

Identical triplets and twins developed from isolated blastomeres of 8- and 16-cell mouse embryos supported with tetraploid blastomeres

ANDRZEJ K. TARKOWSKI*, WACLAW OZDZENSKI and RENATA CZOŁOWSKA

Department of Embryology, Institute of Zoology, Faculty of Biology, Warsaw University, Warsaw, Poland

ABSTRACT We studied the developmental potential of single blastomeres from early cleavage mouse embryos. Eight- and sixteen-cell diploid mouse embryos were disaggregated and single blastomeres from eight-cell embryos or pairs of sister blastomeres from sixteen-cell embryos were aggregated with 4, 5 or 6 tetraploid blastomeres from 4-cell embryos. Each diploid donor embryo gave eight sister aggregates, which later were manipulated together as one group (set). The aggregates were cultured *in vitro* until the blastocyst stage, when they were transferred (in sets) to the oviducts of pseudopregnant recipients. Eighteen live foetuses or pups were obtained from the transfer (11.0% of transferred blastocysts) and out of those, eleven developed into fertile adults (one triplet, one pair of twins and four singletons). In all surviving adults, pups and living foetuses, only diploid cells were detected in their organs and tissues as shown by analysis of coat pigmentation and distribution of glucose phosphate isomerase isoforms. In order to explain the observed high rate of mortality of transferred blastocysts, in an accompanying experiment, the diploid and tetraploid blastomeres were labelled with different fluorochromes and then aggregated. These experiments showed the diploid cells to be present not only in the inner cell mass (ICM) but also in the trophoctoderm. The low number of diploid cells and the predominance of tetraploid cells in the ICM of chimaeric blastocysts might have been responsible for high postimplantation mortality of our experimental embryos.

KEY WORDS: *mouse, 8-16-cell embryo, isolated diploid blastomere, tetraploid carrier blastomere, triplet*

Introduction

Over many decades, a variety of experimental approaches, such as reduction of the volume of the zygote or blastomeres, bisection of morulae and blastocysts to produce twins, aggregation of cleaving embryos to form chimaeras and separation of the embryo into single blastomeres, showed that the early mammalian embryos, including mouse embryos, are very flexible in their developmental potential. Traditionally, in literature, the blastomeres that originate from 2-, 4- and 8-cell embryos are designated as 1/2, 1/4, 1/8 blastomeres and the single blastomeres, which are able to develop into normal fertile adults, are termed 'totipotent'. In animals such as the rabbit, sheep and cattle, normal adults have been obtained from 1/2, 1/4 and 1/8 blastomeres (Moore *et al.*, 1968; Willadsen, 1981; Willadsen and Polge, 1981; Johnson *et al.*, 1995). However, it seemed that in the mouse, only 1/2 blastomeres and not the 1/4 and 1/8 blastomeres, can develop into normal animals (Tarkowski, 1959a,b; Mullen *et*

al., 1970; Hoppe and Whitten, 1972; Tsunoda and McLaren, 1983; Togashi *et al.*, 1987; Papaioannou *et al.*, 1989; Wang *et al.*, 1997a; Sotomaru *et al.*, 1998). It was assumed that the inability of 1/4 or 1/8 mouse blastomeres to develop normally might be caused not by their limited developmental potential but by the low number of cells present in the resulting embryos (blastocysts) at the beginning of cavitation. Tarkowski and Wroblewska (1967) and Rossant (1976) showed that these blastocysts, because of the low total cell number had either very small inner cell mass (ICM), or did not have ICM at all. In order to overcome this obstacle, Kelly (1975,1977) aggregated single 1/4 and 1/8 blastomeres with a few genetically distinct 'carrier' blastomeres, restoring the number of cells to normal or nearly normal level and found that cells derived from the single blastomere were present

Abbreviations used in this paper: BSA, bovine serum albumin; GPI, glucose phosphate isomerase; hCG, human chorionic gonadotrophin, ICM: inner cell mass.

*Address correspondence to: Dr. Andrzej K. Tarkowski, Department of Embryology, Institute of Zoology, Faculty of Biology, Warsaw University, Miecznikowa 1, 02-096 Warsaw, Poland. Fax: +48-22-5541-210 or 203. e-mail: akt@biol.uw.edu.pl

in various tissues of the body and in the extra-embryonic membranes of the chimaeric foetuses/animals. In addition, she found, that several foetuses/pups/animals derived solely from the single blastomere. This indicated that, in the mouse, single 1/4 and 1/8 blastomeres are capable to give rise to a complete animal, provided, however, that they were supported by carrier blastomeres. Subsequently, similar results were obtained by Tsunoda *et al.* (1987) and Pinyopummin *et al.* (1994) when they used parthenogenetic embryos as the source of carrier cells.

