Successive generations of mice produced from an established culture line of euploid teratocarcinoma cells

(microinjection into blastocysts/developmental totipotency/germ-line transmission)

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ABSTRACT The possibility of utilizing mouse teratocarcinoma stem cells as intermediaries for production of new strains of mice with preslected mutant or foreign genes requires that, after propagation in culture (to allow for genetic manipulation and selection), the cells be capable of normalization and orderly development in carrier embryos and, ultimately, of germ-cell formation. Heretofore, no in vitro cell line has fulfilled all these requirements. A karyotypically normal teratocarcinoma culture line was recently established in this laboratory and now has been investigated as a candidate. The line, designated METT-1, is chromosomally female (X/X) and was obtained from the 129 (agouti-colored) inbred strain [Mintz, B. & Cronmiller, C. (1981) Somat Cell Genet 7, 489–505]. The developmental potential of these cells was tested, after prolonged culture and freezing and thawing, by microinjecting them into early (blastocyst stage) embryos of the C57BL/6 (black) strain. Among 312 experimental animals examined at 1 week of age, there were 41 mice (21 females and 20 males) that displayed the coat colors of both strains (13%), as well as the extent of the coat areas derived from the cell line, greatly surpasses the contributions observed in all previous experiments, whether with other in vitro teratocarcinoma cell lines or with in vivo transplant lines. The developmental totipotency of METT-1 cells became evident from the presence of substantial amounts of 129-strain cells (bearing an isozyme marker) in all internal tissues of an individual whose coat was largely agouti. The culture-cell lineage also proved to be capable of giving rise to reproductively functional oocytes. Of nine mosaic-coat females tested, one produced progeny of the diagnostic agouti color in two litters; these heterozygous F1 offspring in turn transmitted their marker genes to F2 homozygous segregants. Thus, the METT-1 teratocarcinoma line bridges the gap between in vitro cell propagation and in vivo development and between the soma and the germ line. This creates the option of producing new mouse strains with predetermined genetic changes designed as probes of developmental regulation or as models of human genetic diseases.

Mouse teratocarcinoma cells from some transplanted tumors and culture lines have been experimentally channeled into normal development, to a greater or lesser extent (1–4), by placing them in early (blastocyst stage) embryos. Although the cells of one in vitro line (NG 2) have proved capable of contributing to all somatic tissues under these circumstances (5), germ-cell differentiation and progeny have resulted only from two in vivo transplant lines, both of which had appreciable subpopulations of cells with a normal karyotype (1, 2, 6). Of a total of three animals with tumor-strain germ cells and offspring, two were males from a chromosomally male (X/Y) ascites line (OTT 6050, of the 129 inbred strain) and one was a female from a chromosomally female (X/X) solid-tumor line (72484–395, of the LT strain). Cell culture lines, on the other hand, have shown various karyotypic anomalies that—even in the best-developing case (5), characterized by a trisomy (6)—would be incompatible with production of viable offspring (7).

Realization of the scheme that has been proposed for utilizing cultured teratocarcinoma cells as vehicles for introducing preselected new genotypes into mice (8–10) therefore depends on first obtaining an in vitro line with not only somatic but also germlinal totipotency. Specific genetic changes could then be introduced during the culture phase, incorporated into entire animals, and transmitted to subsequent generations. Teratocarcinoma stem cells with selected mutations (e.g., refs. 5, 11, and 12) or stem cells that have incorporated cloned foreign genes (13) have in fact been successfully isolated from cultures and found to have retained the capacity for somatic development. It remains for a culture line with germinal potential to become available.

A candidate cell line, designated METT-1, was recently established for the purpose (14). It was obtained from an embryo-derived malignant teratocarcinoma of the 129/Sv (or 129) agouti-colored inbred strain. Its promising features are karyotypic normalcy (the chromosomal sex is X/X female), stability, feeder-independence, and ability to differentiate in cultures or tumors. We now report that METT-1 cells can form all somatic tissues in host embryos; and they can also produce germ-line progeny, even after more than 100 cell doublings in vitro, and after freezing and thawing. Thus, the cells of this line can bridge the gap between asexual propagation in vitro and sexual reproduction in vivo—the necessary preamble for engineering new mouse strains to be used as probes of developmental regulation or as models of human genetic diseases.

