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Original Article

Early changes in embryonic nuclei fused to chemically enucleated mouse oocytes

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ABSTRACT Mouse oocytes were chemically enucleated by subjecting them to etoposide and cycloheximide treatment during the first meiotic division (Fulka, Jr. and Moor, *Mol. Reprod. Dev. 34:* 427-430, 1993) and thereafter electrofused to karyoplasts prepared from: (i) two-cell stage embryos at the G2-phase; (ii) four-cell stage blastomeres (S- or G2-phase); or (iii) embryonic stem (ES) cells. In the first series of experiments we used fusion conditions which do not induce egg activation to define the series of nuclear changes that are initiated immediately following fusion. Although fusion is evident within 5-10 min of induction, nuclei remain visible for up to 20 min prior to chromatin condensation and the formation of metaphase plates (60-90 min post fusion). After activation, the anaphase-telophase transition is completed within 1-2 h, followed thereafter by cleavage of 75% of reconstituted eggs into two equal nucleated blastomeres, irrespective of the origin of the nuclei used for fusion. We conclude from the first study that a protocol involving fusion without activation, followed 90 min later by activation, is likely to be optimal for nuclear transplantation using MII-phase cytoplasts. In the second series of experiments the above optimized protocol was used to study the effects of different cell cycle combinations on chromosome organization in eggs reconstituted by nuclear transplantation. Both G1- and S-phase karyoplasts fused to MII-phase cytoplasts exhibited spindle abnormalities in all eggs studied. Characteristic abnormalities in these cell cycle combinations included chromatin fragmentation and joining or aggregations of chromatin. An entirely different spindle morphology was associated with G2- to MII-phase fusion: morphologically normal spindles were found in 45% of eggs in this group while the remainder showed abnormalities characterized by chromosomes dispersed (36%) or detached (12%) from the spindle. Although elevated MPF levels during metaphase II are considered necessary for nuclear remodelling, our results demonstrate the highly restricted requirements needed for normal spindle organization and subsequent cloning success after nuclear transplantation.

KEY WORDS: *mouse, cytoplasts, karyoplasts, nuclear transplantation*

Introduction

Maturation Promoting Factor (MPF) is central to cell cycle regulation. During meiosis the activity of MPF increases sharply before germinal vesicle breakdown (GVBD), remains high throughout first metaphase, declines slightly during anaphase and telophase and increases again at metaphase II. After activation of the egg by the fertilizing sperm MPF activity falls to basal levels (Fulka *et al.*, 1992). The elevated level of MPF in the egg at the time of sperm entry plays an important role in the formation of the pronuclei while the subsequent decline of MPF allows for progression of the zygote into the G1-phase of the first mitotic cell cycle.

Nuclei transplanted into egg cytoplasts undergo a process of remodelling which includes structural reorganization, reprogramming of gene expression and the synchronization of the cell cycle stages in both karyoplast and cytoplast. Extensive studies using eggs from frogs and mice have suggested strongly that high levels of MPF are essential for this remodelling process (see Hoffner and DiBerardino, 1980). Pioneering work by these workers demonstrated that somatic nuclei transplanted into *Rana pipiens* oocytes at metaphase I, and conditioned thereafter for 24 h in a high MPF environment, were transformed after activation into 'pronuclei'. DiBerardino and Hoffner-Orr (1992) showed further that the developmental capacity of nuclei subjected to metaphase conditioning

Abbreviations used in this paper: MII, metaphase II; MPF, Maturation Promoting Factor; PCC, premature chromosome condensation; M-plate, metaphase plate; hCG, human Chronionic Gonadotrophin.

