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Derivation of completely cell culture-derived mice from early-passage embryonic stem cells

(pluripotency/tetraploid embryos/chimeras)

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ABSTRACT **Several newly generated mouse embryonic stem (ES) cell lines were tested for their ability to produce completely ES cell-derived mice at early passage numbers by ES cell ↔ tetraploid embryo aggregation. One line, designated R1, produced live offspring which were completely ES cell-derived as judged by isoenzyme analysis and coat color. These cell culture-derived animals were normal, viable, and fertile. However, prolonged *in vitro* culture negatively affected this initial totipotency of R1, and after passage 14, ES cell-derived newborns died at birth. However, one of the five subclones (R1-S3) derived from single cells at passage 12 retained the original totipotency and gave rise to viable, completely ES cell-derived animals. The total *in vitro* culture time of the sublines at the time of testing was equivalent to passage 24 of the original line. Fully potent early passage R1 cells and the R1-S3 subclone should be very useful not only for ES cell-based genetic manipulations but also in defining optimal *in vitro* culture conditions for retaining the initial totipotency of ES cells.**

Embryonic stem (ES) cells are now widely used for introducing targeted mutations and other genetic alterations into the mouse germ line (1, 2). One of the basic requirements for the successful application of these new techniques is to maintain ES cell lines in a state compatible with germ-cell formation, when the cells are introduced back into a host embryo. Germ-line contribution is most likely to occur when cells retain broad developmental potential, contributing extensively to a wide variety of somatic tissues. We have recently developed a system to characterize the full developmental potential of ES cells (3); the system is based on aggregation of ES cells with developmentally compromised tetraploid embryos. In such chimeras the tetraploid component is selected against in all lineages where ES cells are able to differentiate normally, allowing the ES cells to take over the embryo proper and relegating the tetraploid component to the extraembryonic membranes.

We tested the developmental potential of several commonly used ES cell lines, such as D3 (4) and AB1 (5), in this system and found that they were able to support fetal development up to term. However, all such completely ES cell-derived newborns died perinatally (3, 6). To investigate whether this failure to generate viable offspring was intrinsic to all cell lines at all stages of culture, we established four new ES cell lines and tested their pluripotency at different stages from very early passage number. One of the lines (designated R1) produced viable, completely ES cell-derived animals at early passages but lost this ability with extended culture *in vitro*. This cell line was used to study different aspects of maintenance of totipotency during *in vitro* culture.

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MATERIALS AND METHODS

Strains of Mice Used for Embryos. CD1 mice purchased from Charles River Laboratories were preselected for *Gpi-1^b/Gpi-1^b* genotype and set up for breeding to produce GPI-BB albino CD1 animals. Embryos for the tetraploid component of the chimeras were obtained from this stock. To establish ES cell lines, chinchilla 129/Sv females were mated with agouti 129/Sv-CP males, and blastocysts were obtained by flushing the uterus at postcoitum day 3.5.

Establishment of ES Cell Lines. Thirty-eight 129/Sv × 129/Sv-CP embryos were plated individually onto a feeder layer of mitomycin C-treated SNL (5) fibroblasts in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum (FCS; Hyclone) and 2000 units of leukemia inhibitory factor (LIF; ESGRO) per ml in 4-well plates (Nunc). Most embryos hatched and attached to the feeders by day 2 after plating. The inner cell masses (ICMs) were left to grow for 4 more days, when they were mechanically disaggregated in their own wells by using drawn-out Pasteur pipettes. Four to five days later, the cells were transferred into new wells either by trypsinizing or by mechanically disaggregating the undifferentiated colonies. We started counting passage number when we were first able to pass the cells into 35-mm plates (passage 1). The cells were first frozen at passage 5, which was ≈3 weeks after the blastocyst stage. Four cell lines were established, designated R1, R2, R6, and R13.

Production of Tetraploid Embryos. The oviducts of superovulated and mated CD1 (GPI-BB) females were flushed 44–46 hr after treatment with human chorionic gonadotropin to collect late two-cell-stage embryos. The embryos were placed one at a time between two platinum electrodes laid 250 μm apart in M2 medium (7) in the electrode chamber (8). The blastomeres were fused by a short electric pulse (9) (90 V for 100 μsec in M2 medium) applied by a pulse generator (CF 100; manufactured by Biochemical Laboratory Service, Budapest, Hungary).