MATERIALS AND METHODS

Injections into Blastocysts. Teratocarcinoma stem cells of the METT-1 line (14) were used after they had been cultured for nine or more passages since the initial explant, frozen and stored in liquid nitrogen, thawed, and cultured for seven or more additional passages. The cells were then partly dissociated in 0.25% trypsin-EDTA and Ca2+- and Mg2+-free phosphate-buffered saline and rinsed. Three to five cells were injected per blastocyst, of the C57BL/6 (or C57) inbred strain (Icr subline), as described (1). After a few hours' incubation in the cell-culture medium (14), the blastocysts were surgically transferred to uteri of pseudopregnant Icr random-bred albino females for development to term.

Analyses of Tissue Genotypes. The agouti-focus hair-follicle marker enabled donor-derived (agouti) areas to be distinguished from blastocyst-derived (nonagouti, or black) regions in the

Abbreviations: GPI, glucosolphosphate isomerase; MUP, major urinary protein complex.

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coats of 1-week-old animals. The A<sup>am</sup>, or so-called white-bellied agouti, allele in the 129 strain produces a relatively light (not white) ventrum compared with the standard agouti. Homogenates of internal tissues were tested by starch gel electrophoresis for Gpi-I-locus strain variants of the ubiquitous enzyme glucosephosphate isomerase (GPI; d-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9) (15). Blood-cell lysates were analyzed by electrophoresis in cellulose acetate (Cellogel) for hemoglobin variants (Hbb locus) (16) or, in a few cases, for GPI isozymes. Liver genotypes were revealed in living animals by testing urine samples for variants of the major urinary protein complex (MUP) synthesized in hepatocytes and encoded by the Mup-I locus. The polyacrylamide gel procedure described for MUP (17) was modified in that the urine was first dialyzed against the running buffer for 12 hr, and the vertical gel electrophoresis apparatus was replaced by a thinner (1.2 mm) slab gel; these changes resulted in minor modifications in the protein patterns.

RESULTS AND DISCUSSION

Survival and development to birth occurred in 31% (390/1258) of the injected and surgically transferred blastocysts. This rate compares favorably with the 26% (48/183) survival to term obtained in an earlier experiment (also involving injections of three to five nonmutant teratocarcinoma cells of the 129 strain) in this laboratory (1); the present yield is all the more noteworthy inasmuch as the cells were from an established line that had undergone many cell doublings in culture, as well as freezing and thawing (Fig. 1), whereas the cells in the previous study had never been cultured and were taken directly from the ascites embryoid bodies of transplant hosts.

The decision was made to abridge the search for 129-strain tissue contributions in the large experimental population by basing the initial screening on the most readily detected marker—agouti color in the coat. Animals with coat mosaicism were then further analyzed. Only a limited number of all-black individuals were screened for internal mosaicism; inasmuch as some positive cases were found, it is likely that additional positives escaped detection.

The recognition of cases with culture-derived cells was probably also skewed toward underestimation by the effects of a severe and prolonged pandemic of Sendai virus infection in the animal colony during the period that most of the animals in this series were born. The 129 strain has been found to be by far the most sensitive to Sendai virus infection, among many strains tested (18). Young animals are at especially high risk, as was evident among controls. Thus, mosaic individuals with appreciable numbers of 129-strain cells may well have been more vulnerable than animals from the same experiment with only C57BL/6 cells. Characteristic lung lesions were revealed in a number of experimental animals at autopsy. Attrition remained high until a stringent vaccination and isolation program began to take effect. Meanwhile, the size of the experimental population had dropped from 390 at birth to 312 at 1 week and 242 at 4 weeks of age; many that died were no longer suitable for GPI tissue analyses.