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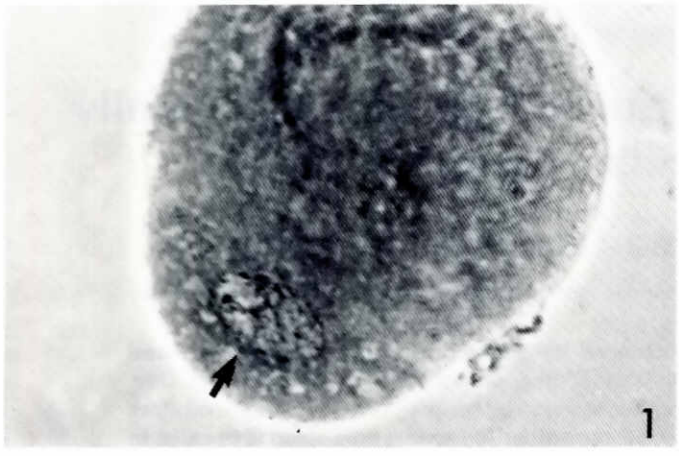
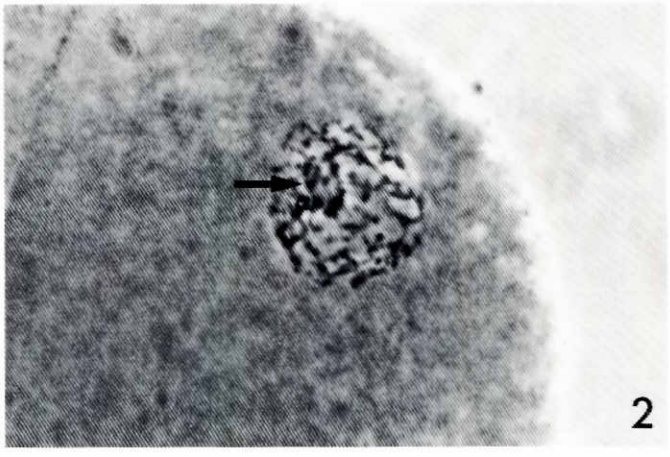
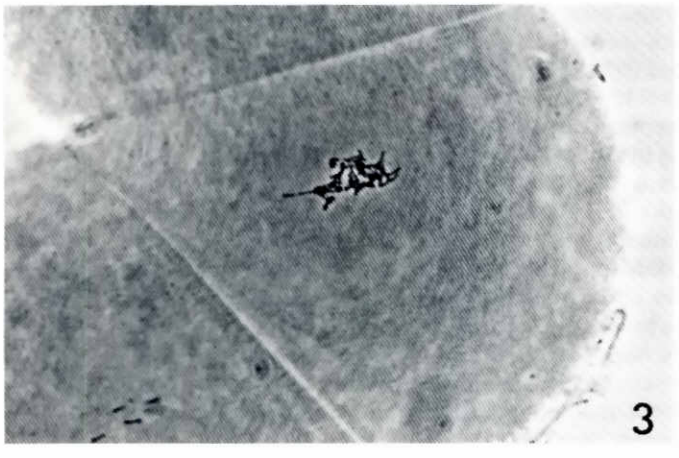
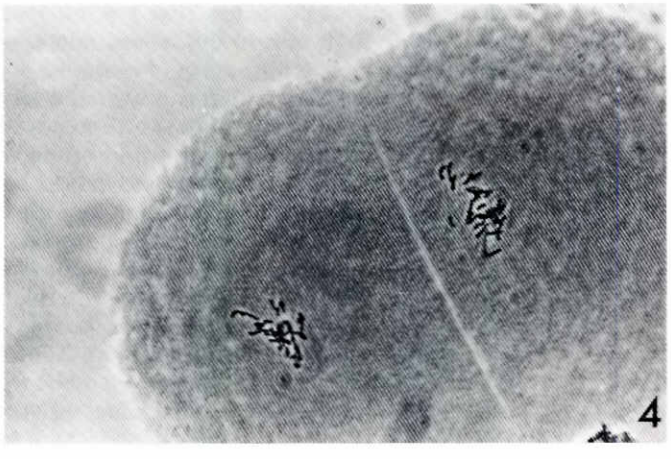








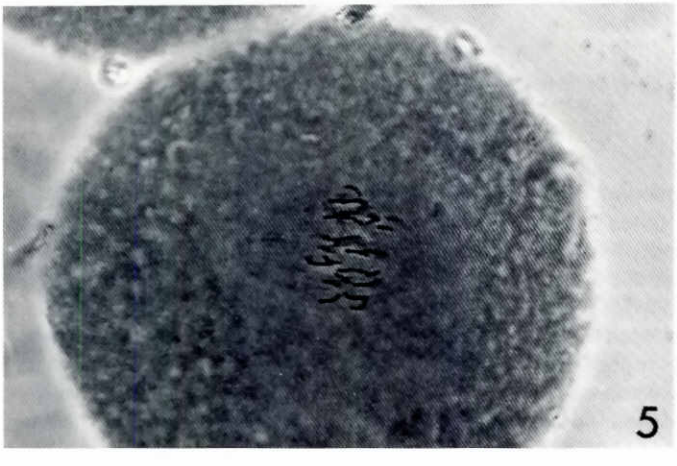


Fig. 1. Four-cell stage blastomere nucleus introduced into a cytoplast produced by chemical enucleation. *After 10 min post induction of fusion the nucleus is still intact (arrow).*

