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Embryonic stem cells alone are able to support fetal development in the mouse

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Summary

The developmental potential of embryonic stem (ES) cells *versus* 3.5 day inner cell mass (ICM) was compared after aggregation with normal diploid embryos and with developmentally compromised tetraploid embryos. ES cells were capable of colonizing somatic tissues in diploid aggregation chimeras but less efficiently than ICMs of the same genotype. When ICM↔tetraploid and ES↔tetraploid chimeras were made, the newborns were almost all completely ICM- or ES-derived, as judged by GPI isozyme analysis, but tetraploid cells were found in the yolk sac endoderm and trophectoderm lineage. Investigation of ES contribution in 13.5 day ES↔tetraploid chimeras by DNA *in situ* hybridization confirmed the complete tetraploid origin of the placenta (except the fetal blood and blood vessels) and the yolk sac endoderm. However, the yolk sac mesoderm, amnion and fetus contained only ES-derived cells.

ES-derived newborns failed to survive after birth, although they had normal birthweight and anatomically they appeared normal. This phenomenon remains unexplained at the moment.

The present results prove that ES cells are able to support complete fetal development, resulting in ES-derived newborns, and suggest a useful route for studying the development of genetically manipulated ES cells in all fetal lineages.

Key words: embryonic stem cells, pluripotency, chimeras, cell selection, tetraploid embryos, mouse embryo.

Introduction

Mouse embryonic stem (ES) cells are the most pluripotent cultured animal cells known. When ES cells are injected into host blastocysts they are capable of contributing to a wide variety of somatic tissues and the germline in resulting chimeras. However, their contributions to different lineages are not necessarily equal. ES cells are able to contribute extensively to the fetus and extraembryonic mesoderm, but their ability to colonize primitive endoderm and trophectoderm derivatives is poor (Beddington and Robertson, 1989). ES cells may thus have restricted potential in certain extraembryonic lineages, suggesting that functional complementation by host cells is required during early stages of development in chimeras. In this study, we asked whether this functional complementation is required at later stages within the embryonic lineages, or whether ES cells can support complete fetal and postnatal development. To test the full developmental potential of ES cells, they were aggregated with cleavage stage embryos that had been placed at a developmental disadvantage by doubling their ploidy. The development of these aggregates was compared with similar aggregates made with ICM cells.

Tetraploid embryos can implant at high frequency, but rarely form embryonic structures (Snow, 1975, 1976; Tarkowski *et al.* 1977), although occasional embryos proceed through organogenesis and reach term (Snow, 1973). Postnatal survival of tetraploid embryos has not been reported. If blastomere stage tetraploid embryos are aggregated with diploid embryos, the tetraploid cells are selected against during development of fetal tissues in most cases (Lu and Markert, 1980), but persist in extraembryonal membranes (Tarkowski *et al.* 1977). Thus, we reasoned that tetraploid cells might complement the deficient extraembryonal differentiation of ES cells while allowing full expression of their potential for fetal development. Production of live fetuses that were entirely ES in genotype was achieved from such aggregates, showing that ES cells have the potential to form all fetal cell lineages.

Materials and methods

Animals

C57×CBA F_1 (LATI) hybrid females were crossed with F_1 males to produce the blastomere stage components of the chimeras analyzed by GPI. In some of the tetraploid↔ES chimeras, the tetraploid embryos were labelled with a DNA marker by crossing F_1(LATI) or CD-1 (Charles River Canada Montreal) females and Tg-MβG-1 males homozygous for a transgenic insertion of about 1000 tandemly repeated copies of plasmid carrying a mouse β-globin gene (Lo, 1986). 129/Sv mice were obtained partly from a laboratory stock and partly from the animal facility of SzBK. The original breeding pairs were a generous gift of Anna Wobus.

Diploid and tetraploid embryos

Electrofusion of late 2-cell-stage blastomeres (22–23 h post-hCG) was used to produce tetraploid embryos as described by Kubiak and Tarkowski (1985). Two 0.1 mm thick platinum ribbons were placed at a distance of 0.1 mm apart on the bottom of a plastic Petri dish. The electrodes were covered by a large drop (0.5 ml) of M2 (Quinn *et al.* 1982) medium. The embryos, one at a time, were placed between them, and oriented so that the plane of attachment of the blastomeres was perpendicular to the electric field. Then a single square pulse of 100 μs and 10 V was applied by a pulse generator (CF-100, Biochemical Labor Service, H-1165 Budapest, Zselyi A. u. 31. Hungary). Shortly after, the embryos were transferred to the incubator at 37°C. The fusion of the two blastomeres took place in 30 min with more than 98% efficiency. The fused embryos were separated and considered as tetraploids. They were cultured overnight (5% CO_2 in air, at 37°C) in M16 (Whittingham, 1971) and in the afternoon of the next day the compacting 4-cell-stage embryos were used for aggregation chimeras.

