Foetal fibroblasts introduced to cleaving mouse embryos contribute to full-term development

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Abstract

Foetal fibroblasts (FFs) labelled with vital fluorescent dye were microsurgically introduced into eight-cell mouse embryos, three cells to each embryo. FFs were first identified in the inner cell mass (ICM) in about one-third of embryos, whereas in three quarters of embryos FFs were located among trophoblast cells. Some elimination of FFs from trophoblast occurred later on. Eventually, in blastocysts' outgrowths, an equally high contribution from FFs progeny (60%) was found in both ICM and trophoblast. Three days after manipulation, FFs resumed proliferation *in vitro*. More than three FFs were found in 46.2% of embryos on day 4. On the 7th day *in vitro* in 70% of embryos more than 12 FFs were found, proving at least three cell divisions.

To study postimplantation development, the embryos with FFs were transferred to pseudopregnant recipients a day after manipulation. After implantation, FFs were identified by electrophoresis for isozymes of glucose phosphate isomerase (GPI). A single 11-day embryo delayed to day 8 proved chimeric by expressing both donor isozyme GPI-1B and recipient GPI-1A. Similar chimerism was found in the extraembryonic lineage of 11% of embryos by day 12. Starting from day 11 onwards, in 32% of normal embryos and in 57% of foetal membranes, hybrid GPI-1AB isozyme, as well as recipient isozyme, was present. Hybrid GPI-1AB can only be produced in hybrid cells derived by cell fusion, therefore, we suggest that during postimplantation development, FFs are rescued by fusion with recipient cells. In the mice born, hybrid isozyme was found in several tissues, including brain, lung, gut and kidney.

We conclude that somatic cells (FFs) can proliferate in early embryonic environment until early postimplantation stages. Foetuses and the mice born are chimeras between recipient cells and hybrid cells with contributions from the donor FFs. Transdifferentiation as opposed to reprogramming by cell fusion can be considered as underlying cellular processes in these chimeras.

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Introduction

Totipotency of cells is their ability to differentiate into any cell type. Mouse embryonic stem (ES) cells (Evans & Kaufman 1981, Martin 1981) have been proven totipotent by obtaining mice developed entirely from these cells (Nagy *et al.* 1990, 1993, Modliński *et al.* 1996). When injected into mouse blastocyst, a single stem cell of teratocarcinoma populated a variety of different tissues in adult mouse (Illmensee & Mintz 1976), showing its pluripotency.

Tissue stem cells have also revealed some potential to participate in the formation of various tissues when injected into blastocysts ('blastocyst complementation test'). The first to conduct such a test was Brinster (1974), who injected bone marrow cells into mouse blastocysts finding a delay in transplant rejection from the donor strain in potentially chimeric mice born. After injecting

adult haematopoietic stem cells (HSCs) into blastocysts, chimeric animals carried the progeny of HSCs in various haematopoietic tissues and their HSCs from bone marrow expressed embryonic and foetal globins (Geiger *et al.* 1998). After injection of HSCs from human cord blood cells into murine blastocysts, human donor contribution persisted to adulthood (Harder *et al.* 2002). Neurospheres obtained by aggregation of adult neural stem cells and introduced into blastocysts contributed to all three germ layers in 11-day-old mouse foetuses showing tissue-specific protein expression (Clarke *et al.* 2000). Multipotent adult progenitor cells (MAPCs) were found in many tissues of adult mice as a result of blastocyst complementation test (Jiang *et al.* 2002). Indeed, MAPCs could give rise to almost all types of cells, which was confirmed by transplanting single MAPC into blastocysts. Kues *et al.*

(2005) have described cells that are derived from fetuses under conditions of overgrowth and oversupply of foetal calf serum in the culture medium as 'foetal somatic stem cells (FSSCs)'. After injection into mouse blastocysts, such cells participated in tissues of the mesodermal lineage in 15-day embryos.

Studies on the potencies of non-stem somatic cells have been limited so far. Non-stem somatic cells can supply nuclei for cloning by nuclear transfer (Wilmut *et al.* 1997, Wakayama *et al.* 1998), which brings evidence in favour of their totipotency. However, it remains to be elucidated, if entire somatic cells can transdifferentiate to become totipotent and to participate in embryonic development. Hakelien *et al.* (2002) have shown that cytoplasmic extracts from one type of somatic cells (T lymphocytes, neuronal precursors) can reprogram other somatic cells (fibroblasts) *in vitro*. Fibroblasts treated with such cellular extracts show expression of transcription factors and antigens typical of donor cell extract. But can the environment of early embryos act similarly, or in other words, can reprogramming occur *in vivo*? Soon after somatic (epithelial) cells were injected into cleaving mouse embryos, gap junctions were found between them and the blastomeres of chimeric morulae and the injected cells were expressing totipotency marker Oct-4 (Burnside & Collas 2002). Interestingly, this early response was absent from fibroblasts after they had been injected. In this paper, we follow fibroblasts injected into cleaving mouse embryos to determine their long-term response to embryonic environment.