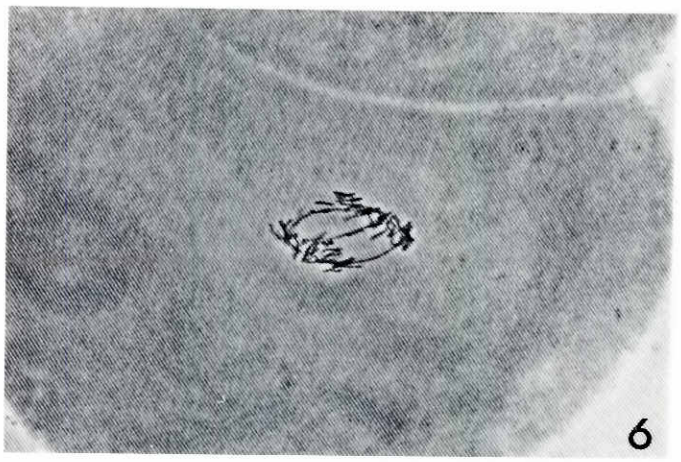
Fig. 2. Intensive chromatin condensation inside the four-cell blastomere nucleus is evident at about 20 min after fusion. *Nucleus still retains its original shape and the nucleolus is also evident (arrow).*

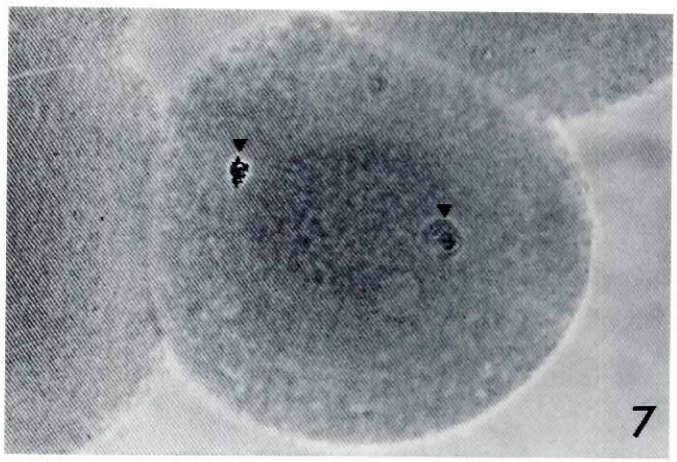
Fig. 3. Nuclear breakdown of the nuclear membrane in the blastomere occurs after 30 min post induction of fusion. *Chromosomes are highly condensed, but still disorganized.*

Fig. 4. Formation of metaphase plate is observed within 45-60 min after fusion.

Fig. 5. Well-formed metaphase with corresponding spindle is typical at about 90 min after fusion of G2-blastomere with enucleated oocyte.

was much greater than that of non-conditioned nuclei. Similarly, thymocyte nuclei transplanted into mouse cytoplasts with high MPF levels (MII stage) undergo extensive and rapid remodelling to a pronuclear-like form (Czolowska *et al.*, 1984; Szöllösi *et al.*, 1988). Surprisingly, incomplete remodelling was detected when 8-cell embryonic nuclei were fused to metaphase II oocytes which were then activated, and minimal remodelling was observed when comparable nuclei were transplanted into already activated cytoplasts with basal MPF levels (Czolowska *et al.*, 1992). Moreover, the beneficial effects of nuclear conditioning on embryonic development in mice has also recently been demonstrated. Thus, blastomere nuclei (late 2-cell stage) transplanted into cytoplasts with high MPF levels undergo subsequent embryonic development while those transplanted into zygote cytoplasts (low MPF) uniformly fail to develop (McGrath and Solter, 1984; Kono *et al.*, 1991, 1992). Moreover a very recent paper by Cheong *et al.* (1993) clearly





Figs. 6-7. When these oocytes are activated, anaphase (6) is observed within 60 min, thereafter the cells exhibit an oval shape with a telophase configuration (arrow heads) inside (7).

demonstrates that even the 8-cell stage blastomere nuclei support full development after fusion to appropriate enucleated oocytes in the mouse. While the advantages of using MII-phase cytoplasts for nuclear reprogramming in domestic species have been reported (First and Prather, 1991; Moor *et al.*, 1992), recent evidence in cattle does not appear to support this concept (Kono *et al.*, 1993; Ushijima and Eto, 1993).

Results from both the early studies using *Xenopus* eggs and more recent experiments in rabbits reveal that large numbers of chromosomal defects occur after nuclear transplantation (DiBerardino, 1979; Gurdon, 1986; Collas *et al.*, 1992; Pinto-Correia *et al.*, 1993). In this paper we test the hypothesis that the initial interaction between nuclei and M-phase cytoplasts after fusion determines chromosomal integrity and, by extension, the future developmental potential of the reconstituted embryos. After identifying optimal methods for the use of MII-phase cytoplasts we show that cytoplasts prepared by non-invasive chemical enucleation (Fulka and Moor, 1993) induce the full range of nuclear reprogramming events and greatly facilitate studies on immediate-early chromatin reorganization after nuclear transplantation. Finally, the results support the concept that in MII-phase cytoplasts normal spindle organization occurs only when the degree of cell cycle synchrony between the karyoplast and cytoplast fulfills precise requirements.

