

Behaviour of blastomere nuclei fused to mouse oocytes is affected by oocyte enucleation and age

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Summary — The influence of oocyte age and presence of oocyte meiotic apparatus on the behaviour of introduced blastomere nuclei was evaluated. Blastomeres from 4-cell mouse embryos were fused to intact (metaphase II) oocytes, demi-oocytes (nucleate) or cytoplasm (anucleate). Fusion and simultaneous activation of the recipient oocytes were accomplished by a single electrical pulse at 20 or 24 h post human chorionic gonadotrophin (hCG) administration. The hybrids were fixed for evaluation 2 h after fusion. There was no difference in the behaviour of blastomere nuclei in whole oocytes and demi-oocytes. Most nuclei fused to the nucleate recipients at 20 h underwent breakdown of nuclear membrane (NMBD), chromosome condensation and consequently proceeded to telophase, in parallel with the resident meiotic chromosomes. Following fusion to cytoplasm, only a small portion of the blastomere nuclei underwent chromosome condensation and the vast majority (83%) of the nuclei remained in interphase. The influence of oocyte age on nuclear behaviour was assessed in oocyte–blastomere hybrids prepared by simultaneous fusion and activation at 20 and 24 h post-hCG administration. The introduced nuclei proceeded to telophase in 63% of the hybrids constructed at 20 h, but in only 28% of those constructed at 24 h. We conclude that nuclei introduced into aged or enucleated oocytes at the time of activation are predominantly remodelled in their interphase configuration.

mouse / oocyte / blastomere / hybrid / fusion / nuclear behaviour

Résumé — Le comportement des noyaux de blastomères fusionnés à des ovocytes de souris est affecté par l'enucléation de l'ovocyte et par son âge. L'influence de l'âge de l'ovocyte et de la présence de son appareil méiotique sur le comportement des noyaux de blastomères a été évaluée. Des blastomères d'embryons au stade 4 cellules ont été fusionnés à des ovocytes intacts (métaphase II) et des demi-ovocytes (nucléés) ou anucléés (cytoplastes). La fusion et l'activation simultanée des ovocytes receveurs ont été obtenues par une seule impulsion électrique à 20 ou 24 h après hCG. Les hybrides ont été fixés pour évaluation 2 h après fusion. On ne constate aucune différence dans le comportement des noyaux de blastomères dans les ovocytes, qu'ils soient entiers ou non. La plupart des noyaux introduits dans les receveurs nucléés à 20 h subissent une rupture de leur enveloppe et

un processus de condensation de la chromatine jusqu'à la télophase établissant un pendant avec les chromosomes méiotiques résidents. Après fusion avec des cytoplastes, seule une faible proportion des noyaux de blastomères subit une condensation chromosomique et le reste (83%) demeure en interphase. L'influence de l'âge de l'ovocyte sur le comportement nucléaire a été démontrée dans des hybrides ovocyte-blastomère préparés par fusion et activation simultanée à 20 et 24 h après hCG. Les noyaux introduits à 20 h atteignent le stade télophasique dans 63% des hybrides, seulement 28% par introduction à 24 h. Les noyaux introduits dans des ovocytes âgés ou énucléés au moment de l'activation sont généralement remodelés dans leur configuration interphasique.

ovocyte de souris / blastomère / hybride / fusion / comportement nucléaire

INTRODUCTION

It is well established that in maturing oocytes maturation promoting factor (MPF) is involved in controlling resumption of meiosis, and the progression to metaphase of the second meiotic division (M II) with arrest at M II until activation (Masui and Markert, 1971; Kishimoto, 1988; Procházka *et al*, 1989; Motlík and Kubelka, 1990; Parrish *et al*, 1992). Changes in MPF concentration affect the behaviour of foreign nuclei introduced into the oocyte. Nuclei exposed to high MPF levels, such as that in the oocyte arrested at metaphase, lose their nuclear envelopes, the chromosomes undergo condensation and become arranged on a mitotic spindle (Tarkowski and Balakier, 1980; Czołowska *et al*, 1984, 1986; Szöllösi *et al*, 1986a; Collas and Robl, 1991). On the other hand, nuclei that are introduced into already activated oocytes, in which MPF level has declined, preserve their nuclear envelopes and are remodelled in interphase configuration (Czołowska *et al*, 1984; Szöllösi *et al*, 1986b). However, if the nuclei are introduced during the metaphase II/telophase II transition, the nuclear membrane breaks down and the chromosomes become condensed; the chromosomes then decondense, the nuclear envelope is reassembled and the swollen nucleus assumes a pronucleus-like appearance (Szöllösi *et al*, 1986b; Szöllösi *et al*, 1988).

The above-described models of nuclear behaviour appear to be important for con-

struction of nuclear transplant embryos (NTE) since they affect DNA synthesis (Barnes *et al*, 1993; Campbell *et al*, 1993) and developmental potential (Collas and Robl, 1991) of the NTE. However, the results of several studies (Procházka *et al*, 1990; Liebfried-Rutledge *et al*, 1992; Hyttel *et al*, 1993) strongly suggest that the behaviour of embryonic nuclei in NTE is not controlled solely by the timing of activation and fusion. The present study was designed to assess influence of the presence of the oocyte meiotic apparatus and the oocyte age on the behaviour of blastomere nuclei introduced in oocytes at the time of activation.

MATERIALS AND METHODS

Oocytes and embryos were collected from 6–8-week-old female C57BL6 mice, superovulated with PMSG (Equinex, AYERST Laboratories, Montreal) and human chorionic gonadotrophin (hCG) (Sigma Chemicals, Saint Louis, MO). The oocytes were released into CZB medium (Chatot *et al*, 1989) buffered with 25 mM HEPES (HEPES-CZB) and freed of cumulus cells by brief incubation in hyaluronidase (50 IU/ml). The denuded oocytes were washed and stored in bicarbonate-buffered CZB medium, pending experimental use. Embryos at the 4-cell stage were flushed from oviducts 49–50 h after hCG administration, the zonae pellucidae removed with 0.5% pronase in HEPES-CZB and the blastomeres dissociated by gentle pipetting in Ca- and Mg-free phosphate-buffered saline (PBS). The individual blastomeres were stored in microdrops of CZB medium until transfer.

A pair of Narishige micromanipulators (Tokyo, Japan) mounted on an Olympus IMT-2 inverted microscope, equipped with Differential Interference Contrast optics was used for oocyte enucleation and blastomere transfer. Oocytes were immobilized on a holding pipette in a droplet of HEPES-CZB covered by paraffin oil and a slit was cut in the zona with a glass needle. Demi-oocytes and cytoplasts were prepared by withdrawing the first polar body and one third of the ooplasm through the slit into a pipette with an internal diameter of 35 μm . The withdrawn material was fixed for 10 min