The Development of Diploid/Tetraploid Mosaic Mouse Embryos Produced by Electrofusion at the 4-Cell Stage

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Abstract: *The developmental ability of diploid/tetraploid mosaic mouse embryos produced by electrofusion at the 4-cell stage was examined in vitro and in vivo. Diploid/tetraploid mosaic embryos amounted to 51.3% of embryos treated. They were cultured for 60 h in BMOC-III medium to examine their development, and 77.8% of them developed to blastocysts. This was about the same rate as in unfused diploid embryos. Compaction of the diploid/tetraploid mosaic embryos started at the 6-cell stage, and the mean number of cells in these blastocysts was about three quarters that in control embryos. These embryos which developed to blastocysts were transferred to the uterus of recipients. By 11.5 days of pregnancy, 90.0% of diploid/tetraploid mosaic embryos had been implanted and 25.9% of them had developed into foetuses, but in these foetuses, however, there were no tetraploid cells, and they contributed to only some foetal membranes in 3 out of 9 embryos examined. On the 19th day of pregnancy, two recipients were delivered of seven pups altogether. These pups were morphologically normal and grew to be adult mice. Chromosome preparation of the bone marrow cells showed that all of these mice had only diploid chromosome constitutions. These results suggest that tetraploid cells show poor developmental ability after implantation.*

Key words: *Tetraploid, Electrofusion, Cell fusion, Mosaic embryos, Embryonic development.*

The technique of cell fusion and check of cleavage of early mammalian embryos is used for producing polyploid embryos. Colchicine, Sendai virus [1], cytochalasin B [2–5] and polyethylene glycol [6] are used for producing mammalian tetraploid embryos, and electrofusion [7–15] in particular is often used nowadays because it takes only a short time and is easy to do. Tetraploid embryos develop into blastocysts, but no investigator has reported that they were carried to term of pregnancy except Snow [2] who induced them with cytochalasin B. Kaufman and Webb [12] reported that the tetraploid mouse embryos produced by electrofusion lived on for 14 days of pregnancy, but they had characteristic craniofacial abnormalities. The strategy of aggregating with normal cells has been used in rescuing semilethal embryonic cells. Lu and Markert [16] succeeded in obtaining few diploid/tetraploid chimeric mice created by aggregating diploid and tetraploid blastomeres with PHA.

In the present study, the developmental ability of diploid/tetraploid mosaic mouse embryos produced by electrofusion at the 4-cell stage was examined *in vitro* and *in vivo* because we expected they had the advantage of diploid/tetraploid chimeric embryos in the damage to aggregation.

Received: July 1, 1996
Accepted: October 11, 1996
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Materials and Methods

Recovery of embryos

Female ICR mice, aged 6–12 weeks, were induced to ovulate (5–7.5 IU each of PMSG and hCG given 46–48 h apart) and mated with males of the same strain. Four-cell embryos were collected 55–58 h post hCG. The embryos were placed in a drop of modified Brinster's medium (BMOC-III) containing 0.5% bovine serum albumin (BSA) under equilibrated paraffin oil in an atmosphere of 5% CO_2 in air at 37°C until the fusion treatment.

Electrofusion

The electrodes for electrofusion which were made of

gold were glued onto a glass slide 1.0 mm apart and attached to a Petri dish. The dish was half filled with 0.3 M mannitol. About five embryos at a time were placed between the electrodes and oriented with only one cleavage plane perpendicular to the direction of the electric field. Square direct current (DC) pulses were generated by a pulse generater (SSH-10, Shimadzu Co.). DC field strength and pulse duration were fixed at 1.5 kV cm^{-1} and 150 μs, but no alternating current (AC) field was applied [8]. Two pulses were applied with an interval of 2 sec.

Embryo culture

The treated embryos were washed immediately three times in BMOC-III containing 0.5% BSA. The embryos were then incubated in drops of the same medium under equilibrated paraffin oil in an atmosphere of 5% CO_2 in air at 37°C. After 1 h of the treatment, the embryos were examined under an inverted microscope and were scored for fusion on the basis of the number and the size of blastomeres: 4-cell=diploids, 3-cell=diploid/tetraploid mosaics for instance. The embryos were further cultured for 60 h to examine their developmental capacity.

Embyo transfer

The embryos which developed into expanded blastocysts after 60 h of culture were transferred to the uterus of female ICR mice on the 2.5 days of pseudopregnancy (day of vaginal plug=0 day) under 0.6 mg of xylazine and 1.5 mg of ketamine anaesthesia. For the chromosome preparation of embryos, mosaic blastocysts were transferred to one horn of the uterus and control blastocysts were transferred to the other horn, and in bone mice, mosaic blastocysts were transferred to each horn of the uterus.