Results

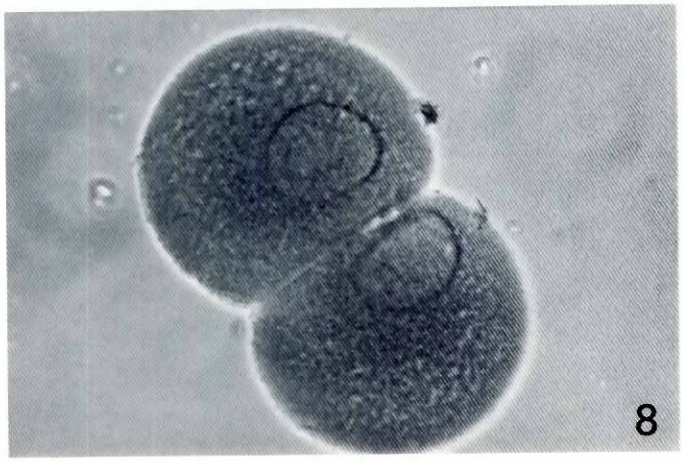
Changes in nuclear organization after G2- to MII-phase fusion

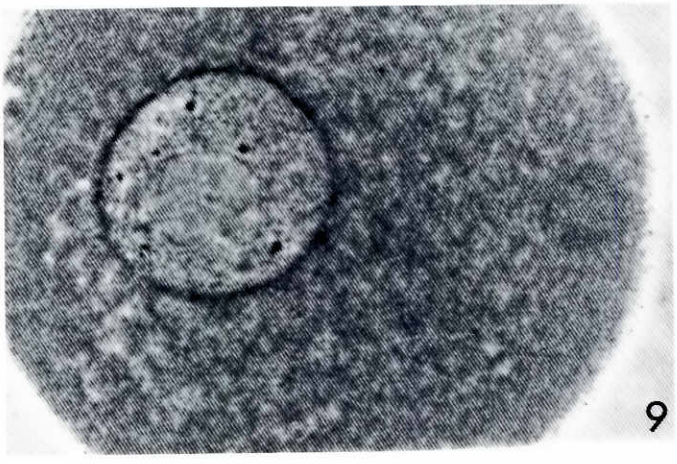
In the first series of experiments, G2-phase blastomeres from 4-cell eggs were fused to MII-phase cytoplasts in order to define the immediate-early sequence of nuclear events. Groups of reconstituted eggs (>20 eggs/group) were fixed and evaluated at 10, 15, 20, 45, 60 or 90 min post fusion. Macroscopic evidence of fusion was apparent 10 min post induction, at which time the fused cells appeared oval in shape (Fig. 1). Eggs fixed and stained at 10 min post fusion contained intact nuclei with distinct nuclear membranes and nucleoli. By 15 min the reconstituted eggs had become rounded in shape but nuclei could still clearly be distinguished in all cells. The first signs of condensation, in the form of spherical areas of heavy condensation of chromatin, were evident 20 min after fusion. As shown in Fig. 2, nucleoli are still visible at this time. By 30 min post fusion no nuclei were visible and a group of condensed chromosomes was evident (Fig. 3). Organization of the metaphase plate occurred between 45-60 min post fusion (Fig. 4) and was definitively established with a clear spindle in almost all cells by 90 min (Fig. 5). Of 100 eggs evaluated 90 min post-fusion, 75% showed a clear G2-phase morphology with sharply demarcated chromosomes. 25% of remaining reconstituted eggs were in S-phase.

The foregoing results not only catalogue the timed sequence of nuclear events following the fusion of G2-staged karyoplasts with MII-phase cytoplasts, but also indicate that 90 min post fusion is the optimal time for evaluation of metaphase plates. Of greater importance is the recommendation that eggs reconstituted by G2- to MII-phase fusion should be activated at 90 min directly after the completion of metaphase plate and spindle formation. The analysis of cell cycle progression after activation revealed a loss of tight cellular synchrony following exit from MII-phase. However, the following general temporal sequence of events occurs after activation. The anaphase-telophase transition (Figs. 6 and 7) was completed within 60 min of electro-activation (30 reconstituted eggs analyzed). Thereafter, early evidence of cytokinesis, including a shape modification from spherical to oval, was observed. Cleavage into two equal cells was observed between 3 to 4 h after activation (78 eggs evaluated), small nuclei were detected at 5 to 6 h, but fully developed nuclei (Fig. 8) were usually evident only at 15 to 18 h post-activation. Of 185 reconstituted eggs exposed to the activation stimulus, 142 (77%) responded to it and cleaved thereafter into two equal cells, each containing a prominent single nucleus; no subnuclei were detected. Exceptionally, we observed after activation a single nucleus without cleavage or unequal division (Fig. 9).