Materials and Methods

Obtaining embryos

All animals originated from the mouse-raising facility in the Department of Experimental Embryology. Mice were kept under a 12-h day starting at 0700 h. Experiments were performed with the permission of the Third Local Ethical Committee in Warsaw. Inbred DBA/2 or MIZ (Swiss albino) females aged 2–3 months mated to 3- to 10-month-old males of the same breeds served as donors of embryos. To obtain eight-cell embryos, females were caged with males in the evening and next morning they were inspected for vaginal plugs. Those females that mated were killed by cervical dislocation 48–50 h later. Oviducts were excised into M2 manipulation medium (HEPES-buffered M16; Fulton & Whittingham 1978) and their contents were flushed with the same medium using a pipette introduced into the infundibulum.

Obtaining foetal fibroblasts

To establish primary cultures of somatic cells (foetal fibroblasts, FFs), females of C57BL10 or CBA/H strains were mated with males of the same or the other strain to

obtain either C57BL10 fetuses or F1 (C57BL10×CBA/H) or F1 (CBA/H×C57BL10) fetuses. At day 13 or 14, p.c. females were killed by cervical dislocation and uterine horns were cut out and placed in sterile PBS. After the fetuses were dissected out of their foetal membranes, they were decapitated and eviscerated. The carcasses were rinsed in PBS, cut into small pieces and transferred to 0.25% trypsin/EDTA for 30 min at 37 °C. Trypsin digestion was stopped by adding Dulbecco's modified Eagle medium (DMEM, Sigma)+10% foetal calf serum (FCS, Gibco). After centrifugation for 10 min at 200 g, the cells were suspended in the same medium and seeded onto tissue culture Petri dishes (cell suspension from single carcass per one 6 cm dish) to be cultured at 37 °C, in an atmosphere of 5% CO₂ in air. Upon reaching confluence, the cells were passaged and were frozen at passages 3–6 to be used later. For the experiments, an ampoule of frozen cells was thawed and the seeded cells were cultured until reaching confluence. For manipulation, confluent culture was trypsinised, centrifuged (in some experiments the cells were stained with PKH26-GL at this stage) and suspended in a small amount (200 µl) of M2 medium to be taken to the manipulation chamber.

Membrane lipophilic dye PKH26-GL

PKH26-GL (Horan & Slezak 1989; Sigma) is a vital fluorescent lipophilic dye, persisting in cell membranes as long as 100 days after staining. Fibroblasts were stained with this dye before being introduced into the embryos, according to manufacturer's instructions. Embryos with stained cells were observed under inverted Opto IM35 microscope (Zeiss) using phase-contrast optics and u.v. light (excitation filter 546 nm).

Manipulation

Before manipulation, the embryos were incubated (30 min–1 h) in M2 containing cytochalasin D (CD, Sigma, 1 µg/ml), at 37 °C in an atmosphere of 5% CO₂ in air. Embryos together with 2 µl fibroblast suspension were placed in a drop of M2 medium with CD under paraffin oil in the manipulation chamber. Manipulation was performed at room temperature, as described by Stewart (1993), under Fluovert (Leitz, Wetzlar, Germany) inverted microscope, with differential interference Nomarski contrast (DIC). Mechanical Leitz (Germany) manipulator, micropumps: Beaudouin (France), connected with injection pipette, and CellTram Air, Eppendorf (Hamburg, Germany), connected with holding pipette, were used. Both holding and injection pipettes were prepared from thin-walled borosilicate capillaries of external diameter 1 mm, made of silica glass (GC 100T-15, Harvard Apparatus Ltd; Edenbridge, Kent, UK). Three fibroblasts were injected into the centre of each eight-cell embryo.

Culture conditions

After thorough rinsing with M2 without CD, the embryos were placed in drops of M2 or KSOM medium (Specialty Media, Phillipsburg, NJ, USA) under paraffin oil (Sigma) in Petri dishes (Corning) and were cultured at 37 °C, in an atmosphere of 5% CO₂ in air. For outgrowth formation, blastocysts were placed in wells of 4- or 24-well dishes (Nunc or Corning) on feeder layer and were cultured in DMEM with high glucose (4.5 g/l), 0.1 mM β -mercaptoethanol (Sigma), 0.1 mM non-essential amino acids (Sigma) and nucleotides (Sigma; Robertson 1987). Media supplements included 20% foetal calf serum (Gibco) and 1000 i.u./ml mouse leukaemia inhibitory factor (LIF; Sigma). For feeder layer production, confluent FFs were inactivated with 10 μ g/ml mitomycin C (Sigma) for 2.5–3 h and were used either directly (5×10^4 cells/cm²) or after freezing.

In vitro studies

MIZ embryos containing F1 (C57BL10 $\times$ CBA/H) or F1 (CBA/H $\times$ C57BL10) fibroblasts stained with PKH26-GL were cultured *in vitro* and were observed every 24 h to localise introduced cells. On day 4 of culture, the embryos were transferred onto feeder layers enabling their settlement and outgrowths formation, and were observed until day 10 (day of fibroblast injection, day 0). To limit deleterious effects of u.v. light, each embryo was observed not more than thrice, usually twice throughout its whole culture period.

