft of normal tissue. There is less hematopoietic tissue here than in the graft of TNT tissue (*Left*). (×450.)

Liver in TNT grafts (Fig. 6 *Left*) had a vacuolated cytoplasm not unlike that of the liver in control grafts (Fig. 6 *Right*).

DISCUSSION

The results of this study demonstrate that the conventional nuclear transplantation procedure in frogs (1, 2, 31, 35) reveals only a portion of the histogenetic potential of the genome of a mature cell. In past studies, conventional TNT resulted in tadpoles with limited differentiation capacity and the inability to feed. In the present study, tissue fragments of TNT embryos survived for at least 40 days as allografts, which grew and differentiated as well as grafts of control embryonic tissue. However, these studies do not reveal whether the developmental limitation of ungrafted TNT embryos should be ascribed to neoplastic changes in the genome of the donor cell or to the inability of egg cytoplasm to reactivate the entire genome of the transplanted nucleus.

The development described here may not be the limit of the histogenetic potential of TNT tissue. The grafts were harvested prior to the time when they would be rejected by the immune response of the host. As noted in *Materials and Methods*, control grafts maintained for periods in excess of 40 days were infiltrated by host lymphocytes and were rejected. Two procedures are available, which may elicit further differentiation to the adult state: (*i*) rendering hosts immunologically compromised by thymectomy (36) and (*ii*) inducing tolerence by simultaneously grafting the hematopoietic stem cell compartment from the donor embryo (37, 38).

Allografting of nuclear transplant animals is not the only method of enhancement of differentiation potential. Another promising procedure is the prior exposure of a mature genome to the cytoplasm of a first meiotic oocyte followed by transfer to egg cytoplasm. Erythrocyte nuclei, so treated, have the competence to program for the development of swimming larvae that feed and manifest limb buds (9). Because there are at least two distinct procedures that allow for added expression of genomic potential, it is yet premature to conclude that selected adult nuclei cannot someday be shown to be totipotent. It is clear, however, that the conventional nuclear transplantation procedure developed almost 40 years ago does not fully characterize the developmental potency of older nuclei.

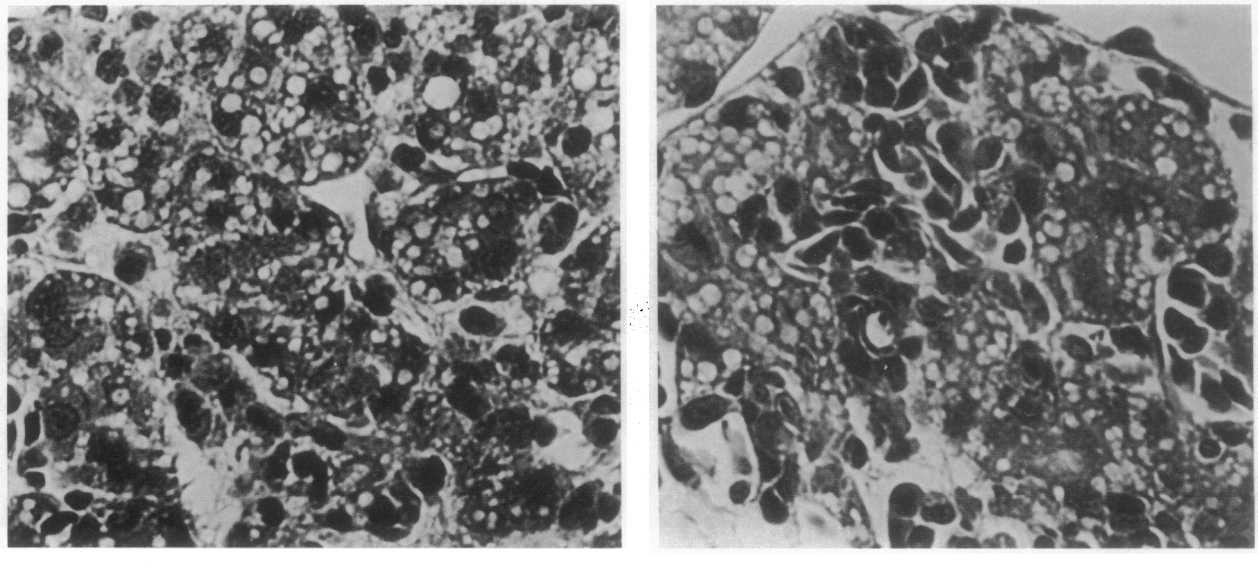

FIG. 6. Hepatic tissue that developed in a graft of TNT tissue (*Left*) and in a graft of normal tissue (*Right*). The hepatocytes have a vacuolated cytoplasm. (×550.)

The present study relates also to the stability of the malignant phenotype. A number of cancers have been shown to be competent to produce mitotic progeny that are benign and, in some cases, fully differentiated (21–24). A common interpretation of cancer studies is that the malignant cells are aberrant stem cells that are blocked prior to terminal differentiation. The various treatments designed to induce differentiation overcome the interrupted steps to tissue maturity. Thus, colon cancer cells can be induced to differentiate to mature colon cells, neuroblastoma cells differentiate to mature nerve cells, and squamous carcinoma cells differentiate to keratin-containing differentiated epithelial cells. Exposure of the Lucké renal carcinoma genome to egg cytoplasm does not induce maturation of a renal stem cell. Rather, the egg cytoplasm reprograms the tumor cell genome to mimic the genome of the zygote. Of course, a zygote is totipotent. We have here augmented the pluripotency of a tumor genome (18) to include the normal development of a number of cell types not previously reported. Further, the survivability of TNT cells is extended ≈3-fold, and histological differentiation of the grafted tissue is similar to that of a 50-day-old free-swimming larva (a 50-day-old larva is approximately halfway to metamorphosis). Totipotency of the rescued cancer genome cannot be claimed until adult cell differentiation of all cell types, including functional gametes, is demonstrated.

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