Cell cycle related chromosomal abnormalities in reconstituted eggs

In most previous experiments on nuclear transplantation no attempts were made to synchronize the cell cycle stages of the karyoplasts and cytoplasts. Using new protocols for cytoplast production (see previous section above), we report in this section on the results of an analysis of the effect of karyoplast cell cycle





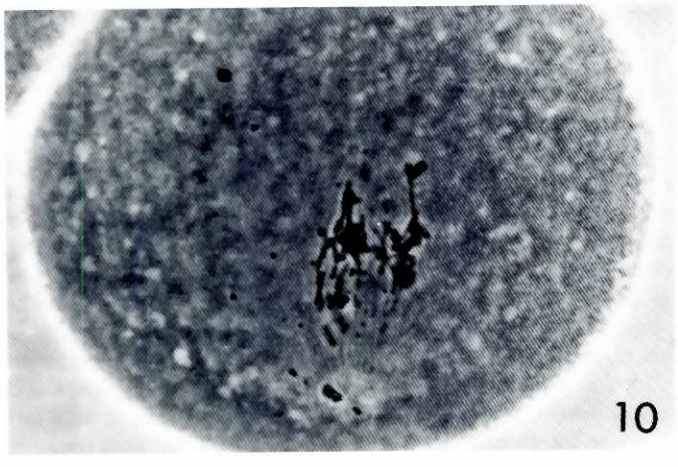


Fig. 8. The activated oocytes cleave into two equal cells in most cases, each containing a well-developed nucleus.

Fig. 9. In a very few cases cleavage does not occur and only one nucleus is formed.

Fig. 10. If an S-phase blastomere (or G-I) is fused to an enucleated oocyte, nuclear membrane breakdown is always induced but the morphology of condensed chromatin is aberrant thus forming the fragments and joined pieces of chromatin. *A spindle is present in these cells.*

stage on chromosome organization during the period of chromatin condensation induced by cell fusion (PCC: premature chromosome condensation). Karyoplasts from G1 (obtained from the two small blastomeres of 3-cell embryos immediately after cleavage), S-phase (blastomeres for 4-cell embryos 5 h post cleavage) and G2-phase (blastomeres 12 h post cleavage) were used. However, we have focused primarily on the S- and G2-phases both because the G1-phase is very short, consequently providing few karyoplasts for transplantation, and because the results from this stage are very similar to those at S-phase (comparison not shown). The results of the S- to MII-phase and G2 to MII-phase fusions together with those using stem cell karyoplasts are summarized in Table 1.

S- to MII-phase fusions

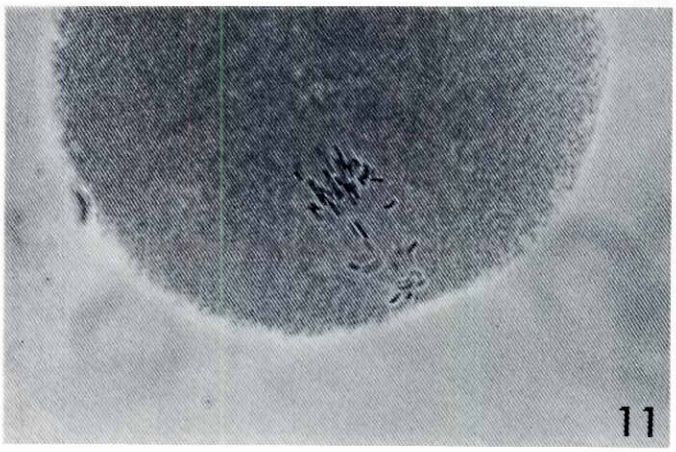
After fusion, chromatin condensation and nuclear membrane breakdown occurred in a uniform manner in all reconstituted cells. However, chromatin alignment on the spindle was aberrant in all 112 eggs studied. The most common abnormalities in this group included chromatin fragmentation, random patches of chromatin and adherent or joined chromatin (Fig. 10).

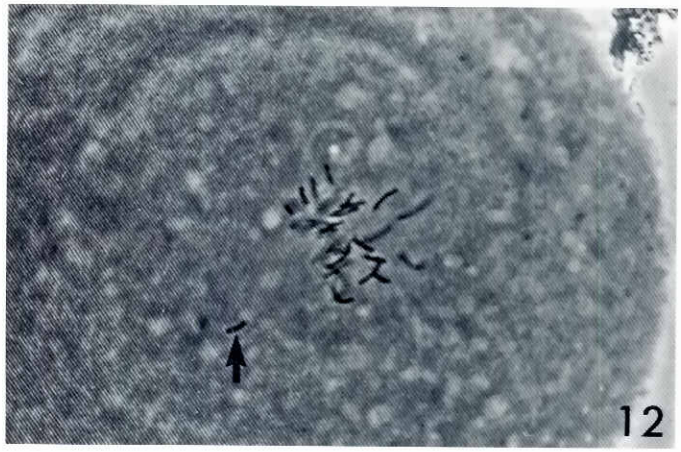
TABLE 1

METAPHASE PLATE ABNORMALITIES INDUCED BY THE FUSION OF M-PHASE CYTOPLASTS TO KARYOPLASTS PREPARED FROM STEM CELLS AND BLASTOMERES AT DIFFERENT STAGES OF THE CELL CYCLE