Embryo transfer

For embryo transfer, F1 (C57BL10 $\times$ CBA/H) or F1 (CBA/H $\times$ C57BL10) females mated to vasectomised F1 males were used as recipients. The embryos (5–12 per transfer) were transferred to the oviducts of recipients during 1st day of pseudopregnancy. Females were anaesthetised by i.p. injections of 0.1 ml/g body weight of 12.5% solution of Vetbutal (Biovet, Pulawy, Poland) in PBS.

In vivo studies

After 24 h of culture, DBA/2 embryos with C57BL10 fibroblasts were transferred to pseudopregnant females and autopsy was performed at days 8–14. Morphological observations were followed by collecting material for electrophoresis of GPI isozymes. Few recipients were left to bear the young. Animals born, after they grew up, were autopsied and tissue samples were frozen for electrophoresis. After 24 h of culture, MIZ embryos with F1 (C57BL10 $\times$ CBA/H) or F1 (CBA/H $\times$ C57BL10) fibroblasts inside were transferred to recipients which were left undisturbed until birth of young. Young born, after

reaching puberty, were crossed with mates from the strain of donors of embryos.

Morphological evaluation of development and collecting tissue samples for GPI analysis

Embryos and foetuses were observed under stereomicroscope in both transmission- and epi-illumination to evaluate their developmental stage (according to Kaufman 1992). After morphological evaluation, 8-day egg cylinders were cleared of Reichert's membrane (which was frozen as a whole) and cut with a glass needle into embryonic and extraembryonic halves to be frozen separately. Samples of amnion, yolk sac and/or the embryos to be frozen separately were taken from 11- to 14-day implantation sites. In case of resorption of the embryo, tissue remnants were taken as samples. From animals born, some organs were dissected out and tissue fragments of about 1 mm diameter were stored.

Electrophoresis of GPI

The isozymes of GPI (DeLorenzo & Ruddle 1969, Lyon & Searle 1989) served as markers of donor and recipient cells in presumed chimeras. Electrophoresis of GPI was performed according to the method by Buehr & McLaren (1985) in samples of DBA/2 strain (GPI-1A) embryos injected with C57BL10 fibroblasts (GPI-1B). Tissue samples were first rinsed in PBS and then in Tris–glycine buffer (0.025 M) to remove any traces of blood. They were placed in 0.5 ml Eppendorf tubes containing 10–200 μ l buffer and were frozen at –20 °C. Samples of blood of animals born taken from tail tips were frozen and thawed thrice in distilled water (1:1 volumes) before being stored at –70 °C. For placing on electrophoretic plate, the samples were thawed and frozen thrice in Tris–glycine buffer (0.025 M, Sigma) and 0.5 μ l liquefied sample was applied. Blood of DBA/2 (GPI-1A) and C57BL10 (GPI-1B) animals served as reference samples. Cellulose acetate-covered plates (Titan III H, Helena Biosciences, Gateshead, Great Britain) were soaked in low molar Tris–glycine buffer (0.025 M) for 20 min at room temperature. Electrophoresis was performed in Tris–glycine buffer (0.1 M) for 60 min, at room temperature, at 200 V in ZipZone chamber (Helena Laboratories, Beaumont, TX, USA).

Results

In vitro development

FFs originally placed in the centre of each embryo were found in either ICM or trophoblast or both of them after 24 h. The percentage of embryos with fibroblasts located within an outer layer was almost twice as high when compared with fibroblasts in the centre of embryos. This ratio did not change throughout 3 days of culture (Table 1), suggesting no migration of introduced cells

Table 1 Contribution of fibroblasts (FFs) and/or their daughter cells to inner cell mass (ICM) and trophoblast.

Embryonic layer	Percentage ^a of embryos harbouring marked cells at subsequent days of culture						
	1st	2nd	3rd	4th	5th	6th	≥ 7th
Inner cells/ICM	38.4	36.3	33.0	38.5	34.5*	60.3*	66.7
Outer cells/TB	74.6*	72.6	60.9* [†]	40.2 [†]	33.3 [‡]	56.9 [‡]	57.9
No. of embryos observed ^b	307 ^c	124	115	122	84	58	57

Values with different superscripts (*, †, ‡) within a row were statistically significantly different ($M < 0.01$) in χ^2 -test.

^aThe sum may exceed 100% or be <100%, because fibroblasts were found in either inner cell mass, trophoblast or both of them. ^bTo limit deleterious effects of u.v. light, each embryo was observed not more than thrice, usually twice throughout its entire culture period. ^cThe majority of these embryos were transferred to recipient females at day 2.

within embryos. The percentage of embryos with FFs in ICM remained constant from days 1 to 5. However, between days 2 and 5 there was a decline in the percentage of embryos with fluorescent cells in trophoblast, indicating elimination of introduced cells and their progeny from trophoblast. Therefore, starting from day 4 onwards, the fraction of embryos with FFs in ICM was comparable with those containing cells in trophoblast (Table 1). At days 6–10, the percentages of embryos with introduced cells either in ICM or in trophoblast had increased (Table 1). This might be the result of enhancing effects of feeder layer and LIF medium on cell division upon outgrowth formation at day 6.

