Original Article

Preimplantation development of manipulated mouse zygotes fused with the second polar bodies: a cytogenetic study

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ABSTRACT   Immediately after fertilization one chromatid of each maternally-derived chromosome is extruded into the second polar body (2PB). We tested the ability of these "extra" chromosomes to support preimplantation development. Micromanipulation and electrofusion techniques were used to fuse 2PBs with diploid, haploid, or enucleated mouse zygotes. Androgenetic haploids, intact embryos, and digynic triploids served as the controls for the reconstructed embryos. Androgenetic haploid zygotes developed to the blastocyst stage only when fused with the 2PBs. This result demonstrates that even when extruded into the 2PB, chromosomes retain their ability to support normal preimplantation development. However, 2PB fusion with diploid zygote impaired preimplantation development. Normal development of experimentally produced digynic triploids (zygotes with one extra maternal pronucleus) indicated that developmental arrests, caused by the 2PB fusion, were not the result of triploidy or micromanipulation procedures. Cytogenetic studies showed that developmental failures of the reconstructed embryos were caused by premature chromosome condensation of the polar body chromosomes. This result indicates that 2PB must be removed from the zygotes' perivitelline space during animal cloning experiments. In addition, we showed that 2PB fusion with enucleated zygote is a reliable method for 2PB karyotyping and may be used in the studies of mammalian meiosis.

KEY WORDS: *mouse embryo, polar body, premature chromosome condensation, triploid, haploid*

Introduction

The purpose of gametogenesis, in essence, is to produce a haploid gamete out of an initially diploid cell. In this respect, oogenesis differs markedly from spermatogenesis with regard to the fate of the extra genetic material present in oogonia. Two meiotic divisions serve to extrude extra chromosomes from the oocyte with a minimal loss of cytoplasm; as a result two polar bodies are formed. Polar bodies never participate in development, as they disintegrate soon after formation. Nevertheless, there are good reasons to believe that the chromosomes of the 2PB have the same developmental potential as their sister chromatids, which are left in the oocyte and form maternal pronucleus. Normal preimplantation development of digynic diploids and triploids, obtained by suppression of the 2PB extrusion (Niemierko, 1975; Niemierko and Komar, 1976, 1985; Borsuk, 1982; Surani and Barton, 1983; Speirs and Kaufman, 1989) and activated MII oocytes (Dyban and Baranov, 1978; Cuthbertson, 1983; Kaufman, 1983) support this conclusion. To date, however, there was only one report indicating that chromosomes retain their ability to support normal development even after extrusion into the 2PB: when osmotic shock was used to incorporate 2PB back into the mouse zygote, 2PB nucleus participated in preimplantation development (Opas, 1977).

The problem of polar body developmental fate attracted our attention since it had been shown that in humans both first and second polar bodies may be used for preconception and preimplantation genetic diagnosis for preembryos at risk of inheriting genetic diseases (Verlinsky *et al.*, 1990, 1992). In contrast to human polar body, mouse first polar body starts to disintegrate virtually at the very moment of extrusion and hence can not be used for preconception analysis (Rodman, 1971; Evsikov and Evsikov, 1995). This study was undertaken in order to investigate the transformations undergone by the 2PB nucleus in the zygote's cytoplasm. We also tested the ability of 2PB chromosomes to support preimplantation development.

Results

By 85 h after ovulation practically all control zygotes (Fig. 1) developed *in vitro* into morphologically normal morulae and blastocysts (Table 1). When left in culture for an extra 24 h, most of the embryos (>70%) reached expanded blastocyst stage and hatched from zonae pellucidae. This result suggested that *in vitro* conditions, used in this study, sustained normal preimplantation