Karyoplasts		PCC morphology at metaphase								
Source	Cell cycle	Normal		Abnormal 1		Abnormal 2		Abnormal 3		Total Nr.
2-cell blastomere	G2	93	(48%)	75	(38%)	27	(14%)	–		195
4-cell blastomere	S	–		–		–		112	(100%)	112
4-cell blastomere	G2	90	(43%)	70	(33%)	21	(10%)	28	(13%)	209
Stem cell	ND	25	(20%)	6	(5%)	2	(2%)	92	(73%)	125

Normal: Normal metaphase spindle; Abnormal 1: Spindle with dispersed chromosomes; Abnormal 2: Chromosomes outside the spindle; Abnormal 3: Joined chromatin, fragmentation.





Figs. 11-12. Typical M-plate abnormalities observed after fusion of G2- four-cell stage blastomeres to cytoplasts. (11) *Chromosomes are dispersed along the spindle.* **(12)** *One chromosome (arrow) is detached from the spindle.*

G2- to MII-phase fusions

Karyoplasts at the G2-phase of the cycle were prepared from embryos in the second and third cleavage cycle. In both groups, nuclear membrane breakdown and premature condensation followed the uniform pattern outlined in «Changes in nuclear organization after G2- to MII-phase fusion» of this paper. However, detailed examination of the spindle organization showed that only 45% of the eggs reconstituted by fusion of G2-phase karyoplasts to MII-phase cytoplasts (183/404) contained morphologically normal metaphase spindles. The remaining eggs displayed spindle abnormalities (see Figs. 11, 12) which differed markedly from those of the S- to MII-phase fusion group and were characterized predominantly by randomly dispersed chromosomes on the spindle (36%: 145/404) or chromosomes detached from the spindle (12%: 48/404). The proportions of normal and aberrant metaphase spindles in reconstituted eggs prepared from G2-staged karyoplasts obtained from 2-cell or 4-cell eggs was similar. However, asynchronous rates of cell cycle progression resulted in a small percentage of blastomeres (13%) lagging in S-phase instead of progressing to the G2-phase by 12 h post cleavage.

Stem cell karyoplasts

Whole cells in this group were scattered randomly throughout the cell cycle. After undergoing chromatin condensation and nuclear membrane breakdown, 73% of spindles in stem cell-derived eggs showed abnormalities characteristic of S-phase fusions, while 27% were of the G2-type.

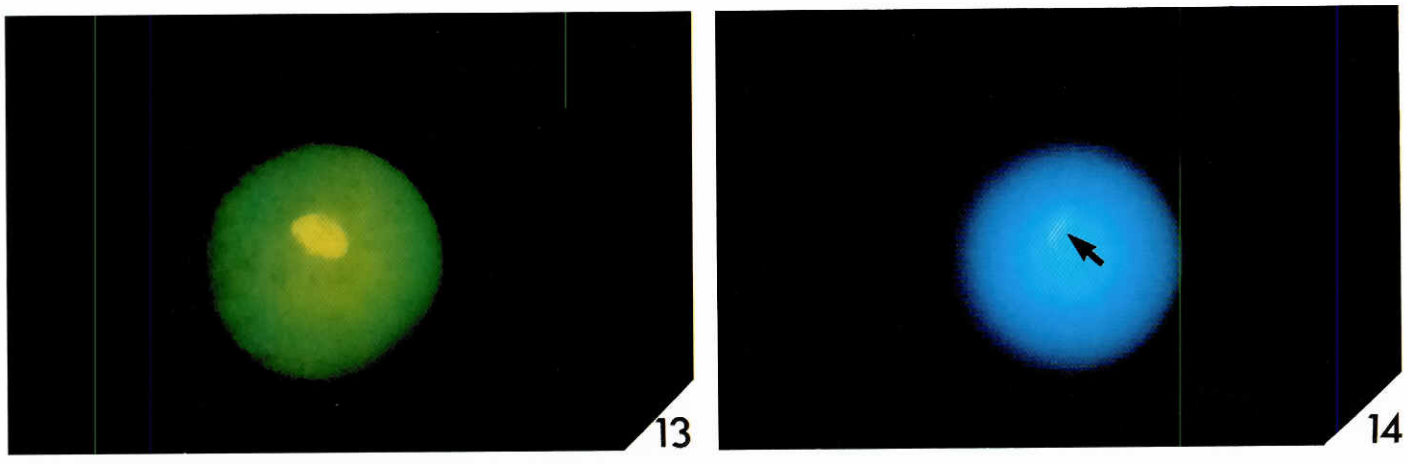
Spindle characteristics and post-activation lesions

