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Mouse singletons and twins developed from isolated diploid blastomeres supported with tetraploid blastomeres

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ABSTRACT The aim of this study was to obtain mice, hopefully identical multiplets, from single diploid blastomeres isolated at the 4-cell stage, or from pairs of sister blastomeres isolated at the 8-cell stage. To this end isolated blastomeres were aggregated with one or two tetraploid carrier embryos produced by electrofusion of 2-cell embryos. Diploid embryos were albino and homozygous for the "a" allele of glucose-phosphate isomerase (GPI-1^a1^a) and tetraploid embryos were pigmented and GPI-1^b1^b. The aggregates were cultured *in vitro* up to the blastocyst stage. Each quartet (occasionally triplet or doublet) of chimaeric blastocysts was transplanted to the oviduct of a separate pseudopregnant recipient. Altogether 62 blastocysts were transplanted to 17 recipients. Eight full-term foetuses (two singletons and three pairs of twins) were rescued by Caesarian section on day 19, 20 or 21 of pregnancy. Three young (one singleton and twins) were successfully reared by foster mothers and proved to be normal and fertile females. All foetuses and animals were albino. In five individuals only the 1-A form of GPI (characteristic for 2n blastomere) was found. In one adult female traces of the 1-B form of GPI (characteristic for 4n carrier blastomeres) were detected in the heart and the lungs while 4 other organs contained only the 1-A form. These observations strongly suggest that the majority of foetuses/animals produced according to our experimental system are 'pure' diploids rather than 2n/4n chimaeras, and that the described method can be used in future to produce twins, triplets and quadruplets in the mouse. Our study confirms earlier work by Kelly (1975, 1977) that 'quarter' blastomeres of the mouse are still totipotent.

KEY WORDS: *mouse, isolated diploid blastomeres, tetraploid blastomeres, chimaeras, twins.*

Introduction

In the mouse single blastomeres of the 2-cell embryos can develop into adult mice (Tarkowski, 1959a,b); occasionally both separated blastomeres can give rise to twin animals (Mullen *et. al.*, 1970). Embryos developed from blastomeres isolated at the 4-cell stage can implant but only sporadically form small egg cylinders (Rossant, 1976), most probably due to the reduced number of cells at cavitation, that results in a small or absent inner cell mass (ICM) (Tarkowski and Wróblewska, 1967). Survival of mouse 'quarter' embryos beyond the early egg cylinder stage has not been described. In other mammalian species, like the rabbit and the sheep, in which at the time of implantation blastocysts consist of a larger total number of cells and, consequently, have more cells in the ICM, single blastomeres of the 4-cell and occasionally of the 8-cell embryo can develop into adults (Moore *et al.*, 1968; Willadsen, 1981). By transplanting sets of four single blastomeres of the 4-cell embryos or sets composed of four pairs of blastomeres of the 8-cell embryos Willadsen (*loc. cit.*) produced 2 pairs of twins, triplets and quadruplets.

Pluripotency and in some cases totipotency of single blastomeres of the 4-cell and 8-cell mouse embryo has been indirectly inferred from experiments in which such blastomeres were aggregated with carrier blastomeres of a different genotype, giving rise to chimaeric blastocysts. In many foetuses developed from these blastocysts the tested blastomeres contributed both to the embryonic tissues and the foetal membranes (Hillman *et al.*, 1972; Kelly, 1975, 1977). In some cases the born animals appeared to be completely derived from tested single blastomeres (Kelly, 1975, 1977). The disadvantage of this experimental system is that it requires meticulous manipulations to surround the tested blastomeres with carrier blastomeres. In spite of this, many of the produced embryos/animals are chimaeric rather than 'pure' blastomere-derived.

Abbreviations used in this paper: GPI, glucose-phosphate isomerase.

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We dedicate this paper to Anne McLaren, a long-time and proven friend of ours and of other Polish mammalian embryologists.