Production of ES Cell–Tetraploid Embryo Aggregation Chimeras. The fused, tetraploid embryos were cultured in M16 microdrops under paraffin oil at 37°C in 95% air/5% CO_2. Twenty-four hours after fusion, most of the tetraploid embryos developed to the four-cell stage. Only these four-cell-stage embryos were used for aggregation. Zonae pellucidae of the embryos were removed by treatment with acid Tyrode's buffer (10). ES cells (plated at low density 2 days prior to aggregation) were briefly trypsinized to form clumps of loosely connected cells. Clumps of 10–15 ES cells were then sandwiched between two tetraploid embryos in aggregation wells made by pressing a darning needle into the plastic

Abbreviations: ES cells, embryonic stem cells; GPI, glucose phosphate isomerase; LIF, leukemia inhibitory factor; FCS, fetal calf serum.

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Table 1. Developmental potentiality of ES cell lines at early-passage numbers (passages 6–8) tested by ES cell–tetraploid embryo aggregates

Cell line	Tetraploid aggregates transferred, no.	Resorptions, no.	Midgestation dead embryos,* no.	Newborns, no.	Recovered newborns, no.
R1	53	21	6	9	3†
R2	31	7	7	0	0
R6	40‡				
R13	37	17	5	2	0

*Resorptions above 4 mm in diameter at term were considered in this category.
†Two of the three recovered newborns survived and reached adulthood.
‡Uteruses with obvious signs of early abortion were recovered from these recipients at term.

bottom of the culture plate (3). The aggregates were cultured overnight in microdrops of M16 before transfer into the uterus of 2.5-day pseudopregnant recipients.

Caesarean Section and Data Collection. Pregnant recipients were routinely subject to a Caesarean section on day 18.5 of pregnancy. Live fetuses were counted as well as the number of early postimplantation resorptions and embryos dying at or after midgestation. Fetuses that were alive at delivery but failed to survive the early postnatal period were subject to complete glucose phosphate isomerase (GPI) analysis. Fetuses that recovered after the Caesarean section were placed with foster mothers, and a small sample of blood and the tip of the tail were taken for GPI analysis. Adult ES cell-derived mice were mated with CD1 females to test for germ-line transmission. After test breeding, two mice were killed and various tissues were dissected for GPI analysis.

Genetic Markers. The polymorphic *Gpi-1* gene was used to distinguish between the tetraploid embryo and ES cell-derived components of the chimeras; the ES cell lines were derived from GPI-AA 129 and the tetraploid components were produced from GPI-BB CD-1 embryos. The isoenzyme analysis was performed as described earlier (11). Briefly, tissue homogenates were diluted in sample buffer and run on cellulose acetate membranes (Helena Laboratories) in Supraheme electrophoresis buffer (Helena Laboratories) for 90 min at 300 V. A 1% agarose overlay containing 15 mg of fructose 6-phosphate, 2 mg of nicotinamide-adenine dinucleotide phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 2 mg of dimethylthiazol-diphenyltetrazolium bromide, and 0.4 mg of phenazine methosulfate per ml of 0.1 M Tris·HCl, pH 7.2/15 mM sodium citrate/30 mM $MgCl_2$ was poured over the gel and incubated at 37°C until the GPI isoenzyme bands appeared. The intensity difference between GPI-AA and GPI-BB electrophoretic bands reflected the composition of chimeric tissues. Coat color also indicated the ES cell origin of the viable ES cells–tetraploid embryo chimeras, since tetraploid embryos were albino, while the ES cell lines were derived from agouti embryos.