Chromosome preparation

Blastocysts: When the embryos developed into blastocysts after 48 h of culture, air-dried preparations were made to determine the number of cells and ploidy [17].

11.5-day embryos: Conceptuses were dissected from the uteri at 11.5 days of pregnancy and chromosomal preparations were made from the embryos and the foetal membranes by using the technique of Evans *et al.* [18].

Born mice: Bone marrow cells were used in karyotype analysis [19]. They were obtained from the femur of the born mice after injection of 1 ml of 0.001% demecolcine.

Results

Reaction to electrofusion and preimplantation development of mosaic embryos

The treated embryos started fusion after 5–10 min of the treatment and completed it after 1 h (Fig. 1). The data on the reaction to electrofusion and the rates of development of the embryos are given in Table 1. Diploid/tetraploid mosaic embryos amounted to 51.3% of treated embryos and 77.8% of them developed to blastocysts. The development rate was about the same as that of the embryos in which the blastomeres failed to fuse (henceforth called 'unfused diploids') and tetraploids, and significantly different from that of diploid/hexaploid mosaics and octaploids. The compaction of diploid/tetraploid mosaics started at the 6-cell stage, and blastocyst formation occurred at the same time as observed in control embryos (Fig. 2).

Ploidy and number of cells in treated embryos at blastocyst stage

The data on the ploidy and the number of cells in treated embryos at the blastocyst stage are given in Tables 2 and 3. Twenty-four out of 76 mosaic blastocysts were confirmed to contain both the diploid and the tetraploid metaphase plates, and the ratio of diploids to

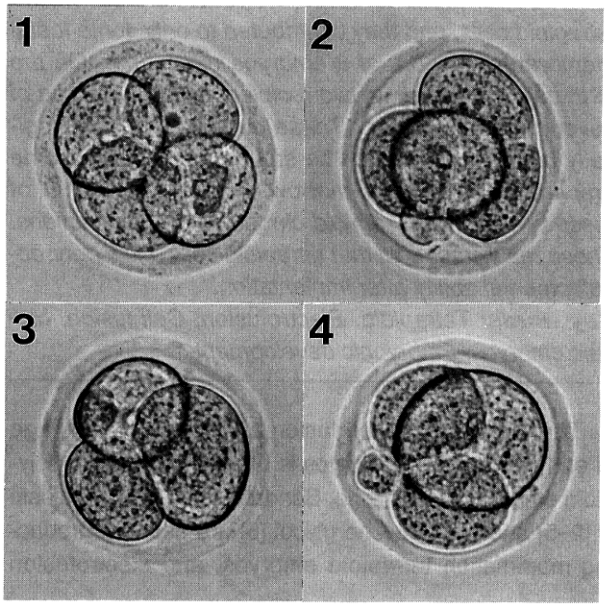

Fig. 1. Time sequence of events in a four-cell embryo after triggering fusion pulse 1; Before the treatment. 2; 10 min. 3; 20 min. 4; 60 min.

tetraploids was 1:0.73. Chromosome preparations underestimated the number of embryos in which mosaicism was confirmed because the blastocysts always contained few cells to have both a diploid and a tetraploid metaphase plate. The mean number of cells in mosaic blastocysts was 30.6 ± 6.3, and was about three quarters that of control blastocysts.

Ploidy of the foetuses and the foetal membranes

Thirty mosaic blastocysts and 17 control blastocysts were transferred to the uterus of four recipients, and 27 mosaics and 15 controls were implanted, but in only one recipient, had seven mosaics and four controls developed to foetuses. These foetuses were morphologically normal and developing at the normal rate. The data on the ploidy of these foetuses and foetal membranes are given in Table 4. There were no tetraploid cells in the foetuses, and they contributed to only some foetal membranes in 3 out of 9 embryos examined. The incidence of tetraploid mitosis in the foetal membranes was 50.0% in one case, but in other cases was very low (0.0–5.2%). Metaphase mitosis from the foetal membrane is shown in Fig. 3.