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In this study we devised a system in which carrier blastomeres were tetraploid (4n) rather than diploid (2n). As a result of their ploidy, these blastomeres were discriminated against in populating the embryo proper of chimaeric conceptuses. The rationale of this experiment was based on our earlier studies on diploid/tetraploid mosaics (Tarkowski *et al.,* 1977). In these experiments we showed that the majority of 3-cell embryos composed of two smaller 2n blastomeres and one larger 4n blastomere developed normally at least until the 13th day of pregnancy, and that in the embryonic body of these conceptuses the 4n cells were either missing altogether or did not exceed 4% of dividing cells. In contrast, the 4n cells occurred in high proportion in the foetal membranes. Selection of tetraploid against diploid cells in foetal tissues was reported also in the majority of chimaeras produced by aggregating 2n and 4n cleaving embryos (Lu and Markert, 1980; James *et al.*, 1995) and embryonic stem (ES) cells with cleaving embryos (Nagy *et al.*, 1990, 1993), or by injecting ES cells into tetraploid blastocysts (Wang *et al.*, 1997).

Our prediction that the 2n/4n experimental system should permit the production of mice from single blastomeres of the 4-cell embryo (or from pairs of sister blastomeres of 8-cell embryos) has proved correct: we obtained eight full-term foetuses (including 3 pairs of twins) and three of these foetuses (including one pair of twins) have developed into normal and fertile adult animals. Only one of these individuals contained traces of tetraploid cells in some tissues.

Results

All isolated blastomeres of 4-cell embryos (henceforth denoted 'quarter' or 1/4 blastomeres) or pairs of sister blastomeres of 8-cell embryos (denoted 2/8 blastomeres) were aggregated with tetraploid embryos in two variants (Table 1). 1) Two 4-cell tetraploid embryos were stuck on the opposite sides of a diploid blastomere(s). 2) One tetraploid embryo (2-, 3- or 4-cell) was aggregated with a diploid blastomere(s).

The rationale of placing the diploid blastomere(s) between two tetraploid embryos was to increase the chance of the 2n component becoming internalized and surrounded by 4n blastomeres and thus contributing predominantly to the ICM of the chimaeric blastocyst. In the majority of cases we succeeded in creating this type of array and we know that it persisted during the next few hours preceding compaction. It is not certain, however, whether this procedure was worth the trouble, because full-term foetuses were produced also by random aggregation of a diploid blastomere(s) with one tetraploid embryo.

To avoid self-aggregation of zona-free chimaeric embryos following their transplantation to recipients, we transplanted only embryos which had developed after 24 h or 48 h *in vitro* to the blastocyst stage, when embryos can no longer aggregate (see McLaren, 1976, p. 14).

TABLE 1

POSTIMPLANTATION DEVELOPMENT OF DIPLOID/TETRAPLOID CHIMAERIC BLASTOCYSTS DEPENDING ON THE NUMBER AND SPATIAL ARRAY OF 2N AND 4N BLASTOMERES

Type of aggregate*	No. of transplanted blastocysts	No. of recipients	No. of pregnant recipients	No. of implantations (foetuses + resorptions)	No. of full-term foetuses/ adults
4n<->2n<->4n	6	2	1	2	2/1
4n<->2n	56	15#	8	13	6/2
Total	62	17	9 (52.9%)	15 (24.2%)	8/3 (14.3%)/(4.8%)

* 4n: 2- or 3- or 4-cell tetraploid embryos; 2n: 1/4 or 2/8 blastomeres
\# one recipient was pregnant rather than pseudopregnant

Postimplantation development of 2n/4n blastocysts

Sixty two chimaeric blastocysts were transplanted to 17 recipients (Table 1). Among the transplanted embryos there were 12 quartets, 4 triplets and one pair of sister embryos. Using criteria described in Materials and Methods we estimated that implantation occurred in at least nine recipients. However, the number of implanted embryos was certainly underestimated because in some females the remnants of resorbed embryos must have disappeared by the time of autopsy. No data of this kind were available in the case of one unique recipient pregnant with their own embryos (see Materials and Methods).

Eight full-term foetuses were recovered by Caesarian section from 5 females. These included: two single foetuses (one recovered on the 19th day, the other on the 20th day) and three pairs of twins (two pairs recovered on the 20th day, one pair on the 21st day).

It appears highly significant that all 8 full-term foetuses developed in recipient females into which 22 chimaeric blastocysts were transplanted on the 1st day (day of plug) before 2 p.m. In this group all 6 recipients became pregnant: 5 had full-term foetuses (+ 4 resorptions) and one had two late resorptions. In contrast, in 11 recipients which were operated on between 2 p.m. and 7 p.m. (40 blastocysts transferred) implantation occurred only in three females and all conceptuses were resorbed.