Karyotype Analysis. ES cells ($\approx 2 \times 10^6$ cells) were arrested in metaphase by adding colcemid (0.2 mg/ml) to the culture medium for 1 hr at 37°C. The cells were collected after trypsin/EDTA treatment and centrifuged at 900 × *g*; the pellet was exposed to hypotonic shock by using prewarmed 0.075 M KCl for 19 min at 37°C. After gentle centrifugation, the hypotonic solution was aspirated, and the pellet was fixed with cold methanol/acetic acid, 3:1 (vol/vol), at least three times prior to spreading on cold microscope slides. The slides were rinsed with Hanks' solution, stained in freshly made 1% Giemsa for 3 min and 50 sec, rinsed with distilled H_2O, and mounted in Permount.

Southern Blot Analysis. DNA was isolated from cell lines by standard procedures and digested with *Eco*RI. The digests were run on a 0.7% agarose gel and blotted onto GeneScreen-*Plus* (DuPont). The blots were hybridized with a Y chromosome-specific probe, pT353/B (12) labeled by random priming, and washed in 15 mM NaCl/1.5 mM sodium citrate, pH 7.0/0.1% SDS at 65°C before exposure to x-ray film.

RESULTS

Developmental Potential of R1, R2, R6, and R13 ES Cells at Early Passages. The cell lines did not differ from each other in their undifferentiated morphology, all of them showing the typical ES cell morphology (13). Differentiation capabilities and developmental potential of cells at passage 6–8 were tested *in vitro* by differentiating the cells into embryoid bodies (4) or *in vivo* by aggregating them with tetraploid embryos (3). All of them developed cystic embryoid bodies at similar frequency *in vitro* (data not shown). However, their *in vivo* developmental potential showed differences (Table 1). R2 and R6 were not able to support development to term. Recipients containing R6 chimeras had only signs that implantation occurred, but no remnants of either fetus or decidual tissue remained. Two of 37 R13–tetraploid embryo aggregates developed to term. Both were completely R13-derived as judged by GPI assay of their main organs. They had extremely high birth weight (*ca.* 3g) and swollen edemic skin. They failed to oxygenize their lungs and died shortly after birth. No detailed pathology was performed. The nine newborns from R1–tetraploid embryo aggregates showed no sign of apparent anatomical abnormalities, although only three of them recovered from Cesarean section. Two of these three reached adulthood. GPI assay of their blood and tail indicated pure R1 origin of one of the survivors. However, the other had about 10–15% tetraploid contribution (data not shown).

Southern blot analysis of the DNA (Fig. 1) prepared from R1, R2, R6, and R13 with a Y chromosome-specific probe (12) revealed that only R1 was a male line.

Developmental Potential of R1 as a Function of Different Culture Conditions and *in Vitro* Culture Time. To investigate the effect of culture conditions on the developmental poten-

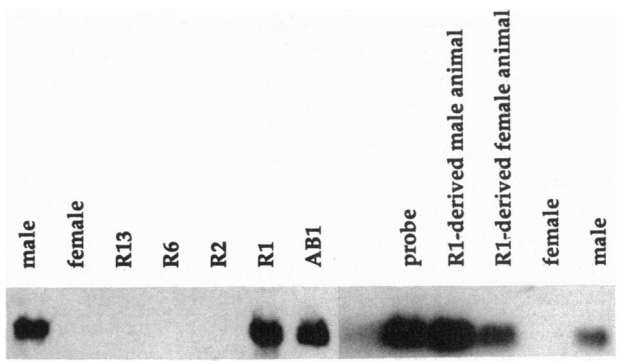

FIG. 1. Southern blot analysis of DNA derived from different cell lines and R1-derived animals. The blots were hybridized with a Y chromosome-specific probe.

Table 2. Prenatal developmental potential of R1 cultured under different conditions between passages 6 and 24 and tested by R1–tetraploid embryo aggregates

Passages, no.	Conditions	Tetraploid aggregates transferred, no.	Implantations, no.	Resorptions, no.	Midgestation dead embryos, no.	Newborns, no.	Recovered newborns, no.	Survivors, no.
6	Feeders + LIF	53	36	21	6	9	3	2
10	Feeders + LIF	15	11	6	1	4	2	1
14	Feeders + LIF	25	13	7	5	1	1	1
	Feeders	13	8	4	3	1	1	1
	LIF	24	19	7	7	5	2	0
16	Feeders + LIF	6	3	2	0	1	0	0
17	Feeders + LIF	18	13	9	3	1	0	0
	Feeders	20	9	5	4	0	0	0
	LIF	15	7	4	2	1	0	0
20	Feeders + LIF	24	20	10	9	1	0	0
	Feeders	25	19	6	10	3	0	0
	LIF	32	24	7	13	4	1	0
24	Feeders + LIF	22	14	8	5	1	0	0

tial of ES cells, we cultured R1 from passage 8 under three different conditions.