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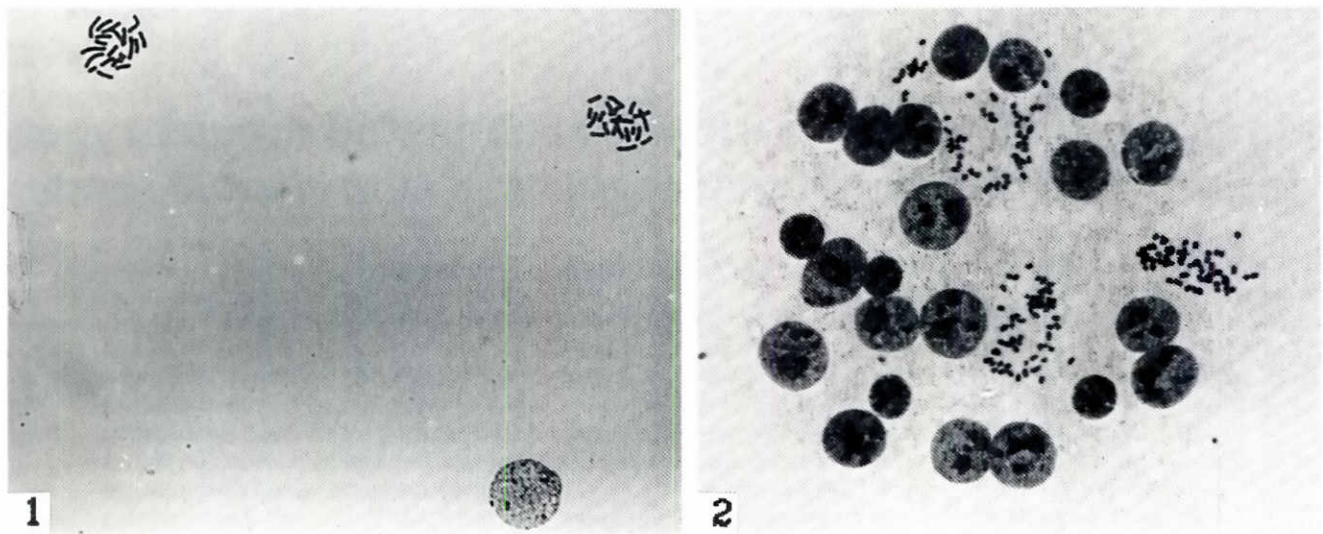
Fig. 1. Colcemide-treated diploid zygote (PPN+MPN) in the first cleavage metaphase. *Haploid metaphase plates of the pronuclei, 2PB interphase nucleus (x500).*

Fig. 2. An air-dried preparation of a digynic triploid blastocyst (PPN+MPN+MPN) produced by the fusion of a pronucleoplast with zygote (x250).

development of the mouse embryos.

When 21-24 h post-hCG old zygotes were fused with the 2PBs, their development was noticeably impaired (Table 1). By 85 h after ovulation only 51% of such triploids (PPN+MPN+2PB) developed to the morula and blastocyst stages, the rate of morphogenesis was 2-3 times lower than that of the control diploid zygotes (28% of blastocysts among morphologically normal embryos as opposed to 69% in the control). This might have resulted from triploidy of the reconstructed embryos or imbalance in the nucleo-cytoplasmic interactions caused by the 2PB nucleus incorporation and/or adverse side effects of the micromanipulation procedures. To distinguish between these possibilities, we produced digynic triploids (PPN+MPN+MPN) by fusing intact zygotes with a pronucleoplast (MPN) from another zygote (Fig. 2). 100% of such triploids devel-

oped to the morula and blastocyst stages, although their development was retarded (Table 1). This is in agreement with previous reports on the parameters of preimplantation development of triploids (Beatty and Fischberg, 1951; Niemierko, 1975; Baranov, 1976; Dyban and Baranov, 1978; Witkowska, 1981; Henery and Kaufman, 1992a, 1993). It became clear that low viability of the zygotes fused with the polar bodies could not be explained by triploidy or by micromanipulation interference alone. Triploidy caused by the 2PB fusion could account for the low rate of morphogenesis but not for the developmental arrest.

