

The 2-cell block occurring during development of outbred mouse embryos is rescued by cytoplasmic factors present in inbred metaphase II oocytes

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ABSTRACT In mice, completion of preimplantation development *in vitro* is restricted to certain crosses between inbred strains. Most of the outbred and inbred strains cease development at the 2-cell stage, a phenomenon known as the "2-cell block". Reciprocal mating between blocking and non-blocking strains has shown that the 2-cell block is dependent upon female, but not male, developmental information. One question that still remains unanswered is whether the genome of the metaphase II (MII) oocyte is genetically programmed to express, during the very early stages of development, some factor(s) required to determine developmental competence beyond the 2-cell stage. In the present study, we have addressed this question by performing reciprocal MII-chromosome plate transfer between MII oocytes of a non-blocking inbred strain and MII oocytes of a blocking outbred strain. Here, we report that development beyond the 2-cell stage does not depend on the MII genome, but instead it relies on a cytoplasmic factor(s) already present in ovulated non-blocking oocytes, but absent, inactive or quantitatively insufficient in blocking oocytes. Further evidence of the ooplasmic origin of this component(s) was obtained by transferring a small quantity of ooplasm from non-blocking MII oocytes to blocking MII oocytes or 2-cell embryos. Following the transfer, a high percentage of blocking oocytes/embryos acquired developmental competence beyond the 2-cell stage and reached the blastocyst stage. This study shows that development beyond the 2-cell stage relies also on a factor(s) already present in the ovulated oocyte.

KEY WORDS: *preimplantation, 2-cell block, nuclear transfer, cytoplasm transfer*

Introduction

In mice, completion of preimplantation development *in vitro* is restricted to certain crosses between inbred strains. Most of the outbred and inbred strains cease development at the 2-cell stage, a phenomenon known as the '2-cell block'. Although it occurs in particular *in vitro* culture conditions (for a review see Biggers, 1998), the 2-cell block may represent a model study that provides important insights on a critical stage of development, when zygotic genome activation (ZGA) occurs (Schultz, 2002). ZGA marks the passage from maternal to embryonic control of development, and represents the first crucial hurdle in the life of the new individual as inhibiting the activation of embryonic genes arrests development (Schultz and Worrall, 1995). Blocks to development at the time

when ZGA occurs have been described in other mammals (Schultz, 2002), including humans at the 4-cell stage, when a high percentage of IVF embryos is lost.

The 2-cell block is rescued when cytoplasm is transferred from 2-cell non-blocking embryos to 2-cell blocking embryos, allowing completion of preimplantation development (Muggleton-Harris *et al.*, 1982). Rescue does not occur when cytoplasm is injected from a 1-cell G1 stage non-blocking embryo to a blocking 2-cell embryo (Muggleton-Harris *et al.*, 1982), suggesting that the component(s) that allows to overcome the 2-cell block is yet not present, it has not been translated or it is not functioning. Instead, rescue occurs

Abbreviations used in this paper: MII, metaphase II

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consistently when injection is performed from a 2-cell G1 or G2 stage, but not S phase, non-blocking embryo to a 2-cell blocking embryo at any phase of the cell cycle (Pratt and Muggleton-Harris, 1988). The blocking activity is not due to a blocking factor, since the transfer of cytoplasm from blocking to non-blocking 2-cell embryos did not impair their ability to complete preimplantation development (Pratt and Muggleton-Harris, 1988).

The cyclic nature of these molecules indicates either a transcriptional control of their expression by the embryonic genome or a translational/post-translational regulation of transcripts/proteins.

Reciprocal mating between blocking and non-blocking strains has shown that the 2-cell block is dependent upon a female, but not male, developmental information (Goddard and Pratt, 1983). One question that still remains unanswered is whether the genome of the MII oocyte is genetically programmed to express, during the very early stages of development, some factor(s) required to determine the developmental competence beyond the 2-cell stage.