Condition 1: feeder layers + LIF. Cells were kept on SNL fibroblast feeders in DME medium supplemented with 20% FCS and 2000 units of LIF per ml. (These were the original conditions used when establishing the cell line.)

Condition 2: feeders alone. The cells were gradually weaned off LIF by decreasing the concentration by 500-unit steps at the first four passages and then maintained on SNL feeders in DME medium supplemented with 20% FCS.

Condition 3: LIF alone. The cells were kept on gelatinized plates in DME medium supplemented with 20% FCS and 2000 units of LIF per ml.

Aggregates with tetraploid embryos were prepared by using cells from passages 6–24. Raw data on the prenatal and perinatal development of these aggregates are shown in Table 2. There was no obvious difference between the different culture conditions in their ability to support development to term. Thus, to obtain a clear idea on the effect of *in vitro* culture time on developmental potential, data from the three culture conditions were pooled into two age groups: early (6–14) and late passages (Table 3).

Interestingly, the groups did not differ in implantation (67%) and resorption rate (35% and 31%, respectively), but the percentage of dead embryos at midgestation increased and the rate of newborns at term decreased significantly by late passage. In the late-passage group, only 1 of the 12 newborns recovered after the Caesarean section, whereas almost 50% of the newborns of the early-passage groups started breathing normally. No animal survived to adulthood from passages later than 14.

Karyotype of R1 at Early and Late Passages. To see if gross karyotypic changes could explain the decrease of developmental potentiality as a function of *in vitro* culture time, chromosome spreads were prepared from an early and a late passage, 11 and 33, respectively. A slight decrease was detected in the frequency of spreads with the normal number of chromosomes (Fig. 2), but the modal number was still 40.

Characterization of Completely ES Cell-Derived Animals. Since the tetraploid component was produced from albino and preselected GPI-BB CD1 embryos and R1 was agouti and GPI-AA, both the coat color and the GPI served as markers to detect occasional tetraploid contribution in the animals derived from R1–tetraploid embryo aggregates. The coat color of all five animals indicated only agouti contribution (Fig. 3). However, GPI analysis of blood from adult animals showed that one animal contained detectable tetraploid contribution (*ca.* 5–10%). This animal was the same one showing tetraploid contribution at birth. The remaining four animals showed no evidence of tetraploid cells in the blood. Further, GPI assay of the organs of two of the five animals—analyzed at 10 months of age—showed exclusive R1 contribution (Fig. 4 shows one of the two analyzed). Thus, within the limits of sensitivity of the GPI analysis, which allowed detection of contributions as low as 2%, no tetraploid cells remained in these animals. A minor contribution (<2%) from tetraploid cells cannot be excluded. The remaining three animals are more than 1 year old. They do not show any signs of premature aging or tumor development. It is likely that the life span of completely ES-derived animals is not affected by their cell culture origin.

The two newborns derived from R13 were female as predicted from Southern blot analysis of the cell line. Nearly all of the completely R1-derived newborns were male as predicted. However, two of these total 32 newborns (Table 3) were female. One of these two newborns reached adulthood but was not fertile. The existence of an R1-derived female may indicate some degree of Y chromosome loss in this cell line even at this early passage number. Y-chromosome loss could not have been complete, Southern blot analysis showed Y-specific sequences in this animal (Fig. 4).