Cytogenetic studies of such triploids revealed the cause of the developmental failures (Fig. 3). The moment of fertilization and 2PB extrusion marks the start of paternal and maternal pronuclei formation. 2PB nucleus never expands to the volume of a pronucleus

TABLE 1

PREIMPLANTATION DEVELOPMENT OF THE MOUSE EMBRYOS *IN VITRO*

| Embryo Types	Karyotype	12 h	24 h	42 h	85 h		
		1-cell	2-cell	4-cell	Morulae and blastocysts	Percent of blastocysts	Mean cell number in morulae and blastocysts
Diploid	PPN+MPN	1031	1003 (97%)	ND	984 (95%)	69	33.5±0.5
Reconstructed diploid	PPN+2PB	81	78 (96%)	37 (46%)	26 (32%)	35	21±2
Digynic triploid	PPN+MPN+MPN	38	38 (100%)	38 (100%)	38 (100%)	32	22±1
Digynic triploid	PPN+MPN+2PB	111	97 (87%)	79 (71%)	57 (51%)	28	22±2
Androgenetic haploid	PPN	110	108 (98%)	44 (40%)	32 (29%)	0	12±2
Reconstructed haploid	2PB	28	27 (96%)	0 (0%)	0 (0%)		

(Timing of development is given in hours after ovulation). ND, not determined; PPN, paternal pronucleus; MPN, maternal pronucleus; 2PB, second polar body.