In the present study we have addressed this question by performing reciprocal MII-chromosome plates (MII-plates) transfer between MII oocytes of a non-blocking inbred strain and MII oocytes of a blocking outbred strain. We report that development beyond the 2-cell stage does not depend on the MII genome, but instead it relies on a cytoplasmic factor(s) already present in ovulated non-blocking oocytes, but absent, inactive or quantitatively insufficient in blocking oocytes. Further evidences of the ooplasmic origin of this component(s) were obtained by transferring a small quantity of ooplasm from non-blocking MII oocytes to blocking MII oocytes or 2-cell embryos. Following the transfer, a high percentage of blocking oocytes/embryos acquired develop-

mental competence beyond the 2-cell stage and reached the blastocyst stage.

Results

The presence in non-blocking embryos of a functional rescuing-factor(s) beginning with the 2-cell stage (Pratt and Muggleton-Harris, 1988) suggests either a developmentally regulated transcription or a translational/post-translational control of transcripts/proteins that could be present already in the ovulated MII oocyte. Since the male genome is unimportant in determining/overcoming the 2-cell block (Goddard and Pratt, 1983), to understand whether the female genome is programmed to express the rescuing activity or whether the rescuing factor(s) is already present in the MII ooplasm, in the present study we have performed reciprocal MII-transfer between MII oocytes of a non-blocking inbred strain (B6C3F1, F1) and MII oocytes of a blocking outbred strain (CD1). We argued that if the MII-plate of a non-blocking strain transferred into an enucleated oocyte of a blocking strain was supporting development beyond the 2-cell stage, whereas the reciprocal transfer was not, then we could assume that the female F1 genome was programmed to control the expression of this factor(s) in a developmentally regulated fashion. On the contrary, we could infer that the factor(s) was already present in the ooplasm of non-blocking MII oocytes.

Reciprocal MII-plates transfer between MII oocytes of a non-blocking inbred strain and MII oocytes of a blocking outbred strain

To this end, four different types of reconstructed MII oocytes were produced: F1^{F1MII} (F1MII-plates transferred into F1 enucleated oocytes), F1^{CD1MII} (CD1MII-plates transferred into F1 enucleated oocytes), CD1^{CD1MII} (CD1MII-plates transferred into CD1 enucleated oocytes) and CD1^{F1MII} (F1MII-plates transferred into CD1 enucleated oocytes). Following MII-transfer (Figure 1), oocytes were inseminated *in vitro* with capacitated spermatozoa isolated from the epididymes of F1 males; 6 hr later, those oocytes that presented two polar bodies (PBs) were transferred to M16 medium for further embryonic development. Control *in vitro* fertilisation (IVF) experiments were conducted in parallel. For each type of experiment, about 10 of the inseminated oocytes with two PBs were fixed, stained with acetic orcein and observed under phase microscopy for the presence of a male and a female pronucleus. The great majority (98%) of all the reconstructed oocytes presented two polar bodies and were correctly fertilised by one single sperm (Fig. 2A). Then, 1-cell embryos were cultured for up to 96 hrs to observe preimplantation development.

The MII-transfer procedure was tested, from fertilisation to birth, on F1^{F1MII} reconstructed oocytes. These experiments showed that the whole procedure did not affect the preimplantation developmental potential of the reconstructed oocytes. The frequency of F1^{F1MII} 2-cell embryos that developed to the blastocyst stage (63.3%; Table 1) was not significantly different compared to that obtained after IVF of normal F1 MII oocytes (71.4%; $p=0.218$, statistically not significant). When eight blastocysts obtained from F1^{F1MII} oocytes where transferred to a pseudopregnant female recipient, three of them reached full-term development giving birth to healthy mice (Fig. 2B). The majority of the zygotes obtained from CD1^{CD1MII} or CD1^{F1MII} oocytes stopped development at the 2-cell