Contribution of FFs and/or their daughter cells to embryos was expressed as the percentage of embryos containing at least one fluorescent cell at subsequent days of culture. The day after introducing three FFs, a decline below this number was found in about 30% of embryos (Table 2, day 1), indicating some deterioration of introduced fibroblasts. Only in 6 and 17% of embryos, more than three fluorescent cells were found at days 1 and 2 respectively. This suggests an inhibition of FFs' divisions concurrent with cleavage delay in recipient embryos. At the fourth day, almost half of the embryos (46.2%) harboured more than three fibroblasts and this percentage increased every day reaching 100% at day 7. A slight decrease occurred later, which might suggest deleterious influence of prolonged culture. Presumed numbers of cell divisions undergone by FFs in cultured embryos are shown in Fig. 1 and Table 2. Embryos with six or more fluorescent cells appear at day 2 indicating

two cell divisions and on day 3, embryos with 12 cells indicating three divisions appear. After 7 days, in 70% of embryos fibroblasts underwent at least three divisions.

A total of 820 embryos were injected with FFs. Over 99% embryos survived until next day. However, they were only at the 8- to 16-cell stages, and they reached the blastocyst stage not earlier than after the following 24 h. Hatching *in vitro* was impaired in some blastocysts, probably due to perforation of the zona pellucida during manipulation. After placing them onto the feeder layer, the embryos attached during an overnight culture and formed outgrowths after next day. Fibroblasts stained uniformly with PKH26-GL and this pattern persisted 3 days *in vitro* (Fig. 2). Later on, fluorescent patches appeared (Fig. 3). Patchy distribution, although normal in accordance with the manufacturer's information (Sigma), interfered in some cases with counting fluorescent cells within outgrowths, thus reducing the numbers of outgrowths analysed at days 5–10.

In vivo development

In 19 transfers, 172 DBA/2 embryos were transplanted, giving rise to 101 implantation sites (58.7%). Out of 72 implantation sites taken for GPI analysis, in 62 the embryos were found (see Table 3).

Chimerism was confirmed by co-occurrence of recipient isozyme GPI-1A with donor isozyme GPI-1B in one embryo recovered at day 11, which was severely delayed to the stage of 8-day egg cylinder and showed signs of degeneration (Table 4). Otherwise,

Table 2 Contribution of fibroblasts (FFs) and/or their daughter cells to embryos cultured *in vitro*.

No. of PKH26-marked cells	Percentage of embryos in which marked cells were found in subsequent days of culture							
	1st	2nd	3rd	4th	5th	6th	7th	> 7th
0	1.8	4.9	4.4	0	1.4	2	0	0
1–2	31.9	41	34.5	22.7	22.2	10	0	11.5
3	60.1	50.0	44.2	30.7	23.6	18.0	0	0
4–6	6.3	3.3	10.6	31.9	15.3	28.0	25.0	15.4
7–12	0	0.8	0.9	4.2	16.7	16.0	5.0	15.4
> 12	0	0	5.3	10.1	20.8	28.0	70.0	57.7
No. of embryos observed ^a	286 ^b	122	113	119	72	51	20	26

^aTo limit deleterious effects of u.v. light, each embryo was observed not more than thrice, usually twice throughout the entire culture period.

^bThe majority of these embryos were transferred to recipient females at day 2.

Figure 1 Diagram showing presumed number of cell divisions undergone by injected fibroblasts (FFs) in mouse embryos cultured *in vitro*. Three fibroblasts were injected to eight-cell embryos and on subsequent days of culture (x-axis), fractions of embryos with PKH26-marked cells were counted. The assumption was that the presence of 4–6 marked cells (yellow) indicates one division, of 7–12 cells (orange) indicates two divisions and the presence of more than 12 cells (brown) indicates three divisions underwent by injected cells. y-Axis shows the percentage of embryos containing marked cells, for example, on day 3, 10.6% of embryos contained 4–6 cells (underwent one division), 0.9% of embryos harboured 7–12 cells (underwent two divisions) and 5.3% of embryos harboured more than 12 cells (underwent three divisions).

co-occurrence of both isozymes was only confirmed in extraembryonic lineage. These comprised: twice – in Reichert's membrane (of 8-day egg cylinders: one normal and one delayed) and five times – in the amnion (once of an 11-day normal embryo and four times of 12-day normal or delayed or abnormal foetuses). The last two

cases of confirmed chimerism were in tissue remnants after resorption.

Surprisingly, in four implantation sites, only the donor isoform GPI-1B was found. Three of them comprised remnants after resorption, found at days 8–12. In one case, some tissue found on day 11 appeared not dissimilar to an egg cylinder (Table 4).

In the samples taken from 11- to 14-day implantation sites, an isozyme was found on electrophoretic plates that migrated intermediately between the slow and the fast variants. Based on its speed of migration, the isozyme was recognised as hybrid GPI-1AB. Hybrid GPI-1AB isozyme was found in morphologically normal embryos (Fig. 4A–C). It was also found in one delayed 12-day embryo, whose amnion contained both GPI-1A and GPI-1B, without GPI-1AB contribution. Among organ samples, GPI-1AB was found in the heart, head and the liver of foetuses. Hybrid isozyme was also detected in the amnion of 12-day (Fig. 4D) and 13-day normal foetuses (six cases) and in the amnion of a 13-day abnormal foetus whose yolk sac was chimeric (one case). GPI-1AB appeared frequently in the yolk sac of 11- to 14-day foetuses (69.3% of 49 samples). Twelve-day embryos showing GPI-1AB in their foetal membranes were morphologically normal in almost 80% of cases. However, among 13-day embryos that contained GPI-1AB in their yolk sac (a total of eight embryos), only 50% were considered morphologically normal. On day 14, GPI-1AB was also found in yolk sacs of resorbed embryos.

