Participation of the female pronucleus derived from the second polar body in full embryonic development of mice

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The second polar body (2PB), extruded from metaphase II oocytes after fertilization or oocyte activation, has a haploid set of female chromosomes like its sister, the fertilized (activated) oocyte. In the present study, the female pronucleus of fertilized mouse oocytes (zygotes) was replaced with the 2PB nucleus from the same or different oocytes to examine the developmental potential of the 2PB nucleus. When the female pronucleus (FPN) was synchronously (FPN and 2PB were same age) replaced with the 2PB nucleus, the rate of reconstructed zygotes developing to blastocysts decreased with the age of donors and recipients (from 70% at 20–21 h to 15% at 26–27 h after hCG injection). When nuclei were replaced asynchronously (FPN and 2PB were of different ages), a higher developmental rate to blastocysts was obtained with young recipient zygotes (20 h after hCG injection) than with aged recipient zygotes (24 h after hCG injection) (64% versus 20%, $P < 0.01$) irrespective of the age of the 2PB. In this second group of embryos, in which nuclei were replaced asynchronously, the 2PB nuclei were prematurely condensed at the time of first mitosis. These findings indicate that after being extruded from the oocytes the cell cycle of the 2PB progressed more slowly than did that of the zygote. After the transfer of reconstructed embryos into pseudopregnant females, normal pups with an expected coat colour were born, indicating the competence of the 2PB chromosomes for full embryo development.

Introduction

The meiotic division of mammalian oocytes differs in several significant ways from that of male germ cells. As a result of male meiotic divisions of single diploid cells (spermatogonia), sets of four round spermatids with an equal volume of cytoplasm are formed. However, the female germ cells (oocytes) undergoing meiotic division sequentially extrude two small polar bodies from the large main egg body. These polar bodies never participate in embryo development and degenerate some time during the preimplantation period (Gilbert, 1991; Evsikov and Evsikov, 1995). The second polar body (2PB), which is extruded after fertilization or parthenogenetic activation, has a haploid set of female chromosomes like its sister, the fertilized (or activated) oocyte. Therefore, the 2PBs have been extensively used to assess the numerical or structural chromosomal abnormalities of the developing embryo in clinical and basic research (Dyban *et al.*, 1992; Wheeler *et al.*, 1995).

So far, many attempts have been made to examine the ability of the 2PB to support embryo development. Zygotes

fused with the 2PB, or diploid parthenogenetic embryos produced by inhibition of the extrusion of the 2PB, readily developed to the blastocyst stage *in vitro* (Opas, 1977; Modlinski and McLaren, 1980; Borsuk, 1982). However, the conclusive evidence for the hypothesis that the chromosomes of the 2PB have the same developmental potential as their sister chromatids is to show full-term development of embryos that contain the 2PB chromosomes. In the present study, diploid mouse zygotes were reconstructed by replacing the female pronuclei with the nucleus of the 2PB to examine their developmental ability *in vitro* and *in vivo*.

Materials and Methods

Preparation of donor 2PBs and recipient embryos

Recipient pronuclear embryos were obtained from B6CBAF1 (C57BL/6 × CBA) females mated with DBA/2 males. Donor 2PBs were obtained from the same recipient embryos, or DBA/2 females mated with DBA/2 males. All females were injected with 5 iu pregnant mares' serum gonadotrophin (PMSG) and 5 iu hCG at a 48 h interval, and caged with males overnight. The pronuclear embryos were collected from the oviducts 15 h after hCG injection. They were freed of cumulus cells by treatment with hyaluronidase (0.1%, w/v) in mKRB

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Fig. 1. (a) Reconstructed mouse embryo that was stained with acetoorcein. These embryos had a large male pronucleus and a small female pronucleus derived from a second polar body. Scale bar represents 25 μm . (b) Reconstructed mouse embryo formed from fusion between an aged zygote and an aged second polar body. The prematurely condensed chromosomes can be observed next to the fully condensed paternal chromosome mass. Scale bar represents 15 μm .

medium (Toyoda *et al.*, 1974) and washed three times with fresh mKRB medium.