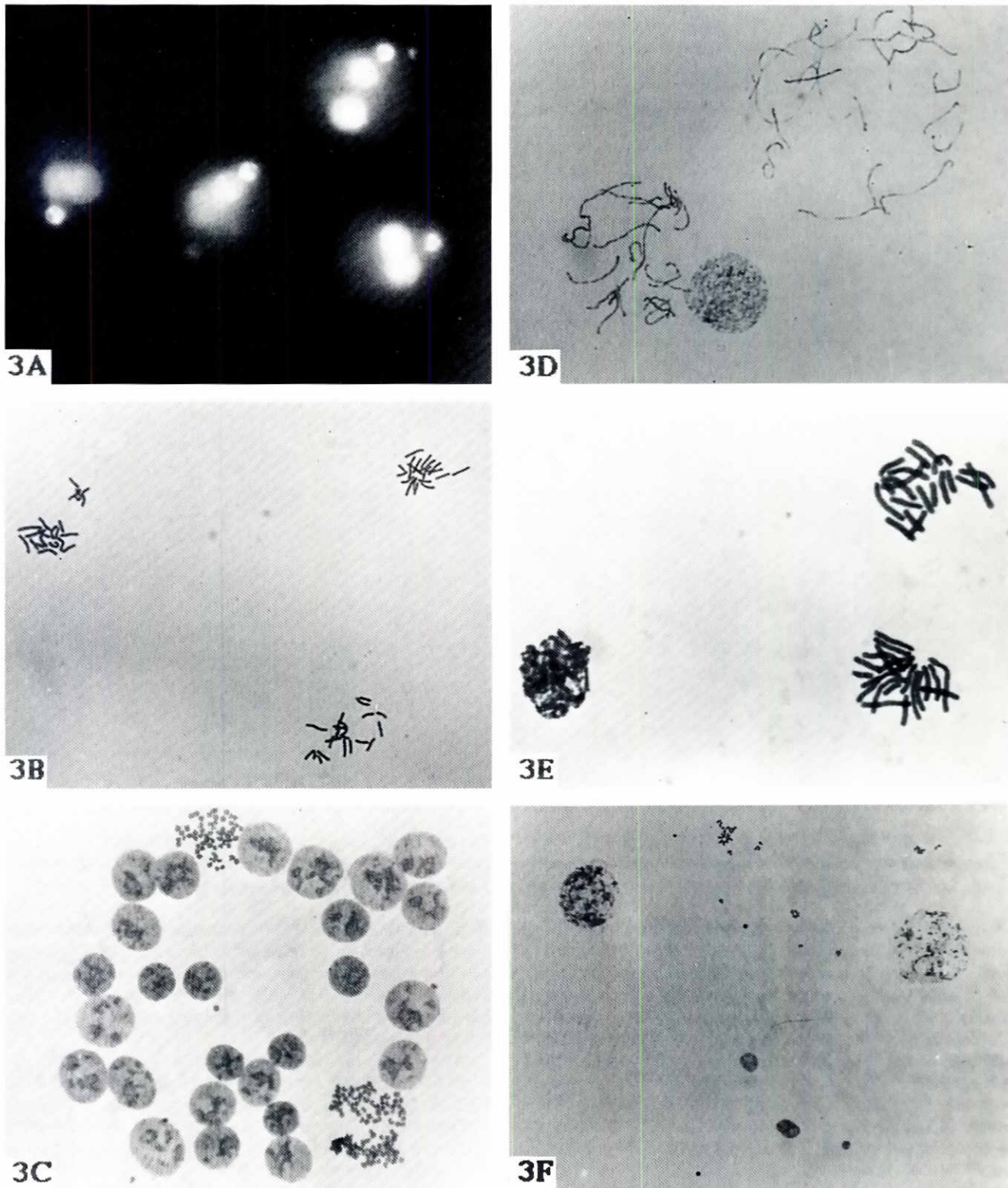
Fig. 3. Developmental fate of the 2PBs fused with zygotes. (A) *Pronuclei and 2PB nucleus in Hoechst 33258 stained zygotes fused with the 2PB;* **(B,C)** *normal preimplantation development of a gynogenetic triploid (PPN+MPN+2PB) produced by the fusion of the 2PB with intact zygote;* **(D-F)** *abnormal development caused by premature chromosome condensation of the 2PB nucleus.* **(B)** *Pronuclei and 2PB formed metaphases (x500).* **(C)** *Blastocyst (x250).* **(D)** *Pronuclei at the prophase stage, interphase 2PB nucleus (x500).* **(E)** *Pronuclei at the metaphase, prematurely condensed chromosomes of the 2PB form a clump of chromatin (x1250).* **(F)** *Embryo arrested at the cleavage stage. 4-cell morphologically abnormal embryo, micronuclei and aneuploid metaphase resulted from the chromosome disbalance (x250).*
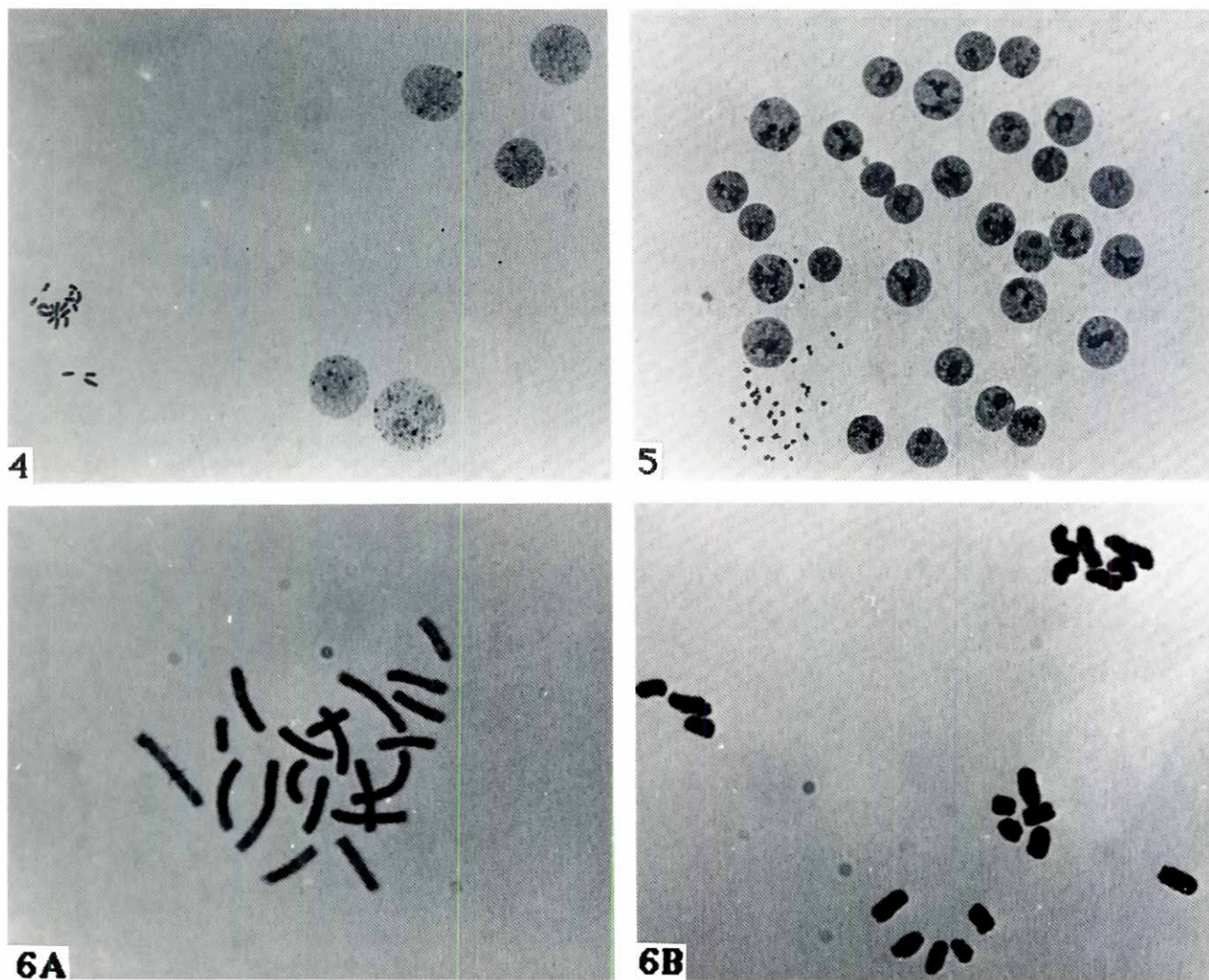
Fig. 4. Androgenetic haploid (PPN) produced by the removal of a maternal pronucleus. *Fixed at the 6-cell stage (x250).*