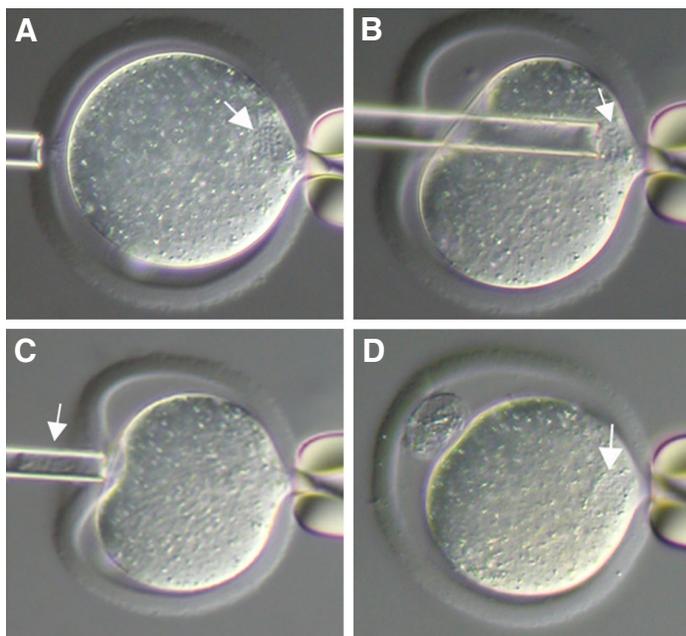


Fig. 1. An MII-plate was removed from an F1 MII oocyte (A,B) and transferred to another, previously enucleated, F1 MII oocyte (C,D). All the other MII-plate transfers were obtained following the same procedure. Arrows: an MII-plate (A); an MII-plate that is being removed from the oocyte (B); an MII-plate enclosed into a small portion of cytoplasm and oolemma, removed using a micropipette (C); an MII-plate few minutes after its transfer into a previously enucleated oocyte (D).

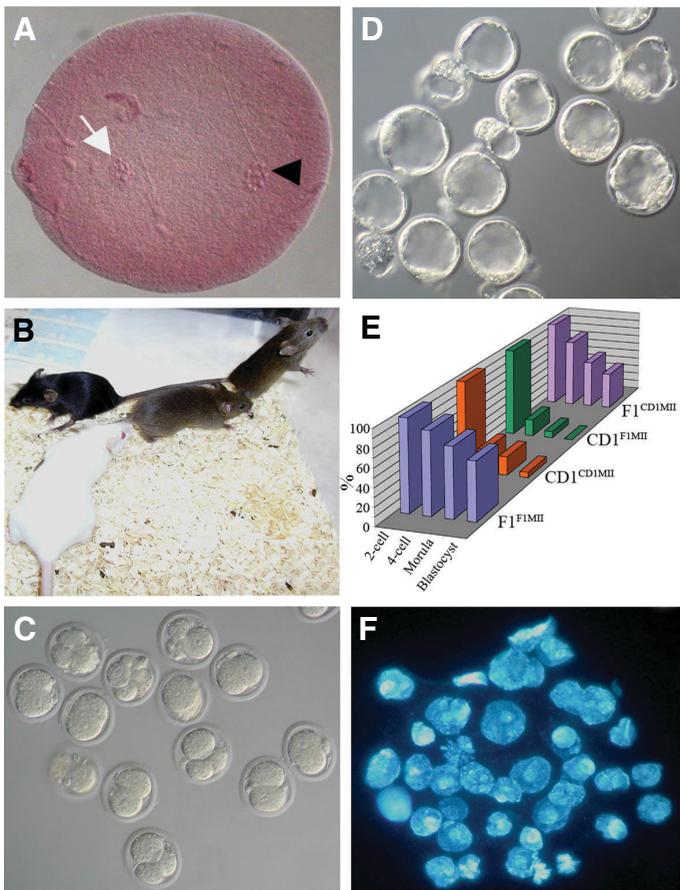


Fig. 2. Development of embryos obtained by reciprocal MII-plate transfer. (A) About ten of the oocytes showing two polar bodies were fixed and stained with acetic orcein and observed under phase microscopy. The micrograph shows the male (black harrowhead) and female (white arrow) pronuclei. The male pronucleus is recognisable for the presence of the sperm tail. (B) Three F2 mice born following IVF fertilisation and embryo transfer of blastocysts obtained after insemination of F1^{F1MII} reconstructed oocytes with F1 sperm. The mouse with the white coat colour is the recipient female. (C) Most of the CD1^{F1MII} stopped development at the 2-cell stage. (D) The majority of F1^{CD1MII} 2-cell embryos developed to the blastocyst stage. (E) Summary of the percentage of development of the 2-cell embryos obtained from the four different types of reconstructed oocytes. (F) Blastomeres of a single blastocyst developed from an F1^{CD1MII} reconstructed oocyte.