Table 1. Effect of electrofusion on four-cell embryos

No. of cells and presumed ploidy	No. of embryos counted 1 h after the treatment (%)	No. of embryos developed to blastocysts	Percentage of embryos developed to blastocysts (%)
3 (2n/4n)	509 (51.3)	396	77.8[a]
4 (2n)	134 (13.5)	99	73.9[a]
2 (2n/6n)	184 (18.5)	111	60.3[b]
2 (4n)	56 (5.6)	42	75.0[a]
1 (8n)	44 (4.4)	20	45.5[b]
disintegrated	66 (6.7)	0	0.0
Total	993 (100.0)	668	67.2

Values with different superscripts are significantly different ($P<0.05$ or 0.01, χ^2-test).

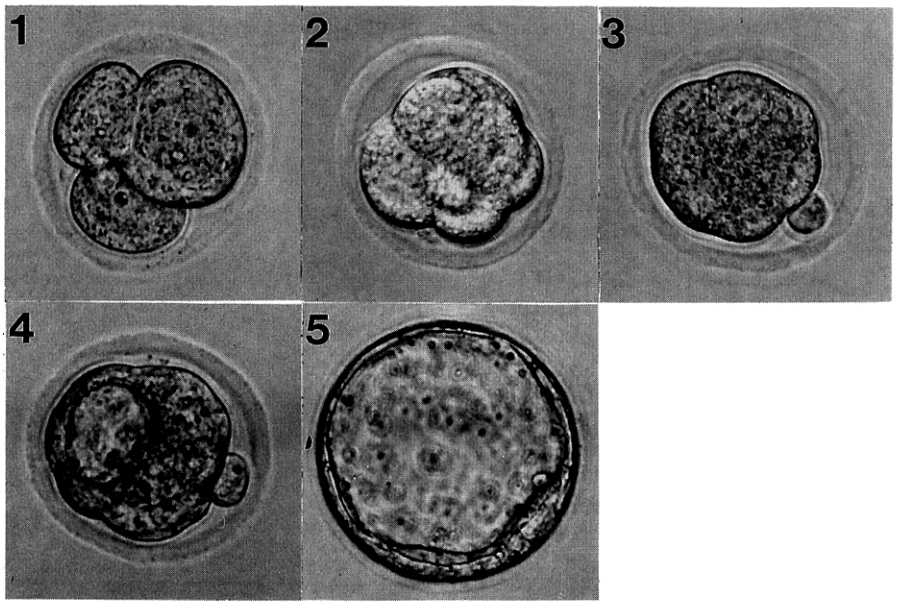

Fig. 2. Development of a diploid/tetraploid mosaic embryo *in vitro*. 1; 1 h. 2; 12 h. 3; 24 h. 4; 36 h. 5; 48 h.

Table 2. Analysis of ploidy at 48 h after the treatment

No. of cells and presumed ploidy determined 1 h after the treatment	Total No. of blastocysts examined	Estimated ploidy
Control (2n)	13	10 (2n) 3 (no mitotic plates)
3 (2n/4n)	76	24 (2n/4n) *2n:4n=1:0.73 15 (2n) 4 (4n) 33 (no mitotic plates)
4 (2n)	20	12 (2n) 8 (no mitotic plates)
2 (2n/6n)	16	2 (2n/6n) *2n:6n=1:1.67 2 (2n) 2 (6n) 10 (no mitotic plates)
1 (8n)	3	1 (8n) 2 (no mitotic plates)

Table 3. Numbers of cells in blastocysts 48 h after the treatment

No. of cells and presumed ploidy determined 1 h after the treatment	Total No. of blastocysts examined	No. of cells (Mean ± S.D.)	Ratio (control=1)
Control (2n)	13	44.2 ± 10.0[a]	1.00
3 (2n/4n)	76	30.6 ± 6.3[b]	0.69
4 (2n)	20	40.3 ± 4.9[a]	0.91
2 (2n/6n)	16	22.0 ± 5.1[c]	0.50
1 (8n)	3	9.0 ± 0.8[d]	0.20

Values with different superscripts are significantly different ($p<0.01$, Student's t-test).

Table 4. Karyology of diploid/tetraploid implanted embryos

Condition	Embryo No.	Tetraploid plates (%)	
		Foetus	Foetal membranes
Fused	1	0/22 (0.0)	2/ 45 (4.4)
	2	0/46 (0.0)	0/ 11 (0.0)
	3	0/ 3 (0.0)	0/ 31 (0.0)
	4	0/ 8 (0.0)	0/ 14 (0.0)
	5	0/31 (0.0)	54/108 (50.0)
	6	0/67 (0.0)	3/ 58 (5.2)
	7	0/34 (0.0)	Not examined
Control	8	0/32 (0.0)	0/ 52 (0.0)
	9	0/43 (0.0)	Not examined
	10	0/42 (0.0)	0/ 39 (0.0)
	11	0/42 (0.0)	0/ 58 (0.0)