Fig. 5. Reconstructed diploid (PPN+2PB) produced by the fusion of androgenetic haploid with 2PB. *Fixed at the blastocyst stage (x250).*

Fig. 6. First metaphases of gynogenetic haploids produced by the fusion of the 2PB with enucleated zygote (x1250).

or completes replication (Howlett and Bolton, 1985). This means that at the very moment of 2PB extrusion its nucleus starts to lag behind the pronuclei. The longer the time elapsed since fertilization the less chance there is that the nucleus of the polar body, after being transferred inside the zygote, will "catch up" with the pronuclei. In this study, 2PBs were incorporated back into zygotes 4-7 h after extrusion. This means that in some cases pronuclei entered metaphase before polar body chromosomes had finished replication (Fig. 3D). Since zygote cytoplasm is the primary driving force of the first cell cycle (Murray and Kirschner, 1989; Smith *et al.,* 1990), it induced chromosome condensation of the 2PB interphase nucleus (Fig. 3E). Prematurely condensed chromosomes of the 2PB randomly distributed among the blastomeres led to chromosome imbalance (Fig. 3F); in 13% of the cases (see Table 1) prematurely condensed chromosomes did not let zygote cleave at all. The need for some degree of synchrony between recipient zygote and introduced nucleus was further supported by the observation that 27-29 h post-hCG old zygotes, if fused with their own 2PBs, never produced triploid morulae or blastocysts. At the

2-cell stage these embryos were aneuploid, and further development was significantly retarded. Those developed to the morula and blastocyst stages were mostly diploids and had on average 14.2±1.4 cells, thus indicating that during the first cleavage only one blastomere had inherited 2PB nucleus and did not participate in further development.