stage (73.5% and 81.2%, respectively) (Table 1, Fig. 2C), a number not significantly different ($p=0.351$, statistically not significant) to that obtained after IVF of CD1 oocytes with F1 sperm (75.6%). As shown in Table 1, 5.9% of CD1^{CD1MII}, none CD1^{F1MII} and 42.2% F1^{CD1MII} 2-cell embryos developed to the blastocyst stage (Fig. 2D).

As reported before (Whittingham, 1974; Pratt and Muggleton-Harris, 1988), the 2-cell block is not an ‘all or none’ phenomenon, but some of the embryos from blocking strains may develop beyond the 2-cell stage and reach blastocyst. In the reconstructed oocytes that we produced with a CD1 ooplasm, the number of blastocysts varied from none (CD1^{F1MII}) to 5.9% (CD1^{CD1MII}). The reconstructed embryos that best developed beyond the 2-cell stage were those with an F1 ooplasm, namely F1^{F1MII} and F1^{CD1MII}.

The percentage of F1^{CD1MII} 2-cell embryos that reached the 4-cell stage (78.9%) was not significantly different ($p=0.085$, statistically not significant) to that of F1^{F1MII} 2-cell embryos (89.4%) (Table 1, Fig. 2E); however, although the majority of F1^{CD1MII} embryos developed beyond the 2-cell stage, further preimplantation development was significantly different comparing the two types of reconstructed embryos as 75.7% F1^{F1MII} and 54.9% F1^{CD1MII} 2-cell embryos ($p<0.05$) reached the morula stage and 63.3% F1^{F1MII} and 42.2% F1^{CD1MII} embryos ($p<0.05$) reached the blastocyst stage (Table 1). In those F1^{CD1MII} embryos that reached the blastocyst stage, the blastomere number was attested at 35 ± 8 (Fig. 2F), a number of cells not significantly different ($p=1$, statistically not significant) to that found in control IVF F1 embryos (35 ± 5).

Cytoplasm transfer from non-blocking to blocking oocytes

Since this rescuing factor(s) is present in the ooplasm of developmentally competent gametes, we then argued whether the transfer of cytoplasm from F1 MII oocytes to CD1 MII oocytes could result in the acquisition of developmental competence by this latter blocking strain. When we injected approximately 8-10 pl of MII-F1 ooplasm into MII-CD1 oocytes, the majority of CD1 2-cell embryos developed to the 4-cell stage (34 4-cell out of 49 2-cell embryos, 69.4%), and 38.7% of them developed to blastocyst (Table 2). Cytoplasm transfer from MII-CD1 to MII-CD1 oocytes did not rescue these oocytes from the 2-cell block (Table 2). This result shows that a small quantity of F1 ooplasmic factor(s) is capable of rescuing the majority of blocking CD1 oocytes to develop beyond the 2-cell stage and also that the CD1 ooplasm does not represent an inhibitory environment since it did not inactivate the functionality of the transferred F1 factor(s). While the transfer of a small quantity of ooplasm from F1 oocytes to CD1 oocytes rescues the latter from the 2-cell block, the transfer of the whole F1 MII-plate to an enucleated CD1 oocyte did not give any developmental improvement, suggesting that the rescuing factor is not present on or around the MII-plate.