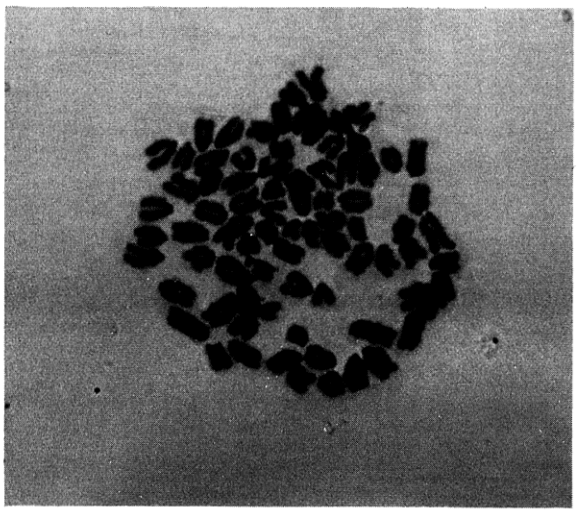

Fig. 3. Metaphase plate of a mosaic foetal membrane (× 600).

Ploidy of the born mice

Fifty-three mosaic blastocysts were transferred to the uterus of seven recipients, and two recipients delivered seven pups (two males and five females) altogether on the 19th day of pregnancy. These pups were morphologically normal and grew to be adult mice. A chromosome preparation of the bone marrow cells at 4–6 months post parturition showed that all of these mice had only diploid chromosome constitutions.

Discussion

Our experiments shows that large proportions of diploid/tetraploid mosaic embryos produced by electrofusion develop to blastocysts and the ratio is about the same as that of unfused diploids. Our experimental system produces 39.9% (No. of blastocysts/No. of treated 4-cell embryos: 396/993) diploid/tetraploid mosaic blastocysts. This rate is higher than that for the embryos produced by cytochalasin B which was reported in Tarkowski's study [20].

Diploid/tetraploid mosaic embryos were observed in only 24 (31.6%) out of 76 embryos examined. The reason for this result is that few cells show 2n/4n mitotic plates in the blastocysts at the same time (Table 2).

Compaction of diploid/tetraploid mosaics, unfused diploids, diploid/hexaploid mosaics and octaploids started at the 6-cell, 8-cell, 4-cell and 2-cell stages respectively, and they occurred at the same time. The ratio of the mean numbers of cells in them was about 3:4:2:1, and this was the same ratio as immediately after the fusion. It has been reported that compaction and blastocyst formation by fused embryos usually occurs at around the same time as observed in the control embryos and that tetraploid blastocysts have only half the number of cells that the control embryos have [9, 11, 13]. Our observations confirm the previous finding that 'compaction and blastocyst formation might be related to the number of mitosis that have taken place.'

According to Eglitis and Wiley [21], the rate of development of tetraploids may be improved by restoring the number of cells to that commensurate with embryonic age by aggregating them with PHA. Nevertheless, as those of diploid/tetraploid mosaics and tetraploids were about the same as that of unfused diploids in our experiments, we conclude that the small number of cells has no influence on the development of the embryos in the preimplantation period.

The embryos which were transferred to the uterus of four recipients had developed to foetuses in only one recipient. In the others, all of the diploid/tetraploid mosaics had died. As all of the control embryos also had died, this was probably caused not by the presence of tetraploid cells but by the health of the recipients or technical failure in embryo transfer. In the foetuses which survived to 11.5 days of pregnancy, there were no tetraploid cells, contrary to Tarkowski's report [20] in which there were either no tetrabloid cells or only a very small proportion. The discrepancy between our results and Tarkowski's report [20] might be due to the difference between mosaic embryos produced by electrofusion in our experiment and ones produced by cytochalasin B in Tarkowski's [20]. Furthermore, the number of tetraploid cells in foetal membranes was of various percentages (0–50%). This result was very similar to that in Tarkowski's report [20]. The elimination of tetraploid cells from mosaic embryos suggests that they have a lower proliferation rate than diploid cells after the implantation stage.

Seven transplanted embryos were carried to term and they grew to be adult mice, but we could not confirm their tetraploidy. As they were morphologically normal, it seems that the stimulus by means of the electric pulse and the presence of tetraploid cells in the preimplantation period does not impede subsequent embryonic development. But, at the same time, the tetraploid cells die out or are gradually excluded. It is necessary to clarify the distribution and fate of the tetraploid cells in the postimplantation period in further studies.

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