Figure 2 Foetal fibroblasts (FFs) in mouse blastocysts. (A) Hatching blastocyst at the third day after FFs injection (phase contrast). (B) The same image after merging with u.v.-light picture; red fluorescent FFs are present in the inner cell mass (left) and in trophoblast (up and middle). (C) Hatched blastocyst at the fourth day after FFs injection. (D) The same image after merging with u.v.-light view; FFs can be seen in inner cell mass (left) and in trophoblast (right).

Figure 3 Foetal fibroblasts (FFs) in mouse blastocysts' outgrowths at days 6 and 7 after injection. Phase-contrast images on the left (A, C and E) and the same images after merging with u.v.-light pictures – on the right (B, D and F). Note a flattened, fluorescent, trophoblast-like cell in F. Patchy distribution of the stain is seen in this cell.

Animals reaching full-term

In 43 transfers, 325 embryos were transplanted giving rise to 70 young born (21.5%). Eleven of them were eaten by mothers soon after birth. Others were allowed to develop. One of the young was killed at 1 month due to developmental retardation and poor health. Males comprised 75% of young. Four females and one male were considered sterile. Fourteen animals (ten females and four males) died at 3–20 months of age for unknown reasons.

Donor FF-derived pigmentation was not found in the skin of adult chimeras. Progeny of fertile animals have not revealed such pigmentation either. Electrophoretic analysis of GPI was performed in tissue samples from five DBA/2 presumed chimeras (Table 5). All these animals revealed hybrid GPI-1AB and recipient GPI-1A isozyme. GPI-1AB was present in the organs originating from all three germ layers: mesoderm – kidneys, heart, gonads and blood; mesoderm/ectoderm – skin; mesoderm/endoderm – liver, endoderm – lung and intestine and ectoderm – brain (Fig. 4E). In some cases, donor GPI-1B

isozyme co-occurred with hybrid and recipient isoforms. In the skin of the newborn and of the 1-month-old chimera hybrid isozyme was found. It was also found in the gonads of all animals except the newborn one. GPI-1AB and GPI-1B were found in the blood of three animals, but two remaining ones showed only recipient isozyme GPI-1A in their blood.

Five females (out of 70 mice born after transfer of manipulated embryos) developed tumours. Four of them (two MIZ and two DBA/2 females) were killed and their internal organs were examined. In all the examined animals, the liver and the spleen were markedly enlarged. One of MIZ females at 10 months of age carried five tumours, four of which were found in four groins. Histopathological examination revealed lymphoblastomas. Liver and kidney were infiltrated with lymphocytes and in the ovary dysplasia of theca and follicular cells was found. The second MIZ female at 6 months of age had a tumour in right proximal groin, which was also diagnosed as lymphoblastoma. The liver

Table 3 Postimplantation development of FFs-injected embryos subjected to GPI analysis.

Day of development	No. (%) of embryos					No. of implantation sites
	Normal	Delayed	N + D	Malformed	Resorbed	
8	4 (36.4)	3	7 (63.6)	1 ^a	3	11
11	4 (44. 4)	2 ^b	6 (66.6)	2 ^c	1	9
12	28 (70)	7 ^d	35 (87.5)	2 ^e	3	40
13-14	6 (50)	2 ^f	8 (66.6)	1 ^g	3	12

^aDegenerating egg cylinder. ^bSeverely delayed 8-day egg cylinder showing signs of degeneration and an embryo, which appeared not dissimilar to an egg cylinder. ^cSmaller than their littermates, with small heads. ^dRetarded 9-day egg cylinder (one case), 10-day embryo (two cases) and 11-day embryo (four cases); two of the latter ones showed signs of degeneration. ^eEmbryos with open head folds; one of them with abnormal limb morphology. ^fDelayed by 1 or 2 days; the latter foetus had poorly blood-supplied heart. ^gHaematoma in the head region.

was severely infiltrated by lymphocytes. In a sterile DBA/2 female (animal 4, Table 5) at the age of 16 months, a tumour was found of a diameter of about 2 cm, located extraperitoneally in the right hind groin under the skin. It was diagnosed as adenocarcinoma type B originating from mammary gland. Electrophoretic analysis confirmed the presence of hybrid isozyme GPI-1AB in the outer, dermal part of the tumour and in other tissues of this animal. Severely enlarged spleen (to 2.5 cm) as well as ovarian cysts (one of them sized 1.5 × 1 cm, filled with blood on the left ovary; the second one 0.7 × 1 cm, filled with translucent fluid on the right) were found. The second DBA/2 female (animal 5, Table 5) at the age of 17 months had a large spherical tumour (Fig. 4F) located subcutaneously extraperitoneally near lymph nodes of left hind leg. This tumour was more solid than the remaining ones. Electrophoretic analysis revealed the presence of hybrid GPI-1AB in the solid part of the tumour as well as in other tissues of this animal. Enlarged spleen as well as right ovary cyst (filled with translucent fluid) was found accompanied by enlargement of kidneys.