When 2PBs were fused with intact zygotes, 51% of such triploids developed to the morula and blastocyst stages (Table 1; Fig. 3B,C). However, this does not necessarily mean that polar body can support normal development. Polar body chromosomes could remain transcriptionally silent and as long as they did not interfere with development, embryo still might develop normally. Haploid androgenetic embryos (Fig. 4) developed very poorly (Table 1, also Modlinski, 1975; Kaufman and Gardner, 1974; Kaufman and Sachs, 1976; McGrath and Solter, 1986; Henery and Kaufman, 1992b); not one developed beyond the morula stage (Table 1). When haploid was fused with its own 2PB – in other words, when maternal pronucleus was substituted for the nucleus of the polar body – 32% of such embryos developed into

morulae and blastocysts (Table 1; Fig. 5). This indicates that the chromosomes of the 2PB can support normal preimplantation development: without these chromosomes haploids had no chance at all of reaching blastocyst stage. Comparison of haploid morulae (PPN) and reconstructed diploids (PPN+2PB) by their cell numbers (Table 1) provides further proof that 2PB, after being inserted inside the embryo, takes part in directing preimplantation development.

When fused with enucleated zygote, polar body never supported development beyond the 2-cell stage (Table 1). Haploidy by itself significantly impairs preimplantation development (see above). It seems that when haploidy is combined with nucleo-cytoplasmic incompatibility, caused by the developmental asynchrony of recipient cytoplasm and introduced 2PB nucleus, developmental arrest at the 2-cell stage occurs. The technique, however, provides an opportunity for karyotyping the 2PB chromosomes. In the absence of the "host" chromosomes, the chances of premature chromosome condensation would appear to be reduced almost to zero. When we karyotyped 2PBs, we achieved metaphase plates from practically all 2nd polar bodies (14 out of 15) fused with enucleated zygotes (Fig. 6).

Discussion

Being extruded only at fertilization, 2PB inherits cytoplasm possessing chromosome-decondensing activity. As a result, chromosomes of the 2PB enter interphase, form a nucleus and even start DNA replication (Howlett and Bolton, 1985). This is as far as 2PB passes through the cell cycle: apparently it does not have enough cytoplasmic substances even to complete replication. 2PB is connected with the zygote and subsequently with one of the blastomeres by a cytoplasmic bridge, midbody (Gulyas, 1986; Evsikov *et al.*, 1994), and remains intact throughout preimplantation development (our observations). There was only one report indicating that 2PB has a biological activity. Surface deformations of the enucleated eggs give an impression that there is some kind of "normalizing" stimulus from the 2PB: cytoplasts connected with the 2PB fragment more slowly and less frequently than the cytoplasts without polar bodies (Waksmundzka *et al.*, 1984).

The results presented in this paper prove that 2PB chromosomes, although destined to degenerate, can not only participate in development but can also direct it. The results obtained on triploid zygotes produced by the fusion of either 2PB or pronucleoplasts with intact zygotes demonstrate the need for a precise synchrony between nucleus and cytoplasm. This fact was also recognized in nuclear transfer experiments (McGrath and Solter, 1984a; Mann and Lowell-Badge, 1987; Dyban *et al.*, 1988; Smith *et al.*, 1988, 1990). At the moment of the 2PB fusion there was a 4 to 7 h difference in the developmental age between 2PB nucleus and pronuclei. This could account for the differences in the developmental fate between two types of triploids presented in the Table 1.