Eggs from both the S- to MII-phase and G2- to MII-phase groups were stained with anti-tubulin antibodies in experiments designed to study spindle structure. Clear spindle formation was observed in 96% (50/52) of reconstituted eggs. No differences in spindle characteristics could be detected between the two groups of reconstituted eggs after tubulin staining (Figs. 13, 14). In addition, studies were carried out on chromosome movement to the spindle poles after activation. Abnormalities in the synchronous movement of chromosomes to the poles during anaphase was observed but no definitive pattern has yet emerged to enable us to correlate post-activation lesions with the different karyoplast-cytoplast combinations.

Discussion

It is generally accepted that MPF exerts a beneficial effect upon the remodelling of transplanted nuclei (First and Prather, 1991; DiBerardino and Hoffner-Orr, 1992). In the mouse, a particularly significant improvement in the results of nuclear transplantation is obtained when the transferred nuclei are exposed to MPF (Kono *et al.*, 1991, 1992). For farm animals the most commonly used protocol for nuclear transplantation involves the fusion of karyoplasts with metaphase II enucleated oocytes, and activation by an electric pulse at the time of fusion, when MPF levels are known to decline (Fulka *et al.*, 1992; Collas *et al.*, 1993). It is therefore inferred that prior to activation, the exposure of nuclei to MPF induces condensation of chromatin (Kanka *et al.*, 1991), and that decondensation recommences soon after as a result of the depletion of MPF. Whether or not the nuclei are remodelled solely by prolonged exposure to MPF is unknown. Czolowska *et al.* (1992) clearly demonstrated that nuclei derived from 8-cell stage blastomeres are not completely remodelled when fused to MII-phase oocytes and thereafter activated. On the other hand, Cheong *et al.* (1993) reported the birth of mice after transplantation of 8-cell stage nuclei to enucleated MII-oocytes. It is therefore possible that morphological analyses of nuclear remodelling do not reflect accurately the subsequent developmental capacity of reconstructed embryos. On the other hand it is equally possible that in the experiments by Cheong *et al.* (1993) complete morphological remodelling was in fact induced. However, since no details of post-activation nuclear configuration were provided by Cheong *et al.* (1993) and no developmental studies were undertaken by Czolowska *et al.* (1992), it is impossible at present to draw firm conclusions from these two studies. Nevertheless, from results obtained recently by us using both EM and autoradiographic analyses it is apparent that prolonged exposure to appropriate cytoplasmic conditioning can return a very advanced nucleus (from a stem cell) to a stage resembling morphologically the pronucleus (Fulka Jr. *et al.*, in preparation).

Our attempts to expose transplanted nuclei to MPF for prolonged



Figs. 13-14. Demonstration of spindle formation after nuclear transplantation. (13) *Well-formed spindle after tubulin staining.* **(14)** *Corresponding metaphase plate after Hoechst staining.*

periods revealed many defects which must be addressed in order to improve the efficiency of the nuclear transplantation procedure. It is immediately apparent that S-phase nuclei are unsuitable for this fusion-activation procedure: the morphology of PCC demonstrated many abnormalities which would be incompatible with further, normal development, confirming the results of Collas *et al.* (1992). Moreover, it is expected that another round of DNA synthesis will occur in such transplanted S-phase nuclei (Wilmut and Campbell, 1992). Nonetheless, it is interesting that these reconstituted oocytes receiving an S-phase nucleus can be activated, resulting in cleavage into two cells of equal size.

Our experiments reveal that many abnormalities occur even after fusion of G2 nuclei, probably arising as the formation of the spindle commences. In the early stages of chromatin condensation the chromatids appear quite compact, with no signs of dispersion. It remains to be determined whether, following activation, the chromatids are pulled regularly to the spindle poles, which would result in the normal karyotype of each nucleus, or irregularly, to give an unequal distribution between the two. The fate of chromosomes outside the spindle could not be determined. However, no subnuclei were observed in activated oocytes.

Under our conditions the fused cells cleaved into two equal daughter cells, a phenomenon which has not been described previously in nuclear transplants (Powell and Barnes, 1992). A major factor in determining the size of resultant daughter cells may be the interval of time between fusion and activation; an adequate interval is probably required to enable the spindle to move to a central position compatible with subsequent equal cell division. That the polar bodies described by Kono *et al.* (1992, 1993) were always larger than normal may therefore be due to the fact that in those studies the oocytes were activated at an early stage when the migration of the spindle was still incomplete.