Description of full-term foetuses

Among 8 full-term foetuses two had breathing problems and died within one hour, two started to breath normally but were eaten by foster mothers, one had a herniated intestine (but otherwise looked normal) and three young (one singleton and twins) were successfully reared by foster mothers and attained adulthood. Organs and tissues of six animals (3 foetuses and 3 adults) were subjected to GPI isoenzyme analysis.

No. 1. Single albino male foetus. Origin: 2/8 diploid blastomeres + one 2-cell tetraploid embryo. A quartet of sister chimaeric blastocysts was transplanted into a pregnant female. Ceasarian section on the 20th day yielded an albino foetus (no traces of pigment in dissected eye-balls) that was small and underdeveloped for one day, and weighed only 0.85 g. The female recipient had also 8 native, much larger, foetuses which weighed between 1.45 and 1.56 g. Since the experimental pup had no chance of surviving, it was killed for GPI analysis. Both 1-A isoenzyme (characteristic for the diploid component) and 1-B isoenzyme (characteristic for the tetraploid component) were present in equal amounts in the foetal membranes (the yolk sac and the amnion were processed together). By contrast only 1-A isoenzyme was observed in all 11 examined foetal tissues and organs (blood, heart, skeletal muscles, tongue, brain, stomach, intestine, liver, kidneys, testes and skin). Since there was no evidence of contribution of tetraploid cells to the foetus itself, its small size and developmental retardation must have been caused by other factors.

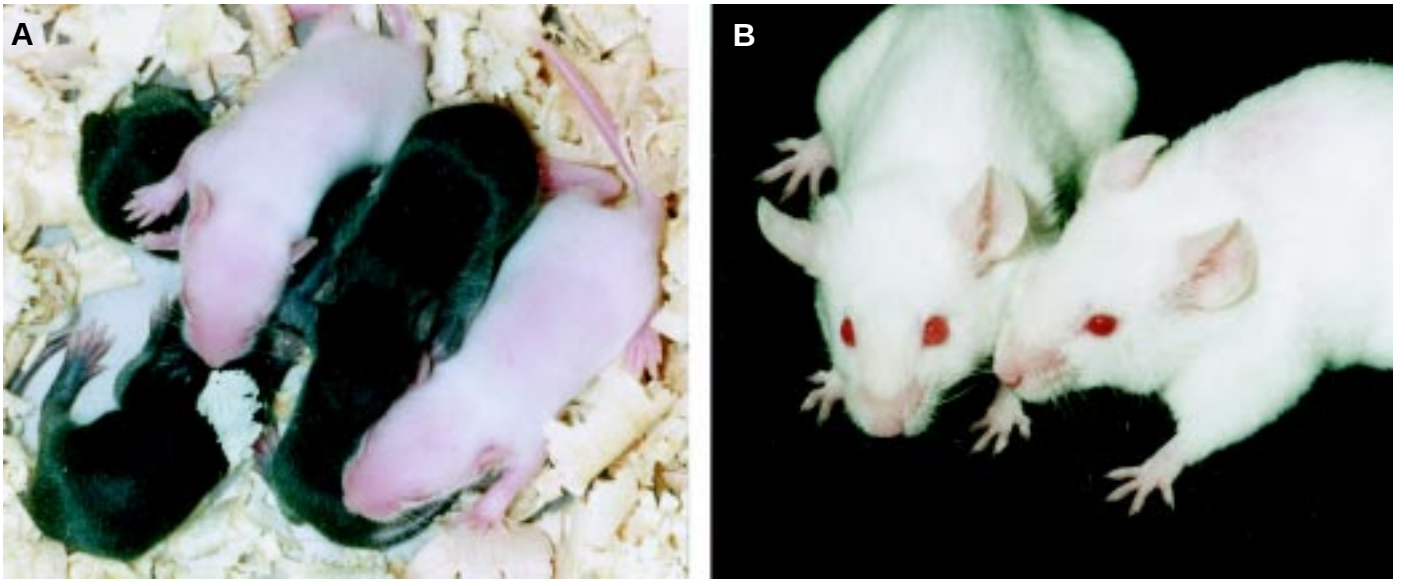

Fig. 1. (A) *Twin albino female young (nos. 5 and 6) at the age of 10 days with pigmented young of the foster mother.* **(B)** *The same twins at adulthood.*