We have described previously, an experimental system in which the carrier blastomeres were tetraploid rather than diploid (Tarkowski *et al.*, 2001). This approach drastically reduced the chance of 'contamination' of the tissues of the resulting embryo with the cells derived from carrier blastomeres and the overwhelming majority of mice thus produced were derived solely from the single, diploid blastomere. Using this system we produced identical twins from 1/4 blastomeres derived from a single embryo (Tarkowski *et al.*, 2001). In the present study we have successfully used this procedure to obtain identical triplet and twin mice from single 1/8 blastomeres and pairs of 1/16 blastomeres. These results show that up to the 8-cell stage, at least some blastomeres retain the potential to develop into an animal, provided that they were supported by tetraploid carrier cells.

Results

Postimplantation development and survival of $2n \leftrightarrow 4n$ chimaeric blastocysts

Total number of 210 chimaeric blastocysts were transferred to 27 recipient females. Each recipient received one set of blastocysts which developed from sister chimaeric aggregates constructed from a single diploid blastomere obtained from a single 8-cell embryo (or a pair of sister blastomeres from the 16-cell embryo) and 4 to 6 tetraploid carrier blastomeres (Figs. 1,2). Thus, within each set of blastocysts, the diploid blastomeres were genetically identical because they originated from one disaggregated cleaving embryo. The tetraploid blastomeres were usually mixed after disaggregation, so they originated from more than one embryo. Implantation occurred only in 21 females, which received altogether 163 blastocysts. Only half of the transferred blastocysts (86/163) implanted (Table 1). In one female all 8 blastocysts constituting one set had implanted. In four females 7 out of 8 blastocysts had implanted and in sixteen remaining pregnant

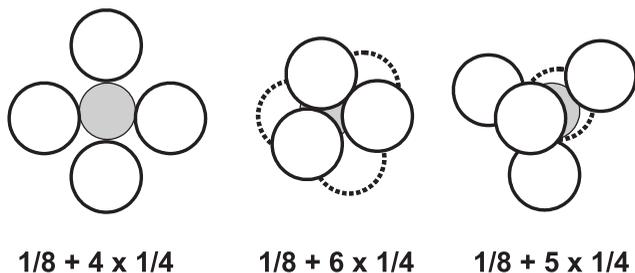


Fig. 1. Schematic representation of the spatial arrangement of a diploid 1/8 blastomere (gray) aggregated with tetraploid blastomeres (white). The aggregates were either 'two-dimensional' (four tetraploid blastomeres) or 'three-dimensional' (five and six tetraploid blastomeres).

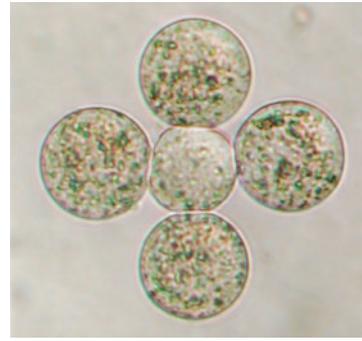


Fig. 2. A diploid 1/8 blastomere from an embryo of BAMIZ origin, surrounded by four tetraploid 1/4 blastomeres from an F1 (C57Bl/10 x CBA/H) embryo. Magnification: $\times 275$.

females 1 to 6 blastocysts had implanted. Seven out of 21 pregnant females terminated pregnancy prematurely (as revealed by replacement of mucus with epithelial cells in the vaginal smears). Two pregnant females were sacrificed on the 15th day of pregnancy, eleven females were sacrificed on the 20th and 21st days and only one female littered naturally. The number of early resorptions in all sacrificed pregnant females was high (69.8% of all blastocysts implanted). The size of resorption sites indicated that embryos must have died before or around the middle of pregnancy. In the second half of pregnancy the mortality was much lower and affected only 9.3% of implanted embryos: six foetuses died between 12th and 16th day of pregnancy and two foetuses shortly before term.

Eleven females, which were sacrificed at term, had very small litters: four females had live twins, four females had just a single live pup and three females had only dead foetuses. The female that littered naturally had three live pups.

Foetuses and pups were obtained from chimaeric aggregates that differed in type of diploid blastomeres (1/8 or 2/16) and in the number of carrier tetraploid blastomeres (4, 5 or 6) (Table 1). Because the five experimental variants differed considerably in the number of embryos produced, transferred and implanted (Table 1), it is difficult to draw any definite conclusion about the possible superiority of any of these variants. One can only conclude that with the exception of the '1/8 + 5 x 1/4' variant, in all other variants some embryos developed to full-term foetuses or pups.