8-cell-stage diploid embryos were recovered from the oviduct of 2.5 day pregnant mice just before the assembly of aggregates.

ICM and embryonic stem (ES) cells

3.5 day 129/Sv blastocysts were subjected to immunosurgery (Solter and Knowles, 1975) in order to isolate inner cell mass (ICM). The intact ICMs were used for aggregation with diploid or tetraploid embryos.

Two embryonic stem cell lines were used in the experiments; D3 (Doetschman *et al.* 1985) and α. Both cell lines are derived from 129/Sv blastocysts, are male and have maintained a normal karyotype in culture. The latter was established in our laboratory following the technique described by Robertson (1987). In the experiments described here, α cells were used within 10 passages from establishment and D3 within 20 passages. In both cases, other experiments have shown that cells at the same passage number can contribute to the germ line after injection into diploid blastocysts (Gossler *et al.* 1989; unpublished observation). ES cells were trypsinized and gently disaggregated 1–2 h prior to assembly of aggregates, when groups of loosely connected 10–15 cells were selected for aggregation.

Aggregation chimeras

The zona pellucida of 8-cell-stage diploid and 4-cell-stage tetraploid embryos was removed by acid Tyrode's solution (pH 2.1). A single isolated ICM or a clump of ES cells was sandwiched between two blastomere stage embryos in microwells prepared as described previously by Nagy *et al.* (1989). After 24 h incubation successful aggregates were transferred to the uterine horns of day 2.5 pseudopregnant F_1 or CD1 foster mothers.

GPI assay

Fetuses were delivered by Caesarian section at 18.5 days of gestation. We found that removing the tail tip did not affect the viability of newborns, therefore this tissue and the blood were chosen to characterize the ES contribution in the newborns before giving them to foster mothers to test their viability. The extraembryonic tissues were also collected. Three tetraploid↔ES chimera newborns were rescued before they died and were eaten by the foster mothers. These animals were dissected and samples of various tissues retained for GPI analysis. The tissues were frozen and thawed at least once before homogenization. The GPI isoenzymes were separated by polyacrylamide gel (5%) electrophoresis, and blotted onto Whatman filter paper and stained for enzyme activity (Peterson *et al.* 1978).

Histology and DNA in situ *hybridization*

Intact conceptuses were dissected at 13.5 days of gestation in PBS then fixed overnight in 3:1 ethanol:acetic acid. They were embedded and sectioned as described by Clarke *et al.* (1988). *In situ* DNA hybridization was carried out according to Rossant *et al.* (1986), except that signal was detected using Avidin–FITC after amplification with biotin-conjugated goat anti-avidin D antibodies (Lichter *et al.* 1988). The slides were stained with propidium iodide and DAPI.

Results

Development of aggregation chimeras

In a pilot experiment, we found that increasing the number of ES cells aggregated between two diploid embryos increased the rate of chimerism and the extent of ES contribution to the chimeras, but decreased the percentage of normal development. The use of 15–20 ES cells aggregated with two diploid 8-cell-stage embryos optimized chimerism and developmental success (data not shown). This cell number was used in both types of ES aggregates, namely diploid 8-cell-stage↔ES cell chimeras (D↔ES) and tetraploid 4-cell-stage↔ES cell chimeras (T↔ES). The ICM of a 3.5 day blastocyst also contains 15–20 cells and so a single ICM was used in the following aggregates; diploid 8-cell-stage↔3.5 day ICM chimeras (D↔ICM) and tetraploid 4-cell-stage↔3.5 day ICM chimeras (T↔ICM).

The fates of aggregates analyzed at term are shown in Table 1. No significant difference was detected among the implantation rates of the four groups. These rates are similar to our usual transfer success with normal, unmanipulated embryos (not shown). The proportion of resorption was much higher in the tetraploid aggregates, reflecting the decreased developmental potential of tetraploid embryos. Presumably these empty implantations result from imperfect aggregation (internalization) of the diploid cells. Retarded fetal development was detected in the ES chimeras (group 2 and 4), but not in the ICM chimeras (group 1 and 3). The final rate of normal development depended on both the rate of empty implantations and retarded