No. 2. Single albino foetus. Origin: 1/4 or 2/8 diploid blastomeres + one 4-cell tetraploid embryo; three blastocysts transplanted. Caesarian section on the 19th day. The pup, that was of normal appearance and size, and showed no pigment in eyes at external inspection, was placed with a foster mother which had littered the previous night but it was not found in the nest on the next morning. Apart from the pup, there were in the uterus two resorptions, one early and one late. Data on the GPI constitution of foetal membranes are not available.

Nos. 3 and 4. Albino twin foetuses. Origin of both: 2/8 diploid blastomeres + two 4-cell tetraploid embryos; three blastocysts transplanted. Caesarian section on the 20th day yielded two female foetuses of normal size and appearance and without pigment in eyes. Both were placed with a litter of pigmented young born one day earlier. On the next morning only one young was found; it was successfully reared by the foster mother and proved to be a normal, fertile female. No pigment could be seen in the eyes and coat. After giving rise to a litter of 10 albino young the female was killed at the age of 12 weeks and its tissues subjected to GPI isoenzyme analysis. All eight tested tissues/organs (blood, heart, spleen, liver, intestine, brain, skeletal muscles, tongue) showed only 1-A form of GPI. Both forms of GPI (with the predominance of 1-A) were detected, however, in the foetal membranes (yolk sac and amnion processed together) of this animal.

The foetal membranes of the other young, which was eaten by the foster mother, contained both forms of GPI with the 1-A variant dominating over 1-B.

Nos. 5 and 6. Albino twin foetuses. Origin of both: 1/4 blastomere + one 2-cell or 3-cell tetraploid embryo; four blastocysts were transplanted. Ceasarian section on the 20th day gave twins that were successfully reared by the foster mother and proved to be normal, fertile females (Fig. 1 A and B). On external inspection both were 100% albino. After giving birth to the first litter of 11 and 12 albino young exactly at the same age of 73 days, they were killed and their tissues were subjected to GPI isoenzyme analysis. In animal no. 5 only 1-A isoenzyme was detected in nine examined tissues/organs (blood, heart, intestine, liver, lungs, skeletal muscles, tongue, brain and kidney). In animal no. 6 in four tissues/organs (blood, intestine, liver, brain) only 1-A isoenzyme was found. However, in the heart and in the lungs both forms of GPI were present, although 1-A (diploid albino component) predominated over 1-B component (Fig. 2).

Foetal membranes (yolk sac and amnion) of both animals were examined electrophoretically but we are not able to ascribe each set of membranes to the particular individual. In one case the two forms of GPI were present in equal amount, and in the other the 1-A form was accompanied only by traces of 1-B form.

Nos. 7 and 8. Albino twin foetuses. Origin: 1/4 or 2/8 diploid blastomeres + one 4-cell tetraploid embryo; four blastocysts were transplanted. Caesarian section on the 21st day gave two foetuses that were very large and weighed 2.24 and 2.0 g probably because of the prolonged pregnancy. The heavier pup (no. 7) had a herniated intestine but otherwise looked normal and had no breathing problems. The second pup (no. 8) looked completely normal but we were unable to sustain its breathing. The twin foetuses were males and had no pigment in the eyes. The young were killed for GPI analysis. Both had only 1-A form of GPI in five examined organs (heart, liver, lungs, skeletal muscles and skin; + brain in foetus no. 8). Foetal membranes were examined only in foetus no. 8 and showed presence of 1-A form only.