Postnatal survival of diploid neonates

Among 15 full term foetuses/pups delivered naturally or by Caesarian section there were four pairs of identical twins and one set of identical triplets. In three pairs of twins only one member from each pair survived (one pup died soon after birth and two pups died within two days, with one of them displaying *varus* deformity of hind legs). From the single litters one pup died at the age of one month. All the remaining 11 pups including one set of triplets (Fig. 3), one pair of twins and 6 singletons survived to adulthood. Among the 11 adults there were four males (including one pair of twins) and seven females (including triplets). All adult animals were fertile and females produced at least one litter.

Distribution of diploid and tetraploid cells in chimaeric foetuses/animals and their foetal membranes

The contribution of diploid and tetraploid components to the organs and tissues and to the yolk sac and amnion of experimental specimens was established by analysis of the distribution of GPI isoforms. Because, in our hands, the sensitivity of the GPI

assay was at the level of about 6%, the potential contribution of the tetraploid component to any examined tissue lower than 6% might have been undetected. We analysed 20 fetuses/pups/adults: three live 15-day fetuses, one dead early foetus (aged about 13 days), four full term fetuses (two dead in womb and two that did not survive the Caesarian section), one 1-month old animal and eleven adults. The following 13 organs/tissues were examined in adults and full term fetuses/neonatal pups: blood, brain, eyes, heart, intestine, liver, lungs, spleen, kidneys, adrenals, gonads, muscles and tongue. The number of examined organs/tissues varied between specimens (4-12), but in the majority of cases ranged between 9 and 11 (in adult triplets 10 tissues were examined; in adult twins, 4 tissues were examined in one twin and 9 tissues in another twin). In 19 specimens (foetuses/pups/adults), in all examined organs/tissues, only the diploid component was detected. One foetus, which probably died around 13th day but was recovered on the 20th day, was examined *in toto*: it contained both GPI isoforms, but the diploid GPI variant predominated.

The yolk sac was examined in 3 adults, 3 living 15-day foetuses and 4 dead foetuses. In two cases only the diploid GPI variant was detected and in the remaining eight cases both the diploid and tetraploid variants were present: in two specimens there ap-



Fig. 3. Female triplets of BAMIZ origin, which developed from sister 2/16 blastomeres, at the age of 5 weeks.

peared to be an equal participation of both variants and in six specimens the tetraploid variant predominated (these estimates were based on visual comparison of the intensity and size of 1A and 1B bands on cellulose acetate plates).

TABLE 1

POSTIMPLANTATION DEVELOPMENT OF CHIMAERIC BLASTOCYSTS

Type of aggregate	A	B	C	D	E
1/8 + 4 x 1/4	13/9	98/67	37	28 + 2 (44.1)	7 ^c /3 (10.3/4.4)
1/8 + 5 x 1/4	3/1	24/8	6	3 + 3 (75.0)	0
1/8 + 6 x 1/4	1/1	8/8	7	5 (62.5)	2/2 ^d (25.0/25.0)
2/16 + 4 x 1/4	7/7	56/56	28	17 + 3 (37.7)	8/5 ^e (14.3/8.9)
2/16 + 6 x 1/4	3/3	24/24	8	7 (29.2)	1/1 (4.2/4.2)
Total	27^b/21	210/163 (100.0)	86 (52.8) (100.0)	60 + 8 (36.8 + 4.9) (69.8 + 9.3)	18^c/11 (11.0 /6.7) (20.9/12.8)

- A Number of recipients (= number of transplanted sets)^a/ number of pregnant recipients
- B Transplanted blastocysts / number of blastocysts transplanted to pregnant recipients
- C Total number of implantations
- D Number of resorptions + dead foetuses (% in pregnant recipients)
- E Number of live foetuses and pups /number of adults (% in pregnant recipients)

^a Each recipient received one set of blastocysts; each set consisted of blastocysts developed from aggregates carrying sister blastomeres from a single diploid embryo.

^b Among 27 recipients 23 recipients received a set of 8 blastocysts, 3 recipients a set of 7 blastocysts and one recipient a set of 5 blastocysts.

^c Including three foetuses recovered in two females on the 15th day of pregnancy.

^d One pair of twins in which both co-twins survived to adulthood.

^e Including one set of identical triplets.

Distribution of diploid and tetraploid cells in chimaeric blastocysts

Our goal was to construct blastocysts in which the diploid cells would contribute solely to the ICM and the trophectoderm would contain only the tetraploid cells. We constructed aggregates that differed in the number of tetraploid blastomeres and their spatial arrangement around the single diploid blastomere (Figs. 1,2). Despite very careful transfer of the aggregates to the drops of culture medium, the original position of tetraploid blastomeres within the aggregate was not always preserved. Moreover, once the aggregates underwent compaction, the relative position of diploid and tetraploid blastomeres was no longer visible. In order to learn what was their distribution within morulae and blastocysts, we produced chimaeric aggregates in which diploid and tetraploid blastomeres were labelled with different fluorescent dextrans.