Table 1. *Prenatal fates of aggregates analysed at term*

Group	Type of aggregates: Blastomere stage embryo	Type of aggregates: Cell	Number of aggregate transferred	Number of implantations (%)	Number of resorptions (%)	Number of retarded fetuses (%)	Number of normal fetuses (%)	Number of chimeras (% fetuses)
1	Diploid F2↔129 ICM		28	21 (75)	8 (29)		13 (46)	12 (92)
2a	Diploid F2↔αES		15	11 (73)	4 (27)	2 (13)	5 (33)	3 (60)
b	Diploid F2↔D3ES		24	21 (87)	7 (29)	1 (4)	13 (54)	7 (53)
c	Diploid B1*↔D3ES		19	19 (100)	7 (37)	2 (11)	10 (53)	7 (70)
3	Tetrapl F2↔129 ICM		21	16 (76)	10 (48)		6 (29)	6 (100)
4a	Tetrapl B2**↔αES		31	19 (61)	11 (35)	2 (6)	6 (19)	6 (100)
b	Tetrapl F2↔αES		28	21 (75)	17 (60)	2 (7)	2 (7)	2 (100)
c	Tetrapl F2↔D3ES		73	32 (44)	24 (33)	2 (3)	6 (8)	6 (100)
d***	Tetrapl CD1↔D3ES		101	70 (70)	45 (46)	10 (10)	15 (15)	15 (100)

F_2=C57Bl/6×CBA
*B1=(C57Bl/6×CBA)×C57Bl/6 (GPI - B/B)
**B2=F_1×Tg-MβG-1 (GPI - A/B)
*** Chimerism was detected by means of eye pigmentation only

development. Therefore, the lowest rate of apparently normal development, 12% (29/233 total), was observed in the T↔ES group and the highest, 46% (13/28), in the D↔ICM group.

All the tetraploid aggregates, both ES and ICM, resulted in newborns with contribution from the added cells. ICMs were also extremely effective (92%) in producing chimeras with diploid embryos (group 1), while 60% of the D↔ES newborns proved to contain ES-derived cells.

Genotypic and phenotypic analysis of chimeric conceptuses

Diploid↔ICM chimeras

ICM-derived cells contributed to newborns and their yolk sacs at high frequency and intensity (Table 1 and Fig. 1). The ICM contribution to placenta was less than that to the blood and tail tip, reflecting the inability of the mature ICM to contribute to the trophectoderm lineage (Rossant and Lis, 1979). All the ICM chimeras recovered normally after Caesarian section on day 19 of pregnancy and reached adulthood. The females were tested for germline chimerism. Three of the four females transmitted ICM-derived germ cells.

Diploid↔ES chimeras

Two different ES lines were used in this group; α for newborns 1 to 3, and D3 for newborns 4 to 10 and in a separate experiment for newborns 11 to 17 (Fig. 2). Some between-experiment variation in the extent of ES contribution was observed.

The proportion of chimeras at term was less (17 of 28) than in the D↔ICM group. Only eight of the seventeen ES chimeras recovered after Caesarian section or reached adulthood. The average ES contribution in the viable and unviable subgroups differed significantly (e.g. for the tails, 47%±11 and 18%±6 respectively, $P<0.05$).

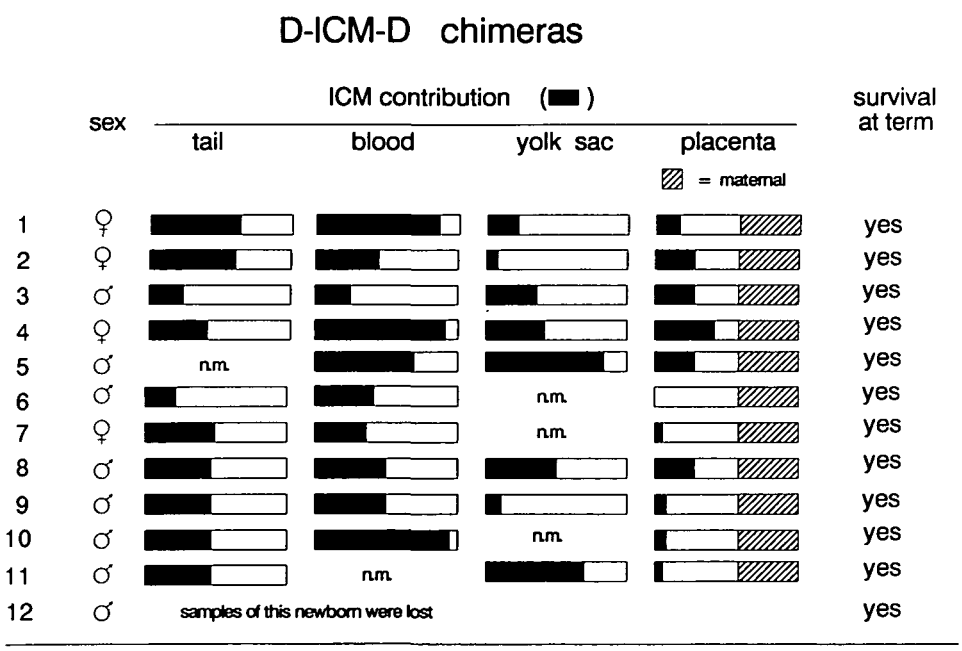

Fig. 1. Contribution of 129/Sv 3.5 day ICM to the tail, blood, yolk sac and placenta of newborns from diploid 8-cell-stage embryo↔ICM aggregates. The generally observed 40% maternal contribution to the placenta is also indicated.