Discussion

Proliferation of fibroblasts in preimplantation embryos

The percentage of embryos harbouring more cells than the introduced three reached 40% only after 4 days of culture. The cell cycle of FFs at low passages takes about 20 h (Hernandez *et al.* 2003), the duration which is sufficient for fibroblasts to divide at least thrice throughout this period. Therefore, we conclude that inhibition of cell division occurs in deposited FFs, possibly due to signals from blastomeres. Burnside & Collas (2002) observed similar block of division of both fibroblasts and epithelial cells 24 h after introduction to cleaving embryos. On the other hand, proliferation of ES cells in blastocysts appears to depend on their number, when a single cell is injected, then it divides within 24 h, whereas two to five cells injected, do not (Saburi *et al.* 1997).

However, after FFs are introduced into embryos, proliferation in the latter is also inhibited. When cleaving embryos obtain ES cells, after 24 h they reach the blastocyst stage (Tokunaga & Tsunoda 1992, Stewart

1993, Saburi *et al.* 1997). This is not the case after somatic cells are injected (Burnside & Collas 2002; and our results). There is experimental evidence for asynchronous blastomeres affecting themselves in chimeras, when 1/2 and 1/8 blastomeres are aggregated, cavitation occurs at intermediate time expected for either embryonic partner (Prather & First 1986). The delay in blastomere proliferation in our experiment might be the effect of prolonged blastomere cell cycle, due to the influence of the longer FFs' cell cycle (fibroblasts *in vitro* – 20 h: Hernandez *et al.* 2003, blastomeres from 8- and 16-cell stages blastomeres – about 13 h: Lehtonen 1980, MacQueen & Johnson 1983). At the end of *in vitro* culture, up to 15 fluorescent cells were present in our chimeric embryos. This is about 3% of cells present in normal 8-day embryos *in vivo* (500 cells; Snow 1978).

Donor-recipient chimerism in egg cylinders and foetal membranes

The only case of chimerism between recipient (GPI-1A) and donor (GPI-1B) cells in the embryonic part was an egg cylinder, delayed 3 days in development and showing signs of degeneration. Otherwise, this type of chimerism was only found in Reichert's membrane and later on, in the amnion at day 12. Thus, it seems that the presence of foreign cells is deleterious for the embryo proper but it does not impair embryonic development

Table 4 Contribution of GPI isozymes to embryos/foetuses and foetal membranes^a.

GPI isozymes ^b	Contribution of GPI isozymes to		
	Embryos/foetuses	Foetal membranes ^a	'Resorptions'
1B	1 ^c	0	3
1B + 1A	1 ^d	7 ^e (11.5%)	2
1AB + 1A	11 ^f (32.4%)	35 ^f (57.4%)	0
No. of samples	34	61	7

^aComprises yolk sac and amnion plus extraembryonic structures: Reichert's membrane and extraembryonic part of egg cylinder.

^bIsozyme GPI-1A migrates slowly on electrophoretic plate and is typical of DBA/2 strain recipient embryos. C57BL/10 strain, donor of fibroblasts, contains fast moving isozyme GPI-1B. ^cTissue which appeared not dissimilar to an egg cylinder. ^d8-Day egg cylinder. ^eUntil the 12th day of development. ^fUntil the 14th day of development.

Figure 4 Postimplantation development of FFs-injected embryos and electrophoretic analysis. (A and B) Normal day 12 chimeric mouse foetuses. (C) Electrophoretic plate showing heterodimer GPI-1AB in such foetuses; 1 and 5, GPI standard: mixed blood samples from DBA/2 and C57BL10 animals; 2, tissue sample from foetus A; 3, tissue sample from foetus B; 4, tissue sample from yet another normal 12-day foetus. (D) Electrophoretic plate showing heterodimer GPI-1AB in foetal membranes of 12-day normal foetuses developed from FFs-injected embryos; these foetuses themselves were not subjected to GPI analysis; 1 and 7, GPI standard: mixed blood samples from DBA/2 and C57BL10 animals; 2, tissue sample from amnion; 3, tissue sample from yolk sac; 4, tissue sample from another amnion; 5, tissue sample from another yolk sac; 6, tissue sample from yet another yolk sac. (E) Electrophoretic plate showing heterodimer GPI-1AB in brain tissue of animals born after FFs injection into mouse embryos; 1 and 5, GPI standard: mixed blood samples from DBA/2 and C57BL10 animals; 2, sample from brain of a fertile male (animal 3 in Table 5); 3, brain of an infertile female (animal 4 in Table 4); 4, brain of a fertile female (animal 5 in Table 5). GPI-1A, recipient isozyme; GPI-1B, donor isozyme; GPI-1AB, hybrid isozyme. (F) A tumour found in chimeric DBA/2 female (animal 5 in Table 5); lateral (left) and frontal (right) view.

when present in the amnion until day 12. Contrary to somatic cells (FFs), when embryo-derived totipotent ES cells were injected into blastocysts, postimplantation chimerism was high not only in extraembryonic mesoderm, but also in embryonic body, germ line included (Beddington & Robertson 1989).