Confocal microscopy analysis showed that in four out of five morulae, the diploid 1/8 blastomere had divided once or twice (giving 2 and 4 cells, respectively) and only one morula contained single undivided diploid blastomere. In these five morulae the number of tetraploid blastomeres varied between 8 and 16. Only in one morula, 2 diploid blastomeres occupied an internal position and were almost entirely surrounded by 8 carrier tetraploid blastomeres (type of aggregate: 1 diploid 1/8 blastomere + 4 tetraploid 1/4 blastomeres) (Fig. 4A). In other four morulae (1/8 + 4 x 1/4 and 1/8 + 6 x 1/4)

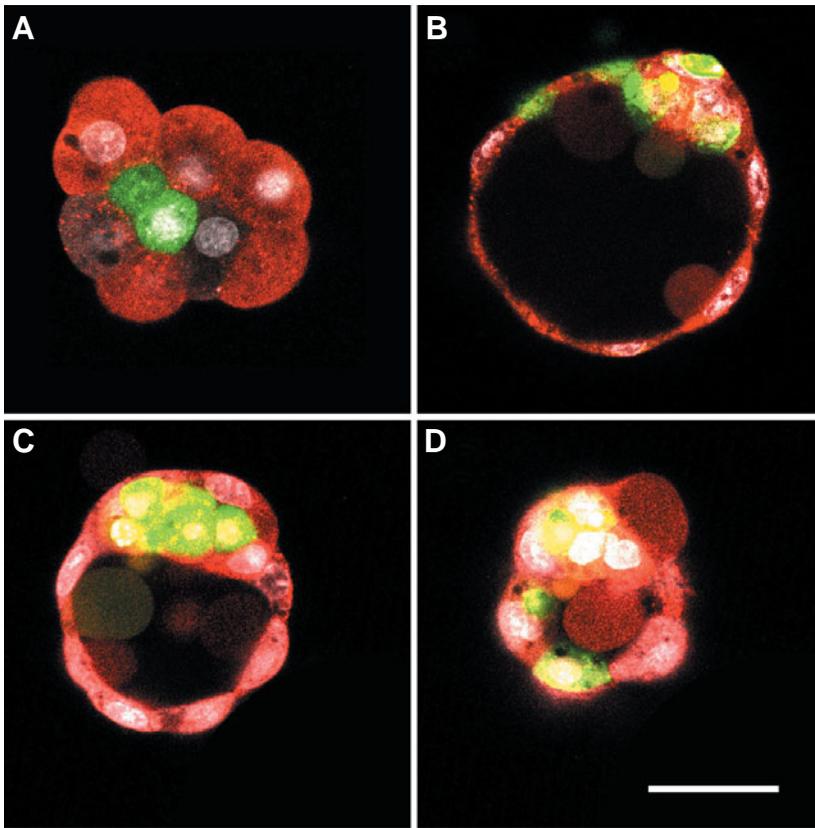


Fig. 4. Embryos which developed from aggregates of diploid blastomeres injected with with FITC-conjugated dextran (green), and tetraploid blastomeres injected with with rhodamine-conjugated dextran (red) and cultured *in vitro*. Fixed embryos were examined with confocal microscope. Bar 50 μ m. **(A)** A morula composed of two diploid blastomeres (green) surrounded by eight tetraploid blastomeres (red) developed from an aggregate composed of one 1/8 blastomere and four 1/4 blastomeres. The diploid blastomeres were almost entirely surrounded by tetraploid blastomeres. The embryo was cultured for 24 h. **(B)** Optical section of a blastocyst developed from one diploid 1/8 blastomere and six 1/4 tetraploid blastomeres, after 48 h in culture. Diploid cells are present in the ICM and polar and mural trophectoderm. **(C,D)** Two optical sections of a blastocyst developed from one diploid 1/8 blastomere and four tetraploid 1/4 blastomeres, after 48 h in culture. **(C)** A group of diploid cells situated in the ICM is covered by tetraploid polar trophectoderm. **(D)** In the tangential section, two diploid cells are situated in the mural trophectoderm. Vesicular structures in the blastocysts' cavities are artifacts.

diploid blastomeres were only partially surrounded by tetraploid blastomeres, or were located outside the tetraploid carrier blastomeres.

We also examined 9 blastocysts. Three blastocysts contained diploid cells in the ICM and in the overlying polar trophectoderm. In five blastocysts, the diploid cells formed a distinct group in the ICM and they were also present in polar and mural trophectoderm (Fig. 4B), or only in mural trophectoderm (Fig. 4 C,D). In one blastocyst the diploid cells were mostly located in the trophectoderm.