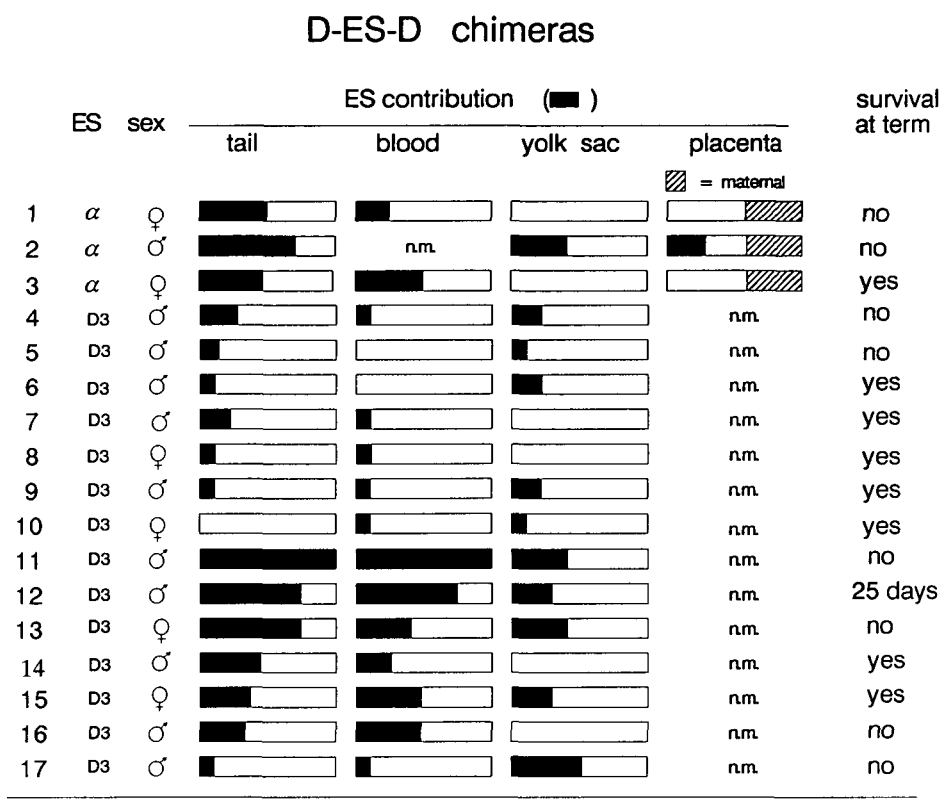

Fig. 2. Contribution of ES cells to the tail, blood, yolk sac and placenta of newborns from diploid 8-cell-stage embryo↔ES cell aggregates. The GPI phenotype of the recipient for D3 chimeras (no.4–no.17) was the same (GPI-A/A) as that of the ES cells, so that the contribution of ES cells to the placenta could not be accurately determined. The genotype of the 8-cell-stage embryos in the aggregates 1 to 10 was F_2, while in aggregates 11 to 17 was F_1XC57Bl/6 backcross.

We have recently set up testcrosses for determining germline chimerism in animals 6, 7, 9 and 14. In separate experiments, we have already produced a germline aggregation chimera from the α line (unpublished data).

Tetraploid↔ICM chimeras

Only one of the six newborns (Fig. 3) contained tetraploid cells in its blood and tail tip (<5%) as assayed by GPI analysis. In all the other cases, the tetraploid cells were apparently absent from the embryo. The presumption that the assay of blood and tail characterized the ICM contribution to the other tissues as well was supported by the coat colour of the animals which uniformly showed the white-bellied chinchilla agouti phenotype of the strain 129/Sv (the origin of ICM cells), and by GPI analysis of internal organs of animals 5 and 6 at four months of age (data not shown).

The yolk sac of tetraploid–ICM chimeras showed two extremes. We found either completely ICM-derived or completely tetraploid-derived yolk sacs. The highest tetraploid contributions were detected in the placentas. Only one of the six did not contain tetraploid cells. Viability of the newborns was reasonable (four out of six survived). To test the germline, we mated animals 5 and 6 with each other. Only 129/Sv offspring were born and litter sizes were normal.