At 1 week of age, despite the handicap to the 129 strain, as many as 41 animals (21 females and 20 males), or 13% of the 312 survivors, clearly had areas of agouti hairs characteristic of the 129 strain in their coats (Fig. 2). Agouti and nonagouti (black) components varied in extent. In regions that were not heavily agouti, the individual narrow transverse bands representing hair-follicle clones could be seen, as in patterns found in allopheic mice produced by aggregating blastomers of two strains with agouti-locus markers (19). This frequency of cases with tumor-lineage contributions in the coat is notably high in comparison with all earlier experiments. In this laboratory, combined results from blastocyst injections with euploid teratocarcinoma cells of the OTT 6050 (129-strain) transplant line yielded 8%, and those with euploid tumor transplant sublines of the LT strain gave 6% of survivors with coat mosaicism (refs. 1–3; unpublished data). In other laboratories, the OTT 6050 ascites transplant line yielded only 1 coat mosaic (out of 60 mice) (4); various aneuploid cell culture lines gave 0–4% (3, 20, 21), and the euploid C143b culture line yielded none (22). Moreover, the actual number, 41, of coat mosaics (among our 312 mice) is much greater than the grand total of all previous studies combined (estimated, from the available reports, at approximately 22 mice among some 1000 animals examined).

Fig. 1. Diagrammatic summary, starting at the upper right, of the experiment. A malignant teratocarcinoma arose from a 6-day embryo of the agouti-colored 129 inbred mouse strain that had been grafted under the testis capsule of a syngeneic host. The solid tumor was dissociated and explanted (without a feeder layer); it yielded a culture line of teratocarcinoma stem cells. The line, later designated METT-1, was karyotypically normal and chromosomally female (X/X) (14). The cells were frozen and stored in liquid nitrogen. After thawing, propagation in culture continued (in this case, until a total of approximately 112 cell doublings since explantation). Blastocysts of the C57BL/6 (black) strain were then microinjected with cells from the culture, and the embryos were transferred to the uterus of a pseudopregnant foster mother for development to term. One of the two-color (striped) mosaic females that was born had tumor-derived normal cells (along with embryo-derived cells) in her germ line (and other tissues): when she was mated to a C57 male, she produced some F<sub>1</sub> progeny with the dominant agouti color of the 129 strain. A subsequent mating between an F<sub>1</sub> female and an F<sub>1</sub> male (both heterozygous for marker genes) yielded F<sub>2</sub> offspring among which were segregants homozygous for the tumor-strain alleles. These animals are wild-type models of a "new strain" that might be similarly derived from METT-1 cells with a preselected specific mutation of interest.
Even more striking is the fact that, in most of the two-color animals from this METT-1-cell series, the proportion of tumor-lineage cells in their coats is substantially greater than has been found in comparable experimental animals produced with teratocarcinoma cells from other sources. Most of the latter have contributed 10% or less of the tumor-strain color, whereas the animals in the present study average 28% agouti. 15 mice were individually graded as having 5–15% agouti, 17 had grades ranging from 20% to 35%, 3 had from 40% to 55%, 5 had from 60% to 75%, and 1 had 85% agouti. Only one mouse in any earlier report (1) has exceeded this, with a grade of 90%. Examples of METT-1-derived animals with 30–75% agouti coats are shown from the ventral or side view in Fig. 2; all have additional agouti on the back.

Developmental totipotency of the METT-1 cells was revealed by GPT analyses of internal tissues from the mouse with 85% agouti in its coat. All 12 tissues tested had substantial amounts of the 129-strain enzyme variant (Fig. 3); amounts ranged from an estimated 10% in the pancreas to 65% in the brain, and averaged 43% overall. It is of interest that approximately 40% of the reproductive tracts (including ovaries, oviducts, and uterus) was of the tumor-strain type. Although such isozymic indicators are not specific for the germ cells (23), the result is consistent with the possibility that the animal, a female, was a germinal mosaic with some oocytes from the teratocarcinoma-cell lineage. The mouse was not mated; it died at 11 days of age, apparently a victim of Sendai virus infection. The METT-1 cells used to produce this animal from an injected blastocyst had a history of 9 passages in culture before freezing in liquid nitrogen and 13 passages after thawing—the total comprising approximately 80 cell doublings in vitro during which totipotency had been retained.