Table 3. Prenatal developmental potential of R1 tested by R1–tetraploid embryo aggregates as a function of *in vitro* culture time (passage number)

No. of passages	Tetraploid aggregates transferred, no.	Implantations, no.	Resorptions, no.	Midgestation dead embryos, no.	Newborns, no.	Recovered newborns, no.	Survivors, no.
6–14	130	87 (67)	45 (35)	22 (17)	20 (15)	9	5
16–24	162	109 (67)	51 (31)	46 (28)	12 (7)	1	0
	χ^2	0.002	0.22	4.10	4.19	8.37	6.23
	P (df = 1)	$\gg 0.2$	$\gg 0.2$	<0.05	<0.05	<0.01	<0.02

The numbers in parentheses are percentages of aggregates transferred.

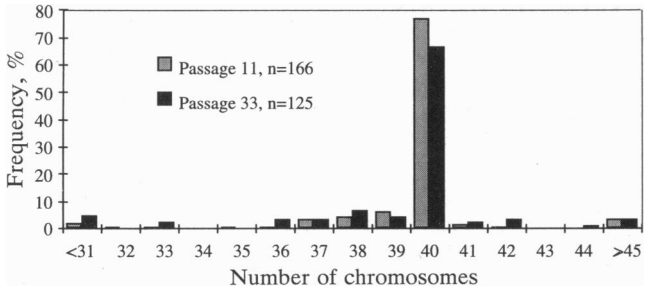

FIG. 2. Distribution of metaphase spreads with different chromosome numbers at passage 11 and passage 33 of R1.

The R1-derived males were all fertile, producing normal-size litters, and exclusively transmitted the R1 genotype to their offspring.

Developmental Potential of Subclones Derived from an Early Passage of R1. *In vitro* culture time negatively affected the developmental potential of R1 ES cells. To further investigate this phenomenon, we tried to distinguish between two models of the changes that may happen during culture. One possibility is that ES cells are not able to replicate exactly their stem-cell state during successive rounds of cell division and gradually lose their full potential. This loss is strictly related to the *in vitro* culture time or the number of cell divisions. The other possibility is that a certain proportion of cells replicate the fully potent stem-cell state at each cell division. The remaining cells move away from this state and form a developmentally restricted compartment. In time this restricted compartment gradually takes over the culture. However, at any time point, there are cells with intact potential. If the first model were true, it should not be possible to retain the full potential of later passage cell cultures by subcloning; but if the second model should be true, it should be possible to establish subclones of the parent cell line that retain full potential after prolonged passage *in vitro*.

To test the two models, we established subclones from single cells of passage 12 by using the original culture conditions and maintained them *in vitro* for a period equivalent to that of passage 24–25 of the original R1 stock before testing for pluripotency by tetraploid embryo aggregation. Table 4 shows the developmental potential of these lines. One of the five sublines, R1-S3, supported completely ES cell-derived development to term and gave rise to viable animals with similar efficiency to the parental R1 cell line at very early passages.

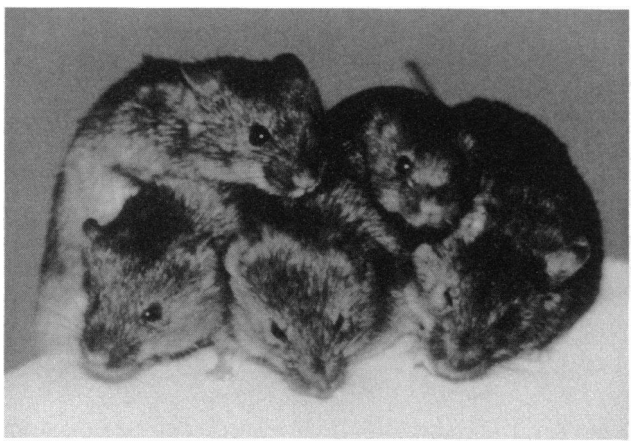

FIG. 3. Group of five R1-derived animals produced by R1–tetraploid embryo aggregates.