These observations show that in the majority of our chimaeric embryos, the diploid cells were present not only in the ICM, but also in the trophectoderm. We believe that the relatively low number of diploid cells in the ICM and a substantial contribution of tetraploid cells to the ICM, were probably responsible for the

high mortality of our experimental embryos.

Discussion

The organisation of the mammalian egg and the early embryo and the mechanisms responsible for the progressive determination and differentiation of embryonic cells, have remained a subject of continuous interest. The results of experimental studies, accumulated during several decades of the last century, point towards the regulative capability of the blastomeres of the early embryos. In mice, the individual blastomeres, or two or more embryos aggregated together during cleavage, are able to develop into normal adult. This led to the idea that in mice, in contrast to many other species where the development is programmed by the polar distribution of organelles and molecules in the egg and early embryo, the embryonic cells are developmentally 'naïve' in the early phases of embryogenesis and their fate is determined epigenetically according to their spatial position in the embryo (the 'inside-outside' hypothesis, Tarkowski and Wroblewska, 1967). However, in the last decade, Gardner (1997, 2001) and Zernicka-Goetz (reviewed in 2002, 2004), suggested that although in extreme experimental conditions the early mammalian embryos can display great regulative capabilities, the normal development relies mostly on an inherent pattern of organisation of the egg and early embryo. We believe, however, that the observed predisposition of certain parts of the egg, or certain blastomeres, to contribute to specific parts of the embryo, does not prove that these cells are already irrevocably committed to specific fates. As long as the fate of blastomeres can be steered experimentally in different directions (for instance into the ICM *versus* the trophectoderm), there is no ground to view them as being irreversibly determined.

The easiest and the most informative way of learning whether a blastomere has the unrestricted developmental abilities of the zygote, (i.e. it is totipotent), or whether its developmental potential is already constrained, is to track the development of an isolated, single blastomere. Mammalian blastomere can be called truly totipotent if it is able to develop into a foetus with extra-embryonic membranes that eventually develops into a fertile adult. However, the blastomere isolated from the embryo in late cleavage may not be able to complete embryogenesis - not because of its restricted capabilities to differentiate into various embryonic and extra-embryonic tissues - but because (as it happens in the mouse) it has no time to produce, before cavitation, enough number of cells to allocate some of them to the ICM. In order to properly implant in the mother's uterus and to start to grow, the mammalian embryo must allocate the large number of cells to the first extraembryonic structure - the trophectoderm. In the mouse, cavitation begins as the embryo approaches the 32-cell stage (Smith and McLaren, 1977) and its ICM consists of about 25% of the total number of cells (Rands, 1985). By the time

of cavitation, single 1/4 and 1/8 blastomeres will divide into 8 and 4 cells respectively and as a result will form the blastocyst with either very small ICM, or trophoblastic vesicles completely devoid of ICM (Tarkowski and Wroblewska, 1967). Consequently, these embryos are unable to develop normally after implantation (Rossant, 1976) and the only tactic available to assess the developmental potential of such blastomeres, is to support them with subsidiary cells. Kelly (1975, 1977) used genetically distinct diploid blastomeres as the subsidiary cells and was able to prove that 1/4 and 1/8 blastomeres can contribute to embryonic and also to extra-embryonic tissues of chimaeras. On some occasions, the single blastomeres were able to form the whole non-chimaeric animals, provided, however that subsidiary blastomeres contributed solely to the extra-embryonic tissues. Although these results are considered to be the evidence of the totipotency of the individual 1/4 and 1/8 blastomeres, none of these blastomeres were self-sufficient in creating the new individuals with supporting foetal membranes. The experiments of Kelly and others who followed her experimental design (Tsunoda *et al.*, 1987; Pinyopummin *et al.*, 1994; Tarkowski *et al.*, 2001; the present study), have demonstrated that the 1/4 and 1/8 blastomeres are able to form the body of the embryo/foetus/animal but only when supported with carrier cells (tetraploid blastomeres or diploid blastomeres derived from genetically distinct, normal or parthenogenetic embryos). Although the classic definition of 'totipotency' does not fully apply in this situation, the extraordinary ability of these isolated blastomeres to give rise to a complete fertile animal, justifies the usage of the word 'totipotency', though in the restricted meaning of this term.

In the present study we used tetraploid blastomeres as subsidiary cells to support the diploid blastomere. This experimental system takes advantage of the negative selection of tetraploid cells against diploid cells in the embryonic (but not extra-embryonic) tissues of 2n/4n mosaics (Tarkowski *et al.*, 1977) and of 2n↔4n chimaeras (Lu and Markert, 1980; Nagy *et al.*, 1990; Goto and Takagi, 1998; Tarkowski *et al.*, 2001).