Cytoplasm transfer from non-blocking MII oocytes to blocking 2-cell embryos

In a third type of experiment we addressed the question of whether the ooplasm factor(s) activates a series of events, comprised between the oocyte and the 2-cell embryo, that lead to completion of the second mitotic division or whether itself can

TABLE 1

RECIPROCAL MII-PLATE TRANSFER						
Enucleated MII oocyte	Transferred MII-plate	N. of reconstructed MII oocytes	% ± S.D. (N.) of preimplantation embryos			
			2-cell	4-cell*	Morula	Blastocyst
F1	F1	89	100 (66)	89.4 ± 10.1 (59)	75.7 ± 9.1 (50)	63.3 ± 13.8 (42)
CD1	CD1	54	100 (34)	26.5 ± 13.2 (9)	20.6 ± 11.2 (7)	5.9 ± 7.3 (2)
CD1	F1	87	100 (69)	18.8 ± 6.3 (13)	7.2 ± 3.6 (5)	0
F1	CD1	89	100 (71)	78.9 ± 10.1 (56)	54.9 ± 9.1 (39)	42.2 ± 10.1 (30)

Reciprocal MII-plate transfer between enucleated MII oocytes of a non-blocking inbred strain (F1) and enucleated MII oocytes of a blocking outbred strain (CD1). Reconstructed oocytes were inseminated with F1 spermatozoa and preimplantation development beyond the 2-cell stage was analysed.

*: the percentage was calculated considering the 2-cell stage as 100%.

TABLE 2

TRANSFER OF OOPLASM FROM F1-MII TO CD1-MII RESCUES THE DERIVED EMBRYOS FROM 2-CELL BLOCK

MII oocyte recipient	MII ooplasm transferred	N. of oocytes	N. (%) of preimplantation embryos			
			2-cell	4-cell	Morula	Blastocyst
CD1	F1	86	49	34 (69.4)	26 (53.1)	19 (38.7)
CD1	CD1	82	32	8 (25)	5 (15.6)	1 (3.1)

participate to unblock the second mitotic division. We have injected approximately 10 μ l of cytoplasm taken from F1 oocytes at the opposite side of the MII-plate, into one single blastomere of early (21 hr post insemination) 2-cell CD1 embryos obtained following IVF with F1 spermatozoa. Out of a total of 58 CD1 2-cell embryos injected, 36 (62%) developed to the 4-cell stage, 26 (44.8%) to the morula and 23 (39.6%) to the blastocyst stage. Development beyond the 2-cell stage in these experiments was significantly ($p < 0.05$) improved compared to that of embryos obtained after IVF of CD1 MII oocytes. These results demonstrate that the same factor(s) present in the F1 ooplasm is capable of rescuing CD1 2-cell embryos and to induce further development, with a percentage of embryos that reached the blastocyst stage similar (39.6%) to that of reconstituted F1^{CD1MII} embryos (42.2%, $p = 0.668$, statistically not significant; Table 1). Also, the small quantity of cytoplasm that is transferred in one blastomere is sufficient to induce the second mitotic division in both blastomeres, as already shown before (Pratt and Muggleton-Harris, 1988) when cytoplasm was transferred between two 2-cell embryos. We think that a passage of molecules from the injected blastomere to the other may occur and induce cell division in the uninjected blastomere. This migration has been demonstrated to occur through gap junctions between blastomeres of cleavage embryos and fibroblasts, these latter shown to express Oct-4, an embryonic-specific transcription factor (Burnside and Collas, 2002).

Discussion

The results of our study show that development beyond the 2-cell stage does not depend on the MII genome, but instead it relies on a cytoplasmic factor(s) already present in ovulated non-blocking oocytes, but absent, inactive or quantitatively insufficient in blocking oocytes. These results also indicate that the CD1 MII-plate, within an F1 cytoplasm, does not gain full developmental competence, suggesting a strain-specificity of this genome. At these very early stages of development, epigenetic modifications from a gametic to an embryonic type of chromatin organisation mold an expression profile required for development. Methylation of cytosines in CpG sites is crucial for regulating the temporal, spatial and parent-specific gene expression patterns (Haaf, 2006). The paternal genome is actively demethylated within a few hours after fertilisation, whereas the maternal genome is passively demethylated after the 2-cell stage (Mayer et al., 2000; Barton et al., 2001). Recent studies have demonstrated a strain-specific difference in the efficiency of methylation reprogramming during preimplantation development (Shi and Haaf, 2002; Haaf, 2006). *In vitro* development of NMRI (an outbred strain that shows the 2-cell block) 2-cell embryos show a higher frequency of abnormal methylation patterns than B6C3F1, suggesting a functional link between methylation reprogramming defects and lower developmental com-

petence (Shi and Haaf, 2002). The transfer of the CD1 genome within the F1 ooplasm clearly improves its developmental competence, but the reprogramming activity is not completely efficient.