Rescue of fibroblasts by fusion with recipient cells?

Starting from the 11th day of development on, both the embryos/foetuses (32%) and their foetal membranes (57%) revealed the presence of hybrid isozyme GPI-1AB. Moreover, the mice born and grown-up revealed hybrid isozyme in their tissues. This can only occur when in one cell both variants (GPI-1A and GPI-1B) are produced to form heteropolymer GPI-1AB, i.e. in heterozygotes or in hybrid cells (Lyon & Searle 1989). Animals used in our experiments were not heterozygous for GPI, and therefore, indication for hybrid cells arises. The presence of the heterodimeric type of GPI was earlier treated as evidence of cell fusion between trophoblast cells (Gearhart & Mintz 1972a) and between myoblasts upon eye muscle formation (Gearhart & Mintz 1972b) in mouse development. We suggest that also in our experimental embryos, hybrid cells arise due to cell fusion. We further hypothesise, that starting from day 11 on, the experimental foetuses and mice are chimeras

between recipient cells and hybrid cells derived from fusion of fibroblasts with recipient cells. In experimentally constructed tetraploid embryos, hybrid GPI-1AB isozyme could only be detected 3 days after cell fusion, since earlier in embryogenesis only the isozymes of both partners were present (Petzoldt 1991). This observation suggests that in our experiment, cell fusion could occur earlier than at day 11, when hybrid isozyme was first demonstrated. Indeed, if we accept that chimeric GPI-1A ↔ GPI-1B embryos only survive until days 7–8, then this is the last moment for fibroblast progeny to be rescued by fusion with recipient cells. The absence of hybrid GPI-1AB isozyme from days 7 to 8 egg cylinders found in this study may be due to delayed expression (like that described above) or to insufficient number of hybrid cells, falling below 1% of the studied sample, which precludes detection in GPI electrophoretic identification (Chapman *et al.* 1972). GPI isozymes were early (Chapman *et al.* 1972, Gearhart & Mintz 1972a, 1972b) and continuously (West & Flockhart 1994, Wang *et al.* 1997, Suwinska *et al.* 2005) used as markers of chimerism in the mouse. In day 9, embryonic chimeras that developed after aggregation of eight-cell embryos varying in GPI isozymes, no heterodimeric form was found thus excluding cell fusion until this stage (Gearhart & Mintz 1972a). Balinsky *et al.* (1983) describe GPI isozyme patterns in chimeric ('allophenic') mice and show that the

Table 5 GPI isozymes in tissues/organs of mice born.

Tissue/organ	Animal 1			Animal 2			Animal 3			Animal 4			Animal 5		
	R	H	D	R	H	D	R	H	D	R	H	D	R	H	D
Brain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lung	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Heart	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Kidney	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Liver	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gonad	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Skin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Blood	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Intestine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Muscle	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tumour	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

R, recipient isoform (GPI-1A); D, donor isoform (GPI-1B); H, hybrid enzyme (GPI-1AB); 1, newborn found dead in the cage; 2, young killed at 1 month of age due to developmental retardation and poor health; 3, fertile healthy male; 4 and 5, females that developed tumours.

^aOnly in the right ovary. ^bPresent in abscess only. ^cOnly in dermal part; n.d., not done.

hybrid isozyme appears in a tissue (lymphocytes) of some (two) of these mice. It is possibly the same phenomenon as we noticed in our study.

It remains to be studied, why mice born in our experiments, which were 75% males and were proven chimeric, did not reveal coat colour chimerism. Overrepresentation of males in the progeny of mice is a strong indication towards chimerism (Tarkowski 1961), since mice chimeric in chromosomal sex (XX↔XY) are not hermaphrodites, but they are fertile males instead. Cells of XY constitution in the gonad determine its differentiation into testis (for review, see McLaren (1984)). Triploid cells in chimeras express coat colour markers (Suwinska *et al.* 2005). Perhaps, hybrid tetraploid cells derived from FFs do not enter the skin or they do not produce pigment there.

Hybrid GPI-1AB isozyme was found in the gonads of chimeric animals, including one fertile male. However, neither GPI analysis of the progeny of this male was done nor can one expect that tetraploid cells can properly undergo meiosis to form functional gametes.

Reprogramming of hybrid somatic cells in the embryos

In our study, hybrid GPI-1AB isozyme was found in tissues originating not only from mesoderm, which is the layer of origin of fibroblasts, but also from two other germ layers; in a 13-day foetus, the heteropolymer was present in partially endodermal liver, and in born animals – in endodermal lungs, ectodermal brain and also in liver. However, in chimeric 10.5- to 15.5-day foetuses obtained after transfer of 'foetal somatic stem cells (FSSCs)' to blastocysts (Kues *et al.* 2005) only in muscle, genital ridge and liver contribution of introduced cells was found, suggesting their common mesodermal roots. 'Foetal somatic stem cells (FSSCs)' were derived *in vitro* from mouse foetuses under conditions of overgrowth and oversupply of foetal calf serum in the culture medium. In our study, FFs were derived from foetuses under standard tissue culture conditions (Freshney 1987). Presumed stem cells comprise only 1% of human FFs grown *in vitro* in standard conditions (Rieske *et al.* 2005). Therefore, it is most likely that 99% of mouse FFs used in our study are somatic cells.