The present study confirms the conclusion of Kelly (1975, 1977) and Tsunoda *et al.* (1987) that at least some blastomeres of the 8-cell mouse embryo and sister pairs of 16-cell embryo, are able to develop into a normal individual. We were able to produce one set of triplets, five pairs of twins and a number of singletons. All eleven adults were fertile. We did not detect the contribution of the tetraploid component to the body of adults, pups and full term foetuses. The type of pigmentation (in coat and/or eyes) was always characteristic of the diploid component. Also, the GPI analysis of four to ten organs/tissues confirmed presence of the diploid GPI isoform only. However, because of the limited sensitivity of the assay, we cannot exclude the possibility of low, i.e. below 6%, presence of the tetraploid component in experimental animals. It was shown previously that conceptuses and animals produced from 2n↔4n chimaeric embryos might occasionally contain traces of tetraploid cells in some tissues (Nagy *et al.*, 1990; Wang *et al.*, 1997b; Tarkowski *et al.*, 2001; Goto *et al.*, 2002). In our experimental chimaeras the tetraploid cells were eliminated from the body of foetuses/pups/adults, even though the tetraploid component initially predominated. The initial ratio between 4n and 2n cells was greatly in favour of the 4n component: 4-6:1 in regard to the number of cells and 8-12:1 in regard to the cellular volume. We used four to six tetraploid blastomeres

in order to enclose the diploid blastomere inside the aggregate and thus to form blastocyst in which diploid cells would be present exclusively in ICM. However, the experiments with blastomeres marked with different fluorochromes showed that the diploid blastomere were often located not only in ICM but also in polar and mural trophectoderm. Thus, the contribution of the diploid component to the ICM was often lower than expected and in consequence might have hampered normal morphogenesis. There was also no evident advantage in making 'three-dimensional' *versus* 'two-dimensional' aggregation chimaeras (Fig. 1). The predominance of tetraploid cells in the ICM, resulting from the excess of 4n blastomeres in the aggregates, might have been another factor contributing to the death of the diploid embryo. It was shown previously that tetraploid embryos of the same genotype as those used in our experiments, initially form apparently normal egg cylinders, but eventually, at the beginning of gastrulation (8th day of development) their morphogenesis deviates from the normal course and the embryos die (Tarkowski, *et al.*, 1977).

In contrast to the embryo proper, the tetraploid cells from the ICM successfully contribute to and survive until birth in the yolk sac. Moreover, in 6 out of 10 examined cases, the tetraploid cells predominated in the yolk sac. Because we observed the same phenomenon in diploid↔triploid mouse chimaeras (Suwinska *et al.*, 2005), this may reflect the general tendency of polyploid cells to colonise preferably the yolk sac and/or survive better in this extra-embryonic structure.

Evaluation of the developmental potential of a single blastomere from a given developmental stage is informative, but much more valuable is the comparison of the developmental potential of all sister blastomeres, i.e. 2 of 2, 4 of 4 etc. In the mouse, Mullen *et al.* (1970), Tsunoda and McLaren (1983), Togashi *et al.* (1987), Wang *et al.* (1997a) and Sotomaru *et al.* (1998) produced twins from isolated sister 1/2 blastomeres, but the efficiency was quite low. So far there are no reports of birth and successful postnatal development of mouse quadruplets. However, the production of quadruplets in the sheep (Willadsen, 1981) and triplets and quadruplets in cattle (respectively Willadsen and Polge, 1981 and Johnson *et al.*, 1995) by separation of blastomeres of the 4- or 8-cell embryos shows that, at least in some mammalian species, all blastomeres at this developmental stage are truly totipotent and do not require support of carrier blastomeres.

The largest set of identical mouse multiples ever produced from single 1/8 blastomeres was four, although one of these animals was overtly chimaeric (Tsunoda *et al.*, 1987). Non-chimaeric mouse triplets described in the present study were generated from pairs of 1/16 blastomeres. Several pairs of twins were produced in mice by Kelly (*loc. cit.*), Tsunoda *et al.* (*loc. cit.*) and by us. There are no similar studies on the 8-cell stage embryos in other mammalian species. So far there is no direct evidence that each blastomere of the 8-cell mouse embryo, or each pair of 1/16 blastomeres, has the full potential to develop into an animal. This type of experiments always raises the question whether the lack of success in the production of 4 animals from all four 1/4 blastomeres and 8 animals from all eight 1/8 blastomeres is due to the unequal developmental potential of sister blastomeres or simply to the shortfall of the techniques. This question has been recently addressed by Piotrowska-Nitsche *et al.* (2005) and Piotrowska-Nitsche and Zernicka-Goetz, (2005). They believe that individual blas-