We have given a clear indication on the presence within the mouse F1 ooplasm of a component(s) involved in conferring developmental competence beyond the 2-cell stage. However, its molecular feature remains unknown. The data of earlier studies and a number of considerations that we may draw out from our results may help its search.

One marked evidence is that this factor(s), or its functional form, has a cyclic nature: it is present in MII oocytes (our data), it disappears in the early 1-cell embryo, reappears in the early 2-cell embryo, disappears in S-phase of the second cell cycle and reappears again in the following G2-phase (Pratt and Muggleton-Harris, 1988). This cyclic appearance speaks for a possible role of this component(s) in the regulation of the embryo's cell cycle and since we have shown that its injection into a blastomere rescues 2-cell blocking embryos from the block, the rescuing molecule is probably the same during the first two cell cycles. At this regard, the maturation promoting factor (MPF) has been considered a possible candidate. MPF is a cell cycle regulator in all eukaryotes and failure of MPF activation leads to arrest of the cell cycle at the G2/M phase (Norbury and Nurse, 1992). MPF is a complex of cyclin-dependent kinase p34cdc2 and cyclin B; p34cdc2 kinase is dephosphorylated and activated by cdc25 phosphatase (King et al., 1994; Lew e Kornbluth, 1996) and cyclin B is phosphorylated by Polo-like kinase I and translocated into the nucleus (Pines and Hunter, 1991; Ookata et al., 1992). Microinjection of MPF purified from *Xenopus laevis* eggs into 2-cell mouse embryos, rescued these embryos from the block (Nakano et al., 2001). An earlier work has proposed that 2-cell blocked embryos contain enough p34cdc2 to induce mitosis, but the mechanisms for its dephosphorylation and activation is inefficient (Aoki et al., 1992). Absence of MPF activation in G2/M-arrested 2-cell embryos occurs in the presence of phosphate in the culture medium, it affects the dephosphorylation of phosphorylated p34cdc2 (Aoki et al., 1992; Haraguchi et al., 1996) and is correlated to a decrease in the levels of cyclin B and cdc25B mRNAs (Haraguchi et al., 1999). When phosphate is absent from the medium, the activation pattern of p34cdc2 kinase during the second cell cycle is similar to that in the first cell cycle, allowing completion of preimplantation development (Haraguchi et al., 1996).

Although a possible involvement of MPF as a crucial molecule in rescuing embryos from the 2-cell block is intriguing and supported by a number of observations, our results suggest that it might not be the sole factor. In mouse MII oocytes MPF is compartmentalised, with the majority of this kinase around the MII-plate (Fulka et al., 1995). The MII-transfer technique that we used removes the whole MII-plate together with some cytoplasm around the spindle (Figure 1), thus MPF from an F1 oocyte should be transferred altogether into the recipient enucleated CD1 oocyte. Since our results clearly show that the reconstituted CD1^{F1MII} oocytes maintained the 2-cell block (Table 1), MPF present in MII oocytes is likely not enough to release 2-cell embryos from the block. MPF is involved in the first mitotic division which occurs also in blocking embryos, but some other molecule(s), perhaps other cell cycle molecules, already present in the F1 ooplasm in an active form (as shown by the transfer of small quantities of F1 cytoplasm to MII oocytes or 2-cell CD1 embryos), is needed to complete the

second cell cycle.