Nuclear transfer and examination of the 2PB-derived pronuclei

All manipulations were performed under an inverted microscope with Nomarski's contrast optics and micromanipulators. The 2PBs and female pronuclei were removed from appropriate zygotes through the zonae pellucidae, which had been partially dissected with a fine glass needle (Tsunoda *et al.*, 1986). The female pronucleus was chosen on the basis of its proximal location to the 2PB and its small size relative to the other pronucleus (male pronucleus). Nuclear transfer was undertaken in a small drop of mKRB containing cytochalasin B ($5 \mu\text{g ml}^{-1}$). The 2PBs were inserted into the perivitelline space of the recipients and the 2PB-zygote pairs were placed in a drop ($10 \mu\text{l}$) of the fusion medium ($300 \text{ mmol mannitol l}^{-1}$, $0.05 \text{ mmol CaCl}_2 \text{ l}^{-1}$, $0.1 \text{ mmol MgSO}_4 \text{ l}^{-1}$, $5 \text{ mg polyvinylpyrrolidone ml}^{-1}$) between the electrodes of a circular electrofusion chamber (Shimadzu, Kyoto). The width and depth of the electrode gap were 0.5 and 2.0 mm, respectively. Electrofusion of the 2PB and nucleated zygotes was induced by applying 20 V cm^{-1} AC for 30 s, 3000 V cm^{-1} DC for 10 μs , and 20 V cm^{-1} AC for 90 s, consecutively.

At 3–13 h after electrofusion, the reconstructed embryos were washed, whole-mounted on glass slides, fixed with 2.5% (v/v) glutaraldehyde for 10 min, and then stained with 0.25% (w/v) acetoorcein (Yanagida *et al.*, 1991). The embryos were examined under a phase-contrast microscope.

Assessment of embryo development in vitro

At 30 min after application of fusion pulse, embryos were examined under a dissecting microscope and those fused were washed and cultured further for 96 h. Two series of experiments were carried out to assess the developmental ability of reconstructed embryos. In the first experiment, the 2PB nuclei were transferred into synchronous recipients (the female pro-

nucleus and the second polar body were the same age) between 20 h and 27 h after hCG injection. In the second experiment, synchronous and asynchronous (the female pronucleus and the second polar body were of different ages) nuclear transfer was performed using 2PBs and recipient zygotes at either 20 h (young) or 24 h (aged) after hCG (2×2 factorial design).

Assessment of embryo development in vivo

After 96 h of culture, compacted morulae or blastocysts were transferred to the day 3 uteri of pseudopregnant ICR females. In this experiment, donor embryos were from DBA (aabbCCdd) females $\times$ DBA males and recipient embryos were from B6CBAF1 (AaBBCCDD) females $\times$ DBA males. Therefore, original recipient embryos would develop to offspring with black (aa) or agouti (heterozygous) coat colour and reconstructed embryos would develop to offspring with the DBA coat colour (dd, diluted). Nineteen days after transfer, recipient females were killed and the uteri were examined for live or dead fetuses. Live fetuses were raised by other lactating foster mothers (ICR mice).

Statistical analysis

The data were analysed by Fisher's exact probability test.

Results

Examination of the 2PB-derived pronuclei

All reconstructed embryos examined (both synchronous and asynchronous) had a well developed pronucleus (male pronucleus) and a small pronucleus (2PB-derived female pronucleus) (Fig. 1a). The 2PB-derived female pronuclei remained small until the first mitotic division (about 34 h after hCG injection).

Table 1. Development *in vitro* of mouse embryos after synchronous transfer of the second polar body at various times after hCG injection (embryos studied 4 days after injection)

Time after hCG injection (h)	Number of eggs	Number (%) of embryos		
		Two-cell	Eight-cell	Blastocyst
20–21	27	7 (25.9)	1 (3.7)	19 (70.4)
22–23	34	19 (55.9)	0 (0)	15 (44.1)
24–25	33	23 (69.7)	0 (0)	10 (30.3)*
26–27	27	22 (81.5)	1 (3.7)	4 (14.8)**

* $P < 0.05$, ** $P < 0.01$ (compared with values at 20–21 h).

Table 2. Development *in vitro* of mouse embryos after synchronous and asynchronous transfer of the second polar body (2PB) after hCG injection (embryos studied 4 days after injection)

Age of male pronucleus (h)	Age of 2PB (h)	Number of eggs	Number (%) of embryos		
			Two-cell	Eight-cell	Blastocyst
20	20	32	11 (34.4)	2 (6.3)	19 (60)
20	24	23	6 (26.1)	1 (4.3)	16 (65)
24	20	42	30 (71.4)	2 (4.8)	10 (20)*
24	24	84	69 (82.1)	0 (0)	15 (18)*

* $P < 0.05$ (compared with value when male pronucleus was 20 h old).

Embryo development in vitro after synchronous nuclear transfer

Most embryos reconstructed by synchronous nuclear transfer could develop to the two-cell stage, irrespective of the time of nuclear transfer (20–27 h after hCG injection) (Table 1). However, later embryonic development was significantly affected by the time of nuclear transfer. The best result was obtained when nuclei were transferred 20–21 h after hCG injection. The developmental rate gradually decreased with age of donors and recipients.