Experimentally produced hybrid mouse cells, obtained *in vitro* by fusion of somatic cells (adult thymocytes) with ESCs and subjected to blastocyst complementation assay, survived in 7.5-day foetuses contributing to tissues originating from all germ layers (Tada *et al.* 2001). In addition, hybrids between foetal neural stem cells and ESCs in blastocyst complementation assay produced overt chimeras and the descendants of injected cells were detected in gut, kidney, heart and liver (where tetraploid cells are probably not eliminated; Ying *et al.* 2002). This comparison shows that FFs are reprogrammed as effectively in mouse embryos as are hybrids with contribution of totipotent ES cells.

Hybrids between ESCs and somatic cells are tetraploid (Terada *et al.* 2002), the condition presumably shared with hybrids between embryonic cells and FFs. Experimental tetraploid embryos develop until mid-pregnancy (Snow 1975, Tarkowski *et al.* 1977, Kaufman & Webb 1990) and in diplo↔tetraploid chimeras 4N cells are gradually eliminated from embryonic body and remain only in extraembryonic structures (Tarkowski *et al.* 1977, Nagy *et al.* 1993). However, participation of tetraploid cells in normal mouse development has been later proven to depend on the way of producing chimeras and/or on the genetic background of the donor. In embryonic chimeras formed by aggregation of two eight-cell diploid embryos with one four-cell tetraploid ones (Goto *et al.* 2002, Suemori *et al.* 1990), they were present in heart, liver, skin and intestinal epithelium of newborns. After embryonic stem cells were aggregated with tetraploid carrier blastomeres, one line, namely D3, promoted survival of tetraploid component in liver, lung and heart (Wang *et al.* 1997). The presence of tetraploid cells in epiblast, earlier considered impossible, has also been confirmed (Eakin *et al.* 2005). These data indicate that tetraploid cells are compatible with mouse development until birth.

Transdifferentiation or reprogramming by cell fusion?

Dedifferentiation of tissue-committed stem cells and their further differentiation into other tissues comprises muscle cells transdifferentiating into blood cells (Jackson *et al.* 1999), neural stem cells – into blood cells (Bjornson *et al.* 1999) and into other tissues (Clarke *et al.* 2000), HSCs – into neural cells (Eglitis & Mezey 1997, Mezey *et al.* 2000, Kopen *et al.* 1999), muscle cells (Gussoni *et al.* 1999) and hepatocytes (Petersen *et al.* 1999, Theise *et al.* 2000a) and bone marrow stromal cells – into neurons (Woodbury *et al.* 2000). Some comments suggest that cases of ‘transdifferentiation’ might result from cell fusion (Wurmser & Gage 2002, Scott 2004, Kucia *et al.* 2005), although in other cases fusion was excluded as the cause of observed transdifferentiation (Jang *et al.* 2004, Wurmser *et al.* 2004). For example, cases of transdifferentiation of bone marrow cells into liver cells (Alison *et al.* 2000, Theise *et al.* 2000b) need to be revised in the light of new experiments, such as fusion *in vivo* between bone marrow and liver cells (Wang *et al.* 2003, Camargo *et al.* 2004) and between bone marrow-derived cells and neural or heart cells (Alvarez-Dolado *et al.* 2003).

Experimental induction of cell fusion revealed that hybrid cells show phenotype, protein expression profile and differentiation abilities characteristic for less differentiated fusion partner (adult thymocytes with ESCs, Tada *et al.* 2001; ESCs with bone marrow-derived cells, Terada *et al.* 2002; ESCs and fibroblasts, Cowan *et al.* 2005; foetal neural stem cells with ESCs, Ying *et al.* 2002; different types of somatic cells with ESCs,

teratocarcinoma cells, Atsumi *et al.* 1982, Forejt *et al.* 1984 and blood stem cells, Camargo *et al.* 2004, Alvarez-Dolado *et al.* 2003). In all these cases, the more differentiated cell was reprogrammed (or at least repressed) by less differentiated fusion partner (see also comment by Surani 1999).

Analyses of hybrids between EGCs and thymocytes show the direction of changes in hybrid cells, their level of methylation and phenotype is similar to that of ESCs (Tada *et al.* 1997). Genome-wide demethylation is characteristic for both PGCs and preimplantation embryonic cells (Monk *et al.* 1987, Kafri *et al.* 1992, Ueda *et al.* 1992).

In our experiments, descendants of FFs were found also in the organs derived from ectoderm and endoderm, which indicates that FFs derived from mesoderm underwent considerable reprogramming. The less differentiated fusion partners for FFs might have been primordial germ cells (PGCs), rare at this stage of development, or cells of early epiblast. These cells do not exhibit totipotency, but still they are less differentiated than FFs.

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