Functional germ cells of the 129 strain could occur here only in phenotypic females: the X/X constitution of the METT-1 cell line would preclude formation of gametes in males (23). Nine of the two-color females remained healthy to breeding age and were test-mated to C57BL/6 males. Progeny from any METT-1-derived oocytes would thus be agouti—the dominant color. The nine had a total of 329 offspring (19–52 per female) during the test period. One of the mosaic females, whose coat was 65% agouti (Fig. 4), did in fact produce 3 all-agouti babies (out of 48 total progeny): 2 females in her second litter and 1 male in her fifth litter. Both F1 females were mated at sexual maturity with their agouti male sib and have had normal F2 offspring that included agouti animals. Members of the successive generations are shown in Fig. 4. The characteristic light-bellied agouti phenotype of the 129 strain is visible in the F1 and F2. The continuity of their germ line is thus traceable to embryo-derived (24).

![Fig. 2](image1.png)

**Fig. 2.** Five examples of animals experimentally produced from C57 blastocysts injected with cultured teratocarcinoma stem cells of the established METT-1 line. All five (four females, one male) show appreciable amounts (30–75%, in these cases) of the agouti color of the tumor-cell lineage (129 strain). (A sixth case is shown in Fig. 4.) Narrow transverse bands of agouti or black (nonagouti) hair-follicle clones (19), in which the agouti-locus genes are expressed, are visible in some areas. (All the animals have additional agouti on the back.)

![Fig. 3](image2.png)

**Fig. 3.** GPI allelic strain variants in starch gel electrophoresis of tissue extracts from an experimental female whose coat was 85% agouti. All 12 internal tissues tested included cells derived from the culture-line source, as seen with the GPI marker. Teratocarcinoma stem cells of the METT-1 line are therefore developmentally totipotent—i.e., they can be normalized and contribute to development of all tissues (see also Fig. 4) in an embryo environment.

![Fig. 4](image3.png)

**Fig. 4.** Three successive generations of mice linked by reproducibly functional germ cells traceable to the METT-1 culture line of teratocarcinoma (somatic) cells. The female at the left, whose coat has some agouti clones of the 129 (tumor-strain) color, was mated to a C57 black male and produced the all-agouti F1 offspring in the center. The F2 agouti animal, at the right, also exhibits the color of the tumor strain that was transmitted from the F1. The relatively light belly in the F1 and F2 is the characteristic phenotype of the A" allele in the 129 strain.
neoplastic somatic cells (25) grown in culture (14)—in this instance, for 9 passages, followed by freezing and prolonged storage in liquid nitrogen, thawing, and 20 more culture passages—or for a total of some 112 cell-doublings in vitro. During that time, although the cells’ genes functioned in the transformed mode, and most of the genes were silent, their capacity for normally regulated expression had apparently remained intact.

Transmission of specific genes from METT-1 cultured cells (via a mosaic female) to F1 heterozygotes and F2 homozygotes in the present instance involves only the 129-strain wild-type alleles. Nevertheless, recovery of those alleles serves as a model for experiments in which tumor cells with preselected mutations might function as “surrogate great-grandmothers” of a new homozygous mouse strain. (A new strain with forced heterozygosity at the locus of interest could of course be initiated earlier, from the F1.) Thus, although the diffuse hemoglobin type of the 129 strain was not found in the germinal mosaic female (whose erythrocytes were entirely of the single or C57 hemoglobin type and therefore all blastocyst-derived), those of her germ cells that were tumor-derived did transmit the diffuse hemoglobin allele to all their F1 descendants and, from these, to homozygous diffuse animals segregating in the F2 (Fig. 5 Left). Similarly, the MUP variant of the tumor strain was absent from the urine of the germinal mosaic female, whose liver at autopsy also contained only C57 cells (with the GPI marker); yet her tumor-lineage oocytes generated F1, heterozygotes (Fig. 5 Right). (MUP tests have not yet been carried out on the F2 mice.)

Following these demonstrations of germinal as well as somatic developmental totipotency, the cell line was assigned the designation METT-1, signifying that it is the first in vitro line of “Mouse Euploid Totipotent Teratocarcinoma” cells.