Tetraploid↔ES chimeras

Only three of the fourteen newborns analyzed by GPI showed any (<10%) tetraploid contribution to the two tissues (blood and tail) examined (Fig. 4). Several major organs of animals 12 to 14 were also assayed by GPI (animals 12 and 13 are shown in Fig. 5). The results were consistent with their blood and tail type, as animals 12 and 14 were completely ES-derived, and animal 13 showed a small tetraploid contribution in all

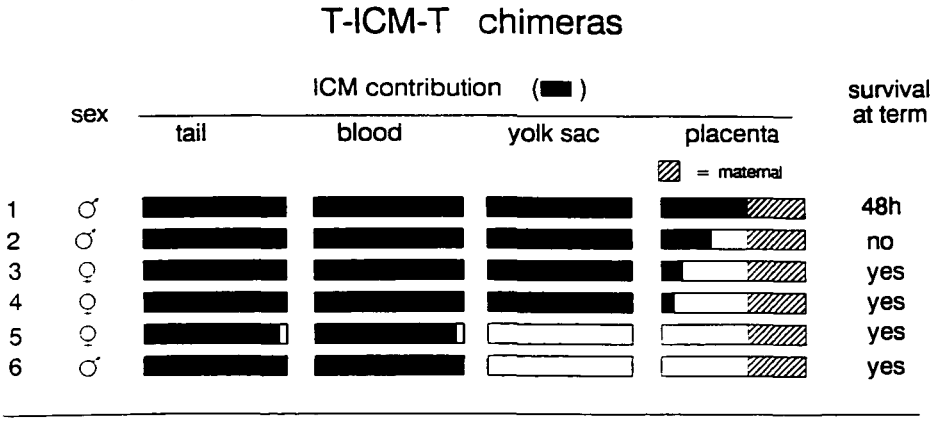

Fig. 3. Contribution of 129/Sv 3.5 day ICM to the tissue of newborns from tetraploid 4-cell-stage embryo↔ICM aggregates.

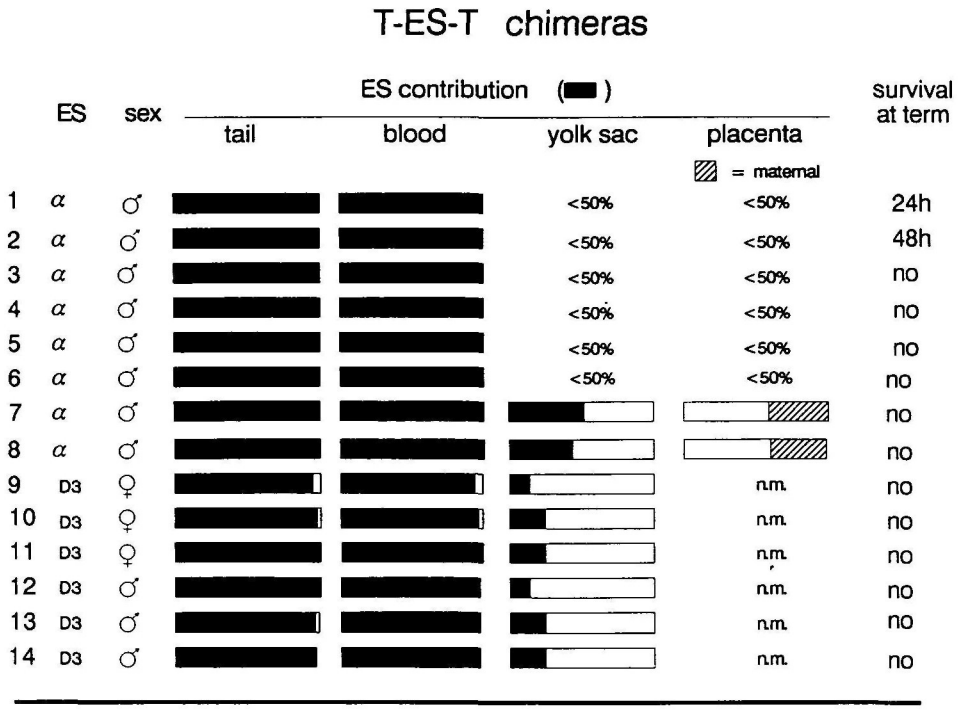

Fig. 4. Contribution of ES cells to the tail, blood, yolk sac and placenta of newborns from tetraploid 4-cell-stage embryo↔ES cell aggregates. In the first five animals, the contributions to the yolk sac and placenta could not be estimated, since the tetraploid components were heterozygous for GPI and therefore overlapped the band derived from the ES cells. However, tetraploid contributions were clearly strong.

organs except tongue, muscle and brain. Newborns 7 to 14 showed extensive tetraploid contributions to the yolk sac (and in the case of 7 and 8 to the placenta as well). In newborns 1 to 5, the GPI assay did not allow us to estimate the contribution of ES-derived cells to the extraembryonic tissues, since the tetraploid component was heterozygous at the Gpi-1 locus (see M&M). Therefore, one fourth of GPI of the tetraploid cells was GPI-A/A, and its electrophoretic band made it impossible to detect a small GPI-A/A ES contribution. The apparent 1:2:1 density of A/A:A/B:B/B, respectively, indicated tetraploid domination in these tissues.