Other candidates might be found focusing on the events that most characterise these early developmental stages. For example, the female gamete accumulates during growth a group of gene transcripts and proteins that become necessary, after fertilisation, for successful embryogenesis. These maternal-effect genes are crucial during the oocyte-to-embryo transition (Zheng and Dean, 2007), since their misregulation determines a developmental block, mainly at the 2-cell stage. Our next experiments will try to compare the pattern of expression of these genes and of their proteins in preimplantation embryos derived from oocytes of blocking and non-blocking strains.

Materials and Methods

Animals

Mice of the strains B6C3F1 (3-4 month-old males and 4-5 week-old females) and CD1 (4-5 week-old females) were purchased from Charles River (Como, Italy). Animals were maintained under controlled room conditions (22°C, with 60% air moisture and 14L:10D photoperiod) and investigations were conducted in accordance with the guiding principles of European (n. 86/609/CEE) and Italian (n. 116/92, 8/94) laws protecting animals used for scientific research.

Reagents

All reagents were purchased from Sigma Chemical Co. (Milano, Italy), unless otherwise stated.

Micromanipulation of metaphase II oocytes

Ovulated MII oocytes were collected from the oviducts of females injected with 3.5 I.U. Folligon (Intervet International, Netherlands), followed 48 hr later by an injection of 3.5 I.U. Corulon (Intervet International, Netherlands). MII oocytes were placed in M2 medium containing hyaluronidase (Type II, 500 IU/ml in BSA-free M2—medium) for 4-5 min., then cumulus-free oocytes were thoroughly washed in Whittingham medium (Whittingham, 1971) and transferred to a drop of fresh Whittingham medium in a 35x10 mm plastic petri dish (Corning, Bibby Sterilin, UK) under mineral oil and placed in incubator at 37°C and 5% CO₂, before further treatment. Groups of 20-30 MII oocytes were then transferred to HEPES-buffered CZB (H-CZB) medium (Chatot *et al.*, 1989) containing 0.1% PVP (Polyvinylpyrrolidone), 5 µg/ml cytochalasin B and 0.1% DMSO, for enucleation. Enucleation and MII transplantation were performed using piezo-driven (PMM 150 FU, Prime Tech, Ibaraki, Japan) borosilicate micropipettes (Clark Instruments, Edenbridge, UK) under an inverted microscope equipped with DIC optics (Olympus, Ibaraki, Japan). Following enucleation, oocytes were transferred in M16 medium (Biggers, 1998) and incubated at 37°C in 5% CO₂ in air for 1 1/2 hr. Then they were transferred in H-CZB containing 1% PVP and 5 µg/ml cytochalasin B and following MII transplantation, reconstructed oocytes were incubated in M16 for 1 1/2 hr, washed in Whittingham medium and finally transferred in fresh Whittingham medium for insemination with capacitated spermatozoa.

In vitro fertilisation and embryo culture

Sperm were isolated as previously described (Zuccotti *et al.*, 1998) and incubated for 60 min in 100 µl drops of Whittingham medium at a final concentration of 1.8x10⁶ sperm/ml. MII oocytes were transferred into the insemination drop and incubated at 37°C under a 5% CO₂ in air for 2 hr. Based on the presence of a second polar body, as a sign that fertilisation had occurred, presumptive 1-cell stage embryos were pooled, transferred from Whittingham medium to a 30 µl drop of M16 medium (Biggers, 1998), for further development.

For each experiment type, about 10 oocytes with two polar bodies were used to check that fertilisation was occurred. Oocytes were placed under

a coverslip suspended with drops of beeswax at each corner; the coverslip was slightly pushed down until the oocytes were held in place. Oocytes were then treated with a series of fixatives and washing. Glutaraldehyde (2.5%) was drawn under the coverslip using a filter paper placed at one side of the coverslip; this fixative was followed by 10% formalin, a wash with distilled water, 95% ethanol and finally 1% acetic orcein.

Statistical analysis

Statistical analysis was done with the SigmaStat 3.0 software, introducing the raw data of each single experiment performed and using the Student's t test. A value of p<0.05 was considered statistically significant.

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A reversible block at the G1/S border during cell cycle progression of mouse embryos.

N Ouhibi, J Fulka, J Kanka and R M Moor
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