It should be noted that taken together the results of the 2PB fusion with manipulated or intact zygotes are similar to those obtained in nuclear transplantation experiments. A high percentage of tetraploid blastocysts was obtained after transfer of a 12-18-cell stage or inner cell mass nuclei into diploid mouse zygotes (Modlinski, 1978, 1981). When maternal or paternal pronucleus was exchanged for a nucleus of the 4-8-cell haploid parthenogenetic or androgenetic embryo, development proceeded to term (Surani *et al.*, 1986). However, the cloning attempts of mouse

embryos, when both pronuclei were substituted for a more advanced, 1/4 or 1/8- blastomere nucleus, had very limited success (McGrath and Solter, 1984b; Solter, 1987; Cheong and Kanagawa, 1993). It seems that the developmental potential of a reconstructed embryo depends on the ratio of the "host" vs "transplanted" genetic material.

Transfer of an advanced-stage nucleus into enucleated zygote is one of the methods proposed for animal cloning. An important conclusion inferred from this study is that 2PB must be removed from the perivitelline space during zygote enucleation. Otherwise 2PB may be fused with the reconstructed embryo leading to triploidy or premature chromosome condensation and aneuploidy, thus distorting the outcome of the cloning experiment.

In addition, our results support the previous report (Modlinski and McLaren, 1980) on the possibility of visualizing 2PB chromosomes. The technique was elaborated to a level that enabled practically all 2PBs to be karyotyped. This technique may be used in addition to the existing methods of detecting chromosomal non-disjunctions occurring during meiotic divisions.

Materials and Methods

Hybrid B6D2F1 and CB6F1 mice were used throughout this study. Females were superovulated by i/p injections, 48 h apart, of 7.5 IU of pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) and were then caged with males overnight.

Experiments were conducted on the pronuclei-stage zygotes. Cumulus cells were dispersed using hyaluronidase (200 IU/ml) in M2 medium. Embryos were cultured to the morula and blastocyst stages in M16 medium supplemented with 100 µM of Na_2-EDTA (Whittingham, 1971; Abramczuk *et al.*, 1977), under light paraffin oil, at 37°C in the presence of 5% CO_2 in air.

Micromanipulation technique of McGrath and Solter (1983), as modified by Tsunoda *et al.* (1986) was used. Prior to electrofusion, manipulated embryos were preincubated for at least 30 min in M16 medium (Kono and Tsunoda, 1988). Fusion was induced with the aid of the electrofusion apparatus (Bams Manufacturers Inc, Chicago, Ill; or GI-2, Puschino, Russia) in a fusion chamber consisting of two platinum wire electrodes glued to the bottom of a glass Petri dish with a gap of 0.33 mm. 10-20 manipulated embryos were transferred in the fusion chamber at 23°C overlaid with 0.3 M mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$ and 0.5% polyvinylpyrrolidone, pH 7.4. 5-15 min later the embryos, 3-6 at a time, were placed between the electrodes and aligned manually. The embryos were further aligned with an AC current (0.5 MHz, 7V for 7 sec), fusion was induced with a single 33V DC pulse of 400 µsec duration. Electrical output was monitored with an oscilloscope. Immediately after fusion treatment, embryos were placed in the culture medium. 30 min later fused embryos were thoroughly washed in M16 and placed for culture. Both manipulated and intact zygotes were fused with 2PBs at the age of 21-24 h post-hCG injection, about 4-7 h after 2PBs extrusion. 67% success rate for the 2PB fusion was achieved in this study: 390 out of 582 polar bodies had been fused with zygotes. To confirm 2PB fusion, in the preliminary series of experiments zygotes were stained with Hoechst 33258 for 15 min at a concentration 10 µg/ml in M2 medium and observed under fluorescence.

Cell number in morphologically normal morulae and blastocysts was determined using Dyban's technique of embryo fixation (Dyban, 1983), followed by staining with Giemsa. In order to estimate the ploidy of manipulated embryos they were cultured in the presence of Colcemid (0.1 µg/ml) before fixation (1- and 2-cell embryos overnight, morulae and blastocysts for 1-3 h).

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