Although both α and D3 are male ES lines, females occasionally appeared among chimeras that were apparently entirely ES-derived (animals 9–11 in Fig. 4 and one of the ten tetraploid CD1↔D3 ES, see Table 1). This finding may indicate that early contribution from tetraploid cells could determine the sex of the embryo before these cells are selected out. However, since this effect was only seen with the D3 cell line, it is more likely that some D3 ES cells may have lost the Y chromosome resulting in XO ES contributions. Unfortunately, DNA was not recovered for testing for the presence of the Y chromosome.

Only two of the eight α-derived newborns recovered after the Caesarian section. The other six tried to

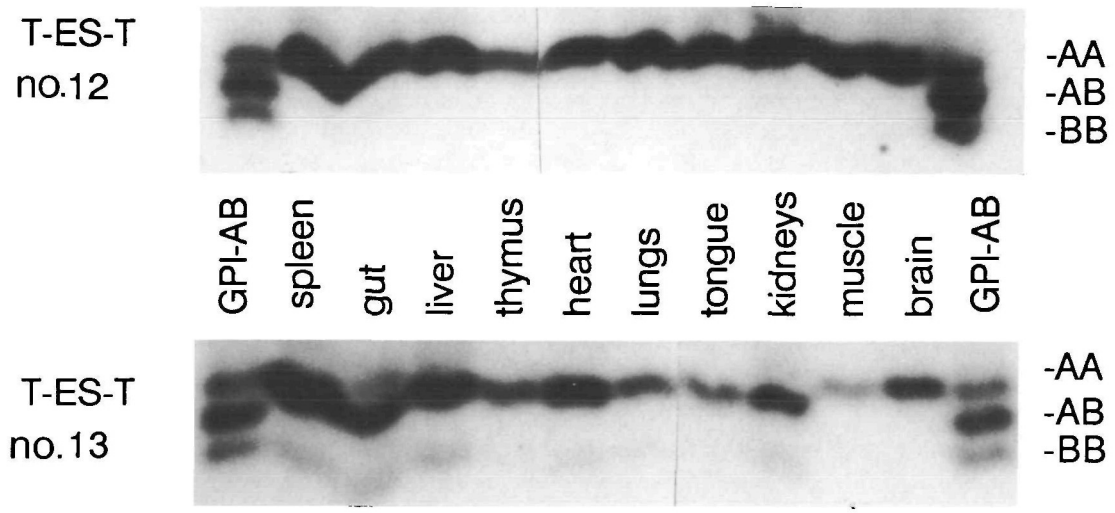

Fig. 5. GPI assay of several major organs of newborns (animal 12 and 13 of Fig. 4) from tetraploid 4-cell-stage embryo↔ES cell aggregates. Animal 11 had no detectable contribution from the tetraploid component (GPI-BB), therefore it was completely ES-derived (GPI-AA). Animal 12 had some minor (<5%) tetraploid contribution except in the tongue, muscle and brain.

oxygenate their lungs after the Caesarian section with several intensive gasps for breath, while the placenta circulation was weakened as normal. After a few minutes, the placenta circulation was re-established, and shortly afterward the animals died of anoxia. The dissection of these newborns did not reveal any apparent abnormality. However, their lungs were either not or only partially oxygenated, and the ductus Botal was still open. The two animals that recovered died one or two days later from apparent dehydration. In the case of animals 8 to 13 oxygen was given directly after Caesarian section. This helped initial recovery, but the animals still died after. To ensure that this failure to survive was not a chance outcome in a small number of offspring, we performed a large series of aggregations of D3 with CD1 outbred mice, and scored offspring by intensity of the eye pigmentation alone: 15 live offspring were produced from this experiment (Table 1), all of which showed complete pigmentation of the eye, suggesting extensive or complete ES contributions. All these offspring showed the same failure to survive as the aggregates analyzed by GPI.