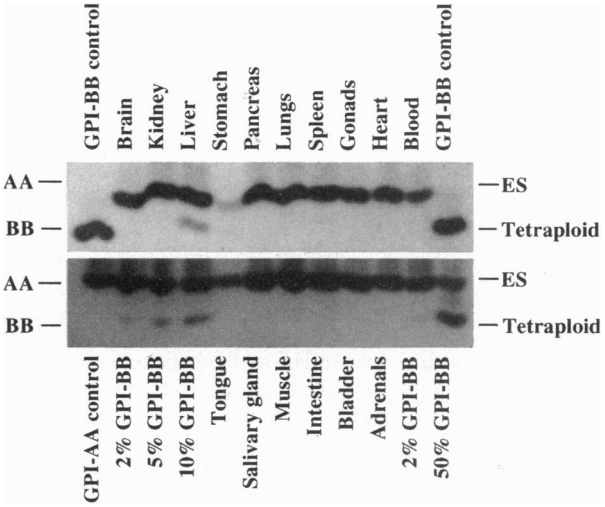

FIG. 4. GPI analysis of the major organs of one of the two R1–tetraploid embryo chimera. The tetraploid component had GPI-BB phenotype, while R1 was GPI-AA. The assay shows the complete R1 origin of the organs. The minor band in the liver sample does not line up with the band of GPI-BB and might be a degradative product of GPI-AA. By overdeveloping the stain, the sensitivity of our GPI test was estimated at 2% on the control samples.

DISCUSSION

Using ES cell–tetraploid aggregates, we have produced viable mice that are entirely ES cell-derived, as judged by GPI and coat color phenotype and germ-line transmission. This success was achieved with a newly derived ES cell line (R1) at early passage number (up to passage 14) and is the fullest demonstration to date of the remarkable potential of these tissue culture cells. Previous experiments using established cell lines had resulted in perinatal death of any totally ES cell-derived fetuses (3, 6). The same effect was observed here with later passages of R1, suggesting that prolonged passage in culture reduces the potential of the ES-cell population as a whole. However, one subclone of R1 isolated at passage 12 and maintained in culture until the equivalent of passage 24 was still able to produce viable ES cell-derived mice after aggregation with tetraploid embryos. This indicates that loss of full developmental potential is not a necessary consequence of passage in culture, but rather that the proportion of cells that retains full potential diminishes with extended passage. Some cell lines lose potential more quickly than others; three cell lines established in parallel with R1 failed to produce viable ES cell-derived embryos even at early passage.

The nature of the factors that cause this variable decline in developmental potential is still unclear. Both genetic and epigenetic changes can accumulate with passage in culture and may vary from line to line. It seems unlikely that major karyotypic changes are the main cause of the decline, since we only observed a small increase in the proportion of aneuploid cells in the R1 population with extended passage, and other established cell lines similarly retain a high proportion of euploid cells. It seems more likely that minor genetic changes or changes in DNA methylation and imprinting underlie the phenomenon. It will be interesting to compare the properties of the apparently stable subline with the parent cell line to see if subtle changes in DNA modification, gene expression, or differentiation capacity can be detected.

Although the R1 cell line can produce viable ES cell-derived mice, this is still an inefficient process; many ES cell–tetraploid aggregates die before reaching term, even when early passage cells are used. Therefore, this approach cannot be considered as a feasible approach for routinely

Table 4. Prenatal developmental potential of R1 sublines tested by ES cell–tetraploid aggregates

Subline of R1	Tetraploid aggregates transferred, no.	Resorptions, no.	Midgestation dead embryos, no.	Newborns, no.	Recovered newborns, no.	Animals reached adulthood, no.
R1-S1	19	7	3	0	0	0
R1-S2	45	9	3	1	0	0
R1-S3	20	6	5	5	3	2
R1-C3	10	4	3	2	0	0
R1-B1	23	9	5	2	1	0
Total	117	35	19	10	4	2

achieving germ-line transmission from genetically manipulated ES cells. However, a cell line, like R1, which retains full developmental potential for extended passage in at least one subclone, is likely to contribute well to the germ line after standard blastocyst injection or morula aggregation. Indeed, the R1 cell line has proved to be an efficient vehicle for transmitting genetic alterations through the germ line after either injection into C57BL/6 blastocysts or aggregation with diploid CD1 eight-cell stage embryos (unpublished data).

Understanding more about the factors that affect the developmental potential of ES cells in tetraploid aggregates may help to improve the technology of genetic manipulation of the mammalian genome.

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