Seven internal tissues of the germinal mosaic two-color female contained GPI of the 129 strain (Fig. 6 Left). (The tested negative tissues, in addition to blood and liver, were thymus, heart, muscle, and salivary gland.) Note that her ovaries show approximately 50% of the 129 type. Two other mosaic-coat females also had substantial GPI of the cell-line type in their ovaries (Fig. 6 Right), although one of these did not reach breeding age and the other (shown in Fig. 2a) developed severe pneumonia (resembling that in Sendai infections) and produced only 33 offspring. Either of these two, or a third case already discussed (Fig. 3), may have had normal germ cells of the 129 strain.

A panel of 12 internal tissues (as in Fig. 3) was analyzed from each of 38 mice with coat-color mosaicism; the 3 remaining animals had died and their tissues could not be analyzed because of autolysis. For each tissue, the number of cases of mosaicism (and the range of percentages of the 129 strain) was as follows: blood, three cases with both 129- and C57-strain cells (5–40% 129-type); spleen, five (15–50%); pancreas, three (10%); liver, one (15%); thymus, three (15–40%); heart, seven (15–60%); lungs, three (10–20%); gonads, four (35–50%); kidneys, two (20–55%); muscle, seven (15–55%); brain, nine (10–65%); salivary gland, two (10–55%). Among these 38 mice, 17 had no detectable mosaicism in tissues other than the coat. This might be accounted for by the fact that all but 1 of the 17 had the lowest grades of agouti in their coats (e.g., 9 had only 5–15% agouti). In the coat, small amounts of agouti are readily seen, whereas with the GPI test small amounts of one strain distributed among various tissues could easily be missed. There is a slight tendency for the mice with higher grades of agouti in the coat to have more internal tissues in which some 129-strain cells are present. However, there are inconsistencies in approximately one-fifth of the cases—e.g., a mouse with 70% agouti in the coat and only one other tissue (heart) with mosaicism; and one with only 20% agouti but four internal mosaic tissues.

Fifteen all-black animals were analyzed and some 129-type GPI was found in the tissues of four. Mosaicism also occurred in 2 of 12 neonates.

A tumor was seen in only one animal in the entire study. It was a large teratocarcinoma in the lower abdominal cavity. Invasion of the dorsal musculature and small metastases in the lung attested to the malignant character of the source cells. The tumor, which was of the 129 genotype, as expected, had formed in an animal with 35% agouti in the coat and no other 129-strain tissues. Presumably, one of the few cells injected into the blastocyst had failed to be integrated into the host embryo and had therefore remained malignant.

The more common event—completely normal embryogenesis of METT-1 cells in blastocysts—is at least partly attributable to their normal karyotype. However, a normal-appearing karyotype could mask many sorts of genetic changes that would be deleterious to development. This in fact may have occurred in other euploid teratocarcinoma cell lines. Five euploid lines have recently been reported. Of these, C145B (22), F1/9, and 1009 all gave only negative results in blastocysts (21), and comparable developmental assays have not been described for the PCC7-S (26) and P10 (27) cell lines.

From our positive results with the METT-1 line, it is apparent that prolonged exposure to a culture environment does not in itself impair the capacity for germinal development of the tumor lineage in host embryos. The frequency of animals with germ-line contributions cannot be estimated from the available data, inasmuch as the single germinal mosaic case was found in
tests of only nine of the candidate females. The entire germ-cell population in a mouse arises from so few primordial germ cells (approximately two to nine) (22), and so few mature gametes are tested in matings of females, that tumor-strain gonias would have to enter the germ line very early in order to be readily detected. Fortunately, however, even a single F1 offspring with the gene change of interest would be sufficient to derive a new strain.

The METT-1 cell line might be used not only to convey pre-selected mutant mouse genes to future generations but also to introduce pure recombinant genes (13). As recently shown (28), recombinant DNA can also be microinjected into eggs and some copies can persist in intact form in the mice that develop. The teratocarcinoma route offers the additional possibility of pre-selection of positive cells and precharacterization of the state and integration site of the DNA while the cells are still in culture. Thus, it may become possible to produce animals with predesigned genetic changes for studies of development and disease.

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