To determine the exact allocation of tetraploid cells on a cellular level and to prove that the fetus could be completely ES-derived, we made some aggregates in which the tetraploid component was marked by the *in situ* detectable transgene of the Tg-MβG-1 line. Eighteen tetraploid↔α ES aggregates were transferred into recipients and their pregnancies terminated on day 13.5. Three apparently normal looking conceptuses and eleven resorptions were found. The normal conceptuses were fixed and subjected to DNA *in situ* hybridization. All three showed the same allocation pattern of tetraploid cells (Fig. 6). The trophoblast layers of the placenta were apparently completely tetraploid (Fig. 6C), although a very small contribution of ES cells could not be ruled out. Within the placenta, epiblast-derived tissues such as the fetal blood cells and blood capillaries were unmarked and therefore ES-derived. Only tetraploid cells could be detected in the endoderm layer of the yolk sac. However, the mesoderm of the yolk sac and the amnion were completely ES-derived (Fig. 6D). Examining serial sections through the fetus, no trace of tetraploid cells was detected (Fig. 6B), indicating that the fetus was completely ES-derived.

Discussion

Tetraploid↔ES and tetraploid↔ICM aggregates developed to term and the latter were viable after birth. In both cases, GPI analysis revealed that most resulting animals were entirely derived from the diploid ICM or ES component. Small residual contributions from the tetraploid cells were observed in a few instances only. GPI analysis could not exclude minor contribution to certain tissues, but *in situ* analysis of mosaicism provided a more sensitive assay. Using a DNA *in situ* marker, we showed that 3 tetraploid↔ES chimeras were entirely ES-derived in all fetal lineages at 13.5 days. Therefore, ES cells have the potential to form all fetal lineages and support normal fetal development. Thus the combination of developmentally compromised tetraploid embryos and ES cells provides a means of forcing development of the entire fetus from the tissue culture-derived ES component.

Extraembryonic lineages in tetraploid↔ES aggregates were mostly tetraploid-derived. This was most apparent in conceptuses studied in mid-gestation, where *in situ* studies showed that most if not all trophoblast and yolk sac endoderm cells were tetraploid-derived. The apparent failure of ES cells to form extraembryonic lineages could mean that ES cells lack the potential to form these lineages. However, Beddington and Robertson (1979) have reported ES contributions to the trophoblast and yolk sac endoderm cells using GPI analysis on ES injection chimeras. We have also occasionally observed ES contributions to these lineages in diploid↔ES aggregation chimeras (unpublished observations, data not shown). This suggests that ES cells have some potential to form the extraembryonal lineages, but they are outcompeted by tetraploid cells in tetraploid↔ES chimeras. Tetraploid cells may be able to function well in trophoblast and yolk sac endoderm lineages, since cells in both lineages undergo endoreduplication in normal diploid embryos (Zybina, 1970; Ilgren, 1980).

The inability of the ES-derived newborns to survive presents an intriguing problem. These pups were apparently normal anatomically and in birthweight. Those that recovered after Caesarian section and lived for one and two days presumably had no gross physiological abnormalities. It seemed to us that they were too weak to suckle and simply dehydrated. The viability of the tetraploid↔ICM chimeras implies that the failure to survive was due to the ES component and not to the tetraploid component within the placenta. The suggestion that the mortality was due to the ES cells was further supported by the frequent death of diploid↔ES chimeras having large ES contributions. The same effect on developmental potency and perinatal mortality was observed with two independent ES lines, suggesting that the effect is not due to mutations accumulated in a specific cell line, although the presence of a proportion of aneuploid cells in the ES population cannot be excluded. Both cell lines were derived from the same inbred strain and it would be of interest to determine whether the effect is a general characteristic of the ES cells by testing more independent ES cell lines from different strains. A trivial explanation for the results would be that both cell lines are infected with an agent that causes perinatal mortality when present at high levels. However, our cell lines have been extensively tested for viral and mycoplasma contamination, making this unlikely. A more fundamental factor that may come into play is the role of genomic imprinting in development (Surani *et al.* 1984, 1988; Solter, 1988). If passage in tissue culture removes elements of the maternal and paternal imprinting pattern, then this may affect development potential of the resulting cell lines.