tomeres of some 4-cell mouse embryos may differ in their fate and that this non-equivalence arises from a particular pattern of cell division during the 2 → 4-cell stage. Aggregates of blastomeres taken from 4-cell embryos develop to term or display developmental abnormalities if they are formed from specific vegetal blastomeres. However, so far there is no evidence that the individual blastomeres in the 4-cell embryo differ at cytological and/or molecular level. The first morphological differences between blastomeres are observed after the 4th cleavage division with the appearance of polar and apolar cells (for review see Johnson and McConnell, 2004). Even then, in the 16-cell embryo the polar and apolar cells though morphologically distinct, are developmentally labile, because aggregates of each cell type can form blastocysts and normal conceptuses (Ziomek *et al.*, 1982). This suggests that at the preceding developmental stage, i.e. in the 8-cell embryo, the majority if not all of the isolated blastomeres (provided each is supported with carrier cells) should be able to develop into normal mouse. We believe that failure to produce octuplets from 8-cell mouse embryo is due to imperfect techniques rather than to a restricted developmental potential of some sister 1/8 blastomeres. However, the confirmation of this belief should await further studies.

Materials and Methods

Animals were kept under 14 h light/10 h dark regime. Embryos were obtained from 1.5 - 6.5 month old females, albino or pigmented and homozygous for *1a* or *1b* allele of glucose phosphate isomerase (GPI).

Diploid (2n) embryos

In most experiments cycling BAMIZ females were used as donors of 2n embryos. These outbred albino mice were obtained from a cross between BALB/c and MIZ mice selected for *Gpi-1a/Gpi-1a* homozygosity. Females were crossed with BAMIZ males and inspected for plugs in the morning (the day of plug = the first day of pregnancy). The plugged females were sacrificed on the 3rd day between 9.30 and 12.30 and the 8- to 16-cell embryos were recovered from oviducts in M2 medium (Fulton and Whittingham, 1978). The zona pellucida was removed by incubation in 0.5% pronase (Sigma) in Ringer solution. The blastomeres were disaggregated by incubation (minimum 15 minutes) in Ca²⁺ - and Mg²⁺ - free M2 medium at 37°C followed by pipetting in the same medium. Each embryo was manipulated individually and the sister blastomeres (octet of 1/8 or 2/16 blastomeres) were stored in a separate M2 drop under liquid paraffin at 37°C in an atmosphere of 5% CO₂ in air, until used for aggregation with tetraploid blastomeres.

In three experiments, cycling F1(C57BL/10 x CBA/H) females mated with F1 males were used as donors of diploid 8- and 16-cell embryos (GPI and coat characteristics given below).

Tetraploid (4n) embryos

In most experiments embryos used for tetraploidization originated from F1(C57BL/10 x CBA/H or a reverse cross) and from F1(C57/BL/6 x CBA/H) females. Females were mated with F1 males of the same genotype. These animals are agouti and have the 1B isoform of GPI. The females were induced to ovulate with pregnant mare's serum gonadotrophin (PMSG; Folligon, Intervet) and human chorionic gonadotrophin (hCG; Chorulon, Intervet) given 47-49.5 h apart in doses of 7.5 - 10 IU each. The females were sacrificed 42-45 h after hCG injection. Recovered 2-cell embryos were electrofused according to the modified technique of Kubiak and Tarkowski (1985) in a fusion chamber filled with 0.25 M glucose supplemented with 100 μM CaCl₂·2H₂O and 100 μM MgSO₄·7H₂O, with the distance between electrodes varying between 220 and 290 μm. Embryos aged 43.5 - 49 h after hCG injection were oriented with the interblastomeric

surface perpendicular to the lines of the electric field and were subjected to two pulses of 40 Volts d.c. and 25 μs duration, given twice (the orientation of embryos was corrected after the first stimulus, if required). Fusion usually took place within the first hour after treatment. Embryos that had not fused were subjected to a second round of pulses. Fused embryos were cultured in KSOM medium (Erbach *et al.*, 1994) (Specialty Media) under standard conditions. Those embryos that had cleaved within one hour after fusion were eliminated. About 24 h later the 4-cell embryos (considered to be tetraploid) were released from zona pellucida and disaggregated as described above. Isolated blastomeres were pooled in drops of M2 medium until aggregation with diploid blastomeres.

In three experiments 2-cell embryos used for tetraploidization originated from hormonally-stimulated BAMIZ females mated to BAMIZ males (GPI and coat characteristics as described for diploid embryos). These were recovered 47 - 51.5 h after hCG injection and at 48-53 h they were subjected to electric pulses as described earlier. Fused embryos were cultured and disaggregated as F1 embryos.