Embryo development in vitro after asynchronous nuclear transfer

For determination of the cause of poor embryo development after synchronous nuclear transfer of aged 2PB nuclei and aged recipient zygotes, asynchronous nuclear transfer was undertaken with young (20 h after hCG injection) and aged (24 h after hCG injection) donors and recipients. Higher developmental rates to the eight-cell and blastocyst stages were obtained with young recipients than with aged recipients, irrespective of the age of the donor 2PBs (Table 2). Reconstructed embryos were cytologically examined at metaphase of the first mitotic division. When young zygotes were fused with young 2PBs, a normal metaphase plate with maternal and paternal chromosomes of mature configuration was formed. In contrast, when aged zygotes were fused with aged 2PBs, the 2PB chromosomes that were prematurely condensed were observed in the vicinity of a fully condensed paternal chromosome mass (Fig. 1b).

Development of reconstructed embryos in vivo

In total, 30 synchronously reconstructed embryos that developed to morulae or blastocysts were transferred to six pseudopregnant females. All six females became pregnant and 18 pups were obtained. One pup was already dead at the time of Caesarian section and three pups failed to be raised by foster mothers. The remaining 14 pups were weaned and grew normally. They all had the DBA/2 coat colour and were shown to be fertile.

Discussion

In mammals, meiosis in females always starts early in life, often before birth, and is arrested at the first meiotic division. After puberty, a very small number of these oocytes are selected to prepare for fertilization and, as a result, the remaining abundant oocytes are wasted (Yanagimachi, 1994). Superovulation by hormone treatment and maturation techniques for oocytes *in vitro* have been developed for many mammals to rescue these oocytes that normally degenerate. Another characteristic of female gamete production is its uneven meiotic division. Here again, as a result of the extrusion of polar bodies, three out of four chromatids are wasted. The present study first demonstrated that the nucleus of the 2PB, which never participates in normal embryo development, potentially has the ability to support full embryo development. We used the laboratory mouse as a model, but it is very probable that the same technique can be applied to other species because in mammals the 2PBs usually persist for more than 24 h after fertilization (Evsikov and Evsikov, 1995).

In this study, we exchanged the nuclei of 2PB and zygotes at different ages after hCG injection. In the first experiment, synchronous nuclear transfer was performed between zygotes and 2PBs obtained 21–27 h after hCG injection. The ability of reconstructed zygotes to develop to blastocysts apparently decreased with time after hCG injection. Two possible reasons can be considered for this dependence on age. First, the developmental rates of zygotes and 2PBs did not progress in parallel, that of the 2PB was probably slower. This might lead to asynchronous development of the male pronucleus and the 2PB-derived female pronucleus within a zygote after nuclear transfer. Second, it is probable that the nucleus of the 2PB gradually loses its developmental ability after being extruded from the oocyte. These possibilities were examined in the second experiment by performing synchronous and asynchronous nuclear transfer using 2PBs and recipient zygotes at either 20 h (young) or 24 h (aged) after hCG injection (2×2 factorial design). The best result was obtained with young recipient zygotes, irrespective of the age of donor 2PBs. This finding clearly demonstrates that the nuclei of the 2PBs did not lose their developmental ability, at least within the age range tested, and that the cell cycle of the 2PBs progressed more slowly than that of normal zygotes. In aged recipient zygotes, the 2PB-derived female pronuclei did not seem to catch up developmentally with the male pronuclei, because the female pronuclei condensed prematurely at the first mitosis. The reconstructed embryos probably entered the first mitosis according to the recipients' schedule; in early embryonic cells, there is no

feedback machinery for detecting the completion of DNA replication (Murray and Hunt, 1993). It is thought that cell division before completion of DNA replication inevitably leads to chromosome damage. This may explain the poor embryonic development after the transfer of 2PBs into aged recipient zygotes. Premature condensation of the 2PB nucleus was also reported by Evsikov and Evsikov (1994) who fused the 2PBs with intact (non-enucleated) zygotes 21–24 h after hCG injection. In their study, development of 2PB-containing embryos to blastocysts was also low (51%) compared with that of zygotes that received another female pronucleus (100%). Their finding and ours clearly indicate the importance of synchronizing the female and male pronuclei for subsequent embryo development.

The fact that the mouse 2PB supported full-term embryo development indicates its potential as an alternative source of female chromosomes. As the 2PBs are smaller cells than oocytes, they can be cryopreserved more easily. A limited gene pool is a general characteristic of most endangered species. In future, *in vitro* fertilization technology for species conservation may be combined with a frozen 2PB resource with genetic diversity. In human infertility clinics, the use of the 2PB might avoid the motherhood problems caused by oocyte donation, although ooplasm donation would raise another question about possible mechanical damages to the oocytes. In every attempt in animal species and humans to transfer the 2PB nuclei, their synchronization with the male pronuclei would be crucial to clinical success, as demonstrated in this study.

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