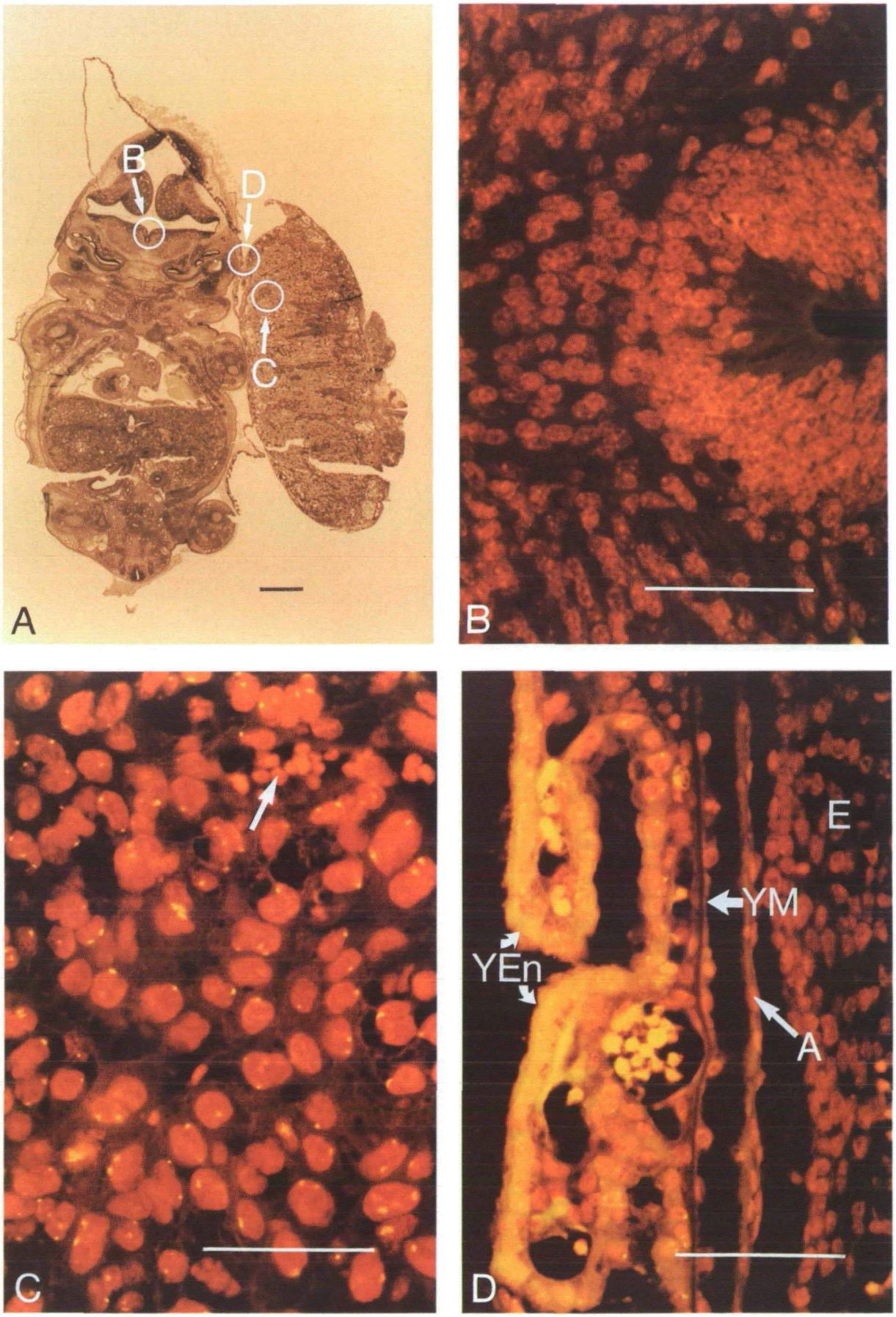

Fig. 6. DNA *in situ* hybridization to a section of a 13.5 day tetraploid↔ES chimera. The tetraploid cells were labeled by two transgenes detected as two fluorescent dots per nucleus. Photograph A (bar=1 mm) of a whole adjacent section indicates the area where the high-power photomicrographs of B (brain), C (placenta) and D (yolk sac, bar=100 μm) were taken. The arrow on photograph C shows a group of negative fetal blood cells. The E, A, YM and YEn abbreviations of photograph D stand for embryo, amnion, yolk sac mesoderm and yolk sac endoderm, respectively.

These studies have shown that embryo aggregation as well as blastocyst injection (Bradley *et al.* 1984) can be used to generate ES chimeras, as previously reported for EC cells (Stewart, 1982). Diploid↔ES aggregates with reasonable ES contribution can be produced and these can survive into adulthood. The ES contributions to the chimeras varied from experiment to experiment, but could reach 70%. We have also achieved germline transmission of the ES genotype from aggregation chimeras (unpublished). In the present study, we have not directly compared the efficiency of embryo aggregation *versus* blastocyst injection for ES chimera production. However, it is clear that ES aggregation chimeras provide a potential route into genetic manipulation of mammalian development for investigators who lack micromanipulation abilities. Tetraploid↔ES aggregates, while not yet allowing production of viable ES-derived mice, can be useful for a variety of studies on the effects of genetically manipulated ES cells on embryonic development. They also provide a pure source of ES-derived progenitors to various adult organs and tissues.

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