Aggregation of diploid and tetraploid embryos

Aggregation was performed between 1.00 and 6.00 p.m. Each set of diploid blastomeres obtained from 8-cell or 16-cell embryos was processed separately. Diploid 8-cell blastomeres (1/8 blastomeres) and pairs of sister diploid 16-cell blastomeres (2/16 blastomeres) were placed with tetraploid 4-cell blastomeres (1/4 blastomeres) in a solution of phytohemagglutinin (300 μg/ml) (Sigma) in BSA-free M2 medium in agar-coated embryological watch glass for 1-3 minutes. Within each set, all sister diploid blastomeres (1/8 or 2/16) were surrounded with four, five or six tetraploid blastomeres (Figs. 1,2). After the blastomeres had stuck firmly together, the chimaeric aggregates were placed in drops of KSOM medium. After 48 h of culture the chimaeric aggregates had developed into large blastocysts which were transferred to recipient females.

Transplantation of chimaeric blastocysts

Each set of chimaeric blastocysts (usually 8, occasionally 7 or 5) was transplanted separately on the first day of pseudopregnancy to the oviduct of a recipient female. The recipients (age: 2.5 - 5 months) were MIZ, BAMIZ and F1 females mated with F1 vasectomised males. Females were anaesthetized with 0.25-0.36 ml of 6 mg/ml of water solution of Nembutal (Serva) injected intraperitoneally.

Monitoring of pregnancy and delivery of young

Starting on the 9th day after mating, vaginal smears were taken daily from each recipient female. Females showing signs of proestrus or oestrus were sacrificed and the uterus was inspected for resorptions. Most of the pregnant females were sacrificed in the afternoon on the 20th day or in the morning on the 21st day and the foetuses recovered by Caesarian section were fostered by females which had littered the same night or 24 h earlier. The experimental animals reared successfully were tested for fertility by crossing with normal males or females and after giving birth to one or more litters they were sacrificed for GPI analysis.

GPI assay

Samples of tissues/organs were frozen in small amount of redistilled water and stored at -20°C. After thawing the samples were homogenized, centrifuged at 14000 rpm and the supernatant was collected. Electrophoresis was carried out on TITAN III-H plates (Helena BioSciences) according to the method of Buehr and McLaren (1985). From 4 to 10 tissues/organs were examined in each experimental foetus/animal.

Labelling with fluorescent dextrans

One-cell diploid and tetraploid embryos were microinjected with 2pl of 2mg/ml solution of dextran conjugated with fluorescein or rhodamine (70000 MW; Molecular Probes) in 2 mM PIPES buffer (pH 7.4; Sigma) and 0.140 mM KCl (Sigma).

Diploid zygotes (BAMIZ x BAMIZ) were collected on the first day of

pregnancy. After removal of cumulus cells with hyaluronidase (Sigma) (500µg/ml in Ca²⁺- and Mg²⁺-free phosphate buffered saline (PBS, Biomed) the zygotes were injected with FITC-conjugated dextran and transplanted to the oviduct of MIZ females mated to F1 vasectomized males (1st day of pseudopregnancy). 48 h after transplantation they were recovered as 8-cell embryos and disaggregated as described previously.

Embryos used for tetraploidization originated from hormonally-stimulated F1 females mated with F1 males. They were recovered between 42 and 45 h post hCG injection as two-cell embryos and subjected to electrofusion 2-4 h later. Fused (now 1-cell) embryos were injected with rhodamine-conjugated dextran and cultured *in vitro* in KSOM medium for about 24 h. Embryos that divided into four cells were disaggregated and the single blastomeres were used for aggregation with diploid blastomeres.

Diploid and tetraploid blastomeres were aggregated as described earlier and cultured in KSOM medium for 1-2 days. They were fixed as morulae or blastocysts for 20 min in 2% paraformaldehyde in Ca²⁺- and Mg²⁺-free PBS, stained for 10 min at 37°C in fluorescent chromatin-specific dye DRAQ-5 (10 µM in PBS; Biostatus Ltd) and transferred to droplets of PBS in a viewing chamber. The embryos were examined with confocal laser microscope LSM 510 Zeiss.

Acknowledgements

This work was partly financed by a grant from the State Committee for Scientific Research (Grant 3 P04C 074 22). We wish to thank Professor Marek Maleszewski and Dr. Ewa Borsuk for injecting eggs with fluorescent dextrans, Mr. Darek Maluchnik for help in examining chimaeric embryos in confocal microscope and in preparing figures, Dr. Anne McLaren for helpful discussions and Professor Michael Bedford for making comments on the manuscript. We are most thankful to Professor Malgorzata Kloc for final shaping of the manuscript.

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Received: April 2005

Reviewed by Referees: August 2005

Modified by Authors and Accepted for Publication: August 2005