It is deduced from these experiments that: (i) the cytoplasm of chemically-enucleated oocytes is physiologically equivalent, in nuclear transplantation, to mechanically-enucleated cytoplasm; (ii) for the transplanted nuclei to be exposed for prolonged periods to MPF, it is most suitable for them to be in the G2 phase, rather than the S-phase; and (iii) further modifications of nuclear transplantation, and critical evaluation of very early stages, are crucial to the understanding and increased efficiency of this technique.

Materials and Methods

The mouse oocytes, (F1 CFLP a/a), were enucleated exactly as described by Fulka, Jr. and Moor (1993). Briefly, after 6 h of culture oocytes are sequentially exposed to etoposide (50 μg/ml) and thereafter to medium containing etoposide and cycloheximide (50 μg/ml). This procedure yields fully enucleated oocytes with the entire chromosome complex expelled into the polar body. Oocytes displaying polar bodies were then washed thoroughly in normal medium, and cultured in that medium for 10-12 h before use for fusion. The embryos were collected from the same strain. The females were injected with 5-10 I.U. of PMSG and 44 h later with 5 I.U. of hCG, before being placed with fertile males. Successful mating was verified by the presence of a vaginal plug. Late 2-cell embryos were collected 48-50 h post hCG. Four-cell stage embryos in mid S-phase were collected approximately 5 h after cleavage from 2 to 4-cell stage; G2 4-cell stage embryos were obtained at 12 h post cleavage (Pratt, 1987).

The whole 4-cell stage blastomeres and stem cells were fused to enucleated oocytes. When 2-cell stage embryos were used, the karyoplasts were prepared as follows. The embryos were incubated in Pronase (0.5%) to dissolve their zonae pellucidae; immediately after this treatment they were incubated in Glycine-EDTA solution (Van Blerk *et al.*, 1991) to induce the dissociation of blastomeres which were then completely separated by gentle pipetting (the same procedure has been applied when 4-cell stage blastomeres were isolated). Two-cell stage blastomeres were then briefly incubated in medium supplemented in cytochalasin D (5 μg/ml) and thereafter quickly sucked into a very narrow pipette. This treatment results usually in the division of the blastomere into two parts. The part containing the nucleus was used for fusion. The enucleated oocytes and karyoplasts were incubated in a phytohemagglutinin solution (PHA, 300 μg/ml in PBS) and contact between them achieved using a narrow-bore pipette, whose diameter was slightly lower than that of both components closely apposed. Fusion was induced by a single pulse in isotonic glucose solution (1 kV/cm, 50 μsec). This treatment induced fusion in about 75-90% of treated cells. The cells were thereafter cultured in TC 199 (5% CO_2 in air, 37°C) before fixation in acetalcohol (acetic acid 1:ethanol 3) and staining with orcein. The activation of fused cells was induced in TALP Hepes medium by 4 pulses at 1 min intervals (0.75 kV, 50 μsec). In order to verify the presence of the spindle, some cells were labeled with rat monoclonal anti-tubulin antibodies using the following procedure. Briefly, the fixed cells were incubated for 30

min in PBS with 10% of goat serum and thereafter overnight (4°C) in PBS with antibody (1:40). Then they were washed in PBS and incubated for 30 min in anti-rat IgG (1:100), washed again and stained with Hoechst for 10 min before being mounted onto slides (glycerin:PBS, 1:1). The cells were evaluated under the Zeiss fluorescent microscope.

Media used for oocyte culture: TC 199 supplemented with Na-pyruvate (100 μg/ml), Gentamicin (25 μg/ml) and BSA (3 mg/ml:BDH) was used. All manipulations were performed in TALP HEPES (Bavister *et al.*, 1983). Unless otherwise stated, all chemicals were purchased from Sigma.

ES cell line and feeder cell line

The mouse ES cell, AB1, derived from a murine blastocyst of the 129 strain, and the CHO feeder cell line, SNL76/7, were kindly provided by Allan Bradley (Baylor College of Medicine, Houston, Texas). The ES cells were maintained in the undifferentiated state by culturing on feeder cells (as described by Robertson, 1987) in Dulbecco's modified Eagle's medium supplemented with 1 mM glutamine, mercaptoethanol, and 10% newborn and 5% fetal calf serum (both heat-inactivated). Prior to nuclear transplantation experiments, cultures of AB1 cells, in which no signs of differentiation into endoderm were apparent, were dissociated into single-cell suspensions using a solution of 0.25% trypsin (w/v) in 0.94% (w/v) EDTA (Robertson, 1987).

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