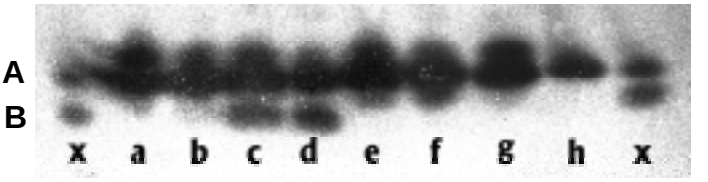

Fig. 2. *GPI-1 electrophoresis plate [tissues/organs of female no. 6 (one of those presented in Fig. 1)]. a, skeletal muscle; b, tongue; c, heart; d, lung; e, liver; f, intestine; g, brain; h, blood. As a control, a mixture of identically diluted blood samples from homozygous animals of* **A** *and* **B** *allelic variants were applied on both borders of the plate (x).*

Discussion

Although we did not obtain - as we had hoped - mouse quadruplets, we were able to produce normal and fertile mice (including twins) from single 1/4 or 2/8 blastomeres. We therefore have confirmed the earlier finding of Kelly (1975, 1977) that at this developmental stage at least some of the blastomeres are still totipotent. However, like in the experiments of Kelly (*loc. cit.*), totipotency could be demonstrated only with the help of an artificial system in which 1/4 blastomeres were supported by carrier blastomeres. If totipotency of a blastomere is defined in the strict sense as the ability to develop - like an intact zygote -into a complete organism then we have to conclude that in the mouse single 'quarter' blastomeres are no longer totipotent because they are not able to develop *by themselves* into a mouse. Although neither Kelly's nor our experimental system fulfills such stringent criteria, both can provide acceptable evidence in favour of totipotency. However, for the interpretation to be valid it is necessary that the carrier component either does not contribute at all to the body of the experimental animals, or only coexists with (in negligible amount) but not replace any of the donor blastomere-derived tissues. In other words, it is important to know whether the alien (carrier-derived) tissue represents just a superfluous 'contamination' and is not essential for the existence of the blastomere-derived organism. This is not just an academic issue, because the continuous presence of the alien tissue (even in negligible amount) makes the animal a chimaera and this may have various consequences, for instance of immunological nature. Even if it is vestigial, chimaerism raises the question of whether animals developed from sister blastomeres - like animals nos. 5 and 6 described in this study (pp. 593) - can be considered as identical twins.

In practice it may be very difficult, if at all possible, to prove absence of any carrier blastomere-derived alien cells in any of the tissues. Consequently, one should use a system devised to make the chance of such 'contamination' minimal. In this context, it seems that our experimental system is superior to Kelly's system because tetraploid carrier blastomeres are developmentally handicapped as compared to diploid carrier blastomeres in populating the embryo proper (Tarkowski *et al.*, 1977; James *et al.*, 1995). As shown in this study our system greatly increases the probability of obtaining foetuses (animals) composed exclusively of cells derived from the tested 'quarter' diploid blastomeres, although it does not guarantee this status. None of the eight individuals had pigment (the trait characteristic for the tetraploid component) and only one out of six individuals available for GPI analysis contained slight amount of tetraploid cells in two organs (out of six organs/tissues inspected). It is noteworthy that this particular animal was a healthy and fertile female. Among 36 animals produced by Kelly (1977) and suitable for this type of analysis as many as 16 (44.4%) were chimaeras; taking into account that only three organs/tissues were examined for chimaerism: eyes and coat (pigmentation) and blood (GPI) the incidence of chimaerism might have been even higher.

The 1-B (tetraploid) component was, however, present in four out of five examined sets of foetal membranes, but even in these tissues the 1-A (diploid) component either dominated or had equal share with the tetraploid component. As shown by James *et al.* (1995) in 2n/4n conceptuses the tetraploid component is abundant in the endoderm of the yolk sac and only rarely is present in the yolk sac mesoderm and amnion. Probably this was also the case in our conceptuses, but we do not have information on this issue because the two membranes were processed together.

In conclusion, we believe that the majority of the foetuses/animals that we have produced were 'pure' diploids rather than diploid/tetraploid chimaeras. We are reinforced in this view by the fact that in mice developed from chimaeric blastocysts constructed from embryonic stem (ES) cells and tetraploid cells (Nagy *et al.*, 1990, 1993; Wang *et al.*, 1997) the tetraploid cells were detected only in the minority of animals and usually in small amount.

The rare and very limited contribution of tetraploid cells to the body of experimental foetuses and adult animals described in this study is astonishing in view of the great predominance of 4n over 2n components in the chimaeric blastocysts (8:1 or 4:1). The most extensive studies on the allocation and fate of diploid and tetraploid cells in chimaeric pre- and postimplantation embryos have been carried out by John West and his colleagues (see James *et al.* 1995; Everett and West, 1996, 1998; Tang *et al.,* 2000). It has to be stressed, however, that the embryos they investigated had been produced by aggregating one complete tetraploid embryo with one complete diploid embryo (initial volume ratio 1:1) and, therefore, are not fully comparable with our experimental embryos in which 4n blastomeres initially predominated. According to Everett and West (*loc. cit.*) and Tang et al. (*loc. cit.*) in 2n/4n chimaeric blastocysts tetraploid blastomeres are preferentially allocated to the mural trophectoderm and, as early as between 3.5 and 4.5 days, there is a selection against 4n cells. It would follow that already at the time of implantation the proportion of diploid versus tetraploid cells in the ICM is relatively greater than in the initial aggregate. Whether and to what extent these processes help to allocate diploid cells to the ICM of our experimental blastocysts that develop from aggregates in which these cells are in minority, remains to be experimentally demonstrated. On the basis of very exhaustive studies on postimplantation development of 2n/4n chimaeric embryos James *et al.* (1995) conclude that whatever the mechanisms responsible for the absence of 4n cells in strictly embryonic tissues (differential allocation and/or cell selection) they must operate soon after implantation and certainly before 7.5 day of development. Even if the mechanisms observed by James and her colleagues do operate also in our experimental embryos, their effect is likely to be weakened by the great initial predominance of the tetraploid component in the aggregates and, consequently, in the ICMs of the resulting blastocysts. This could lead to intense foetal mortality, not only before and at midpregnancy when most of mouse tetraploid embryos die (Snow, 1973, 1976; Tarkowski *et al.*, 1977) but also late in pregnancy. In fact, we found apart from 8 foetuses 7 resorptions including some with large placentae, and we have evidence that these figures underestimate the number of implantations. Survival of our experimental embryos till term (14.3% of transplanted blastocysts) is definitely suboptimal. However, there are two encouraging observations. First: in 5 pregnancies which successfully proceeded till term 12 out of 18 transplanted blastocysts implanted and in three cases twins developed. Second: when blastocysts were transplanted to recipients before 2 p.m., as many as 36.4% (8/22) developed into full-term foetuses. It remains to be determined to what extent the efficiency of our experimental system is self-limited by its very nature (for instance by variable and noncontrolable location of diploid cells in chimaeric 2n/4n blastocysts) and to what extent it could be increased, for instance by discovering the optimal quantitative ratio of diploid to tetraploid

blastomeres and the optimal relative developmental age of the two components. If these conditions could be optimized, the method described in this study might, perhaps, be used for obtaining small clones of mice, i.e. twins, triplets and quadruplets.

Materials and Methods

Diploid embryos

4-8-cell embryos were obtained from albino animals homozygous for the 'a' allele of glucose phoshate isomerase (GPI-1^a1^a). These animals were of two genetic types: first - BALB/c inbred strain and, second - a line obtained from a cross between BALB/c and our colony of outbred albino mice (MIZ, homozygous for 'b' allele of GPI) and selected for homozygosity of 'a' allele at the GPI locus (and henceforth called BAMIZ).

Cycling BALB/c and BAMIZ females kept under a 12 h light/12 h dark lighting regime (middle of the dark period centered at midnight) were crossed with BALB/c or BAMIZ males respectively and inspected for plugs early in the morning (the day of plug = first day). The plugged females were killed on the 3rd day between 9.00 a.m. and noon and the embryos were recovered from the oviducts in M2 medium (Fulton and Whittingham, 1978) supplemented with bovine serum albumin (BSA). The collected embryos represented all stages from late 4-cell to early 8-cell. After removing the zona pellucida with 0.5% Pronase (Sigma) or acid Tyrode solution (Nicolson *et al*., 1975) the blastomeres were incubated in Dulbecco A solution with 0.02% EDTA and BSA for at least 20 min at 37°C and finally disaggregated by gentle pipetting. The isolated sister blastomeres (1/4 and/or 2/8) were stored in M2 medium until used for aggregation.

Tetraploid embryos

Carrier tetraploid embryos (GPI-1^b1^b) originated from F1 (C57BL/10 x CBA/H) females crossed with F1 males of the same genotype. The females either ovulated spontaneously or were induced to ovulate with pregnant mare's serum gonadotrophin (PMSG; Intervet) and human chorionic gonadotrophin (hCG; Intervet) given 43 - 51 h apart in doses of 10 IU each. The females that ovulated spontaneously were killed on the second day of pregnancy, around 2 p.m. Those induced to ovulate were killed between 32 and 45 h post hCG. The recovered 2-cell embryos were electrofused according to the slightly modified technique of Kubiak and Tarkowski (1985). In brief: the embryos were oriented with the interblastomeric surface perpendicular to the lines of the electric field in the fusion chamber filled with 0.25 M glucose supplemented with 100 μM $CaCl_2 \cdot 2H_2O$ and 100 μM $MgSO_2 \cdot 7H_2O$ and subjected to two pulses of 40 Volts d.c. of 25 μs duration. The gap between the electrodes was 116 μm. Fusion usually took place very rapidly (within 20 minutes) and the embryos were placed in separate drops of KSOM I (Erbach et al., 1994) or KSOM II medium (Summers *et al*., 1995) under liquid paraffin at 37°C in an atmosphere of 5% CO_2 in air. About 24 h later the embryos were at the 2-, 3- and 4-cell stage. The zona pellucida was removed with acid Tyrode solution and the embryos were stored in M2 medium on the surface of 2% agar in 0.9% NaCl.

Aggregation of diploid blastomeres with tetraploid embryos

Each quartet of sister 1/4 and/or 2/8 blastomeres was processed separately. The diploid blastomere(s) and 2-, 3- or 4-cell carrier 4n embryos were placed in a solution of phytoheamagglutinin (300 μg/ml) (Sigma) in BSA-free M2 medium for about one minute allowing the diploid blastomere(s) to aggregate with one or two tetraploid embryos. The aggregates were placed in separate drops of KSOM medium (*loc. cit*.) under liquid paraffin and cultured at 37°C in 5% CO_2 in air for 24 or 48 h.

Transplantation of chimaeric blastocysts

After 24 h of culture the aggreagates were advanced morulae or early blastocysts and after 48 h large blastocysts. Usually 4, but occasionally only 3 or even 2 sister blastocysts were transplanted to the oviduct on the day of vaginal plug (= first day). Sixteen recipients were F1 (C57BL/10xCBA/H) or MIZ albino females mated with vasectomized F1, MIZ or BAMIZ albino males, and one recipient was a MIZ female mated with a fertile F1 male. In the latter combination the native progeny were pigmented and thus could be distinguished from foetuses/neonates developed from albino blastomere(s). Females were anaesthesized with 0.35 ml of water solution of Nembutal (60 μg/ml; Serva) injected intraperitoneally.

Starting on the 11-13th day after mating vaginal smears were taken daily from each recipient female to determine whether implantation had taken place and the pregnancy was being continued. If a smear was typical of proestrus or oestrus the female was killed and the uterus inspected for resorptions; otherwise the females were observed until the 19th, 20th or 21st day and then autopsied.

The foetuses recovered be Caesarian section were either frozen for GPI isoenzyme analysis or fostered by females which had littered the same night or one day earlier. Those successfully reared (3 females) were tested for fertility by crossing with normal albino males. After giving birth to their first litter they were killed for GPI analysis.

GPI assay

Samples of tissues were frozen in small amount of redistilled water and stored at -20°C or -85°C. After thawing the samples were homogenized mechanically, centrifuged at 14000 rpm and the supernatants collected. Electrophoresis was carried out on TITAN III-H (Helena Laboratories) plates using Tris-glicine buffer (0.1 M in chambers and 0.025 M for soaking plates) (Buehr and McLaren, 1985). As control samples we used a 1:1 mixture of blood of adult GPI-1^a1^a and 1^b1^b animals. Electrophoresis was run for 1 h at 200 V. Plates were stained in a mixture of magnesium acetate 2.7 mg, MTT 0.5 mg, PMS 0.05 mg, NADP 0.5 mg, glucose 6-phosphate dehydrogenase 0.25 units, dissolved in 2 ml of redistilled water and mixed (1:1) immediately before use with 2% water solution of agar. Plates were photographed at different times of staining. From five to eleven organs/tissues were examined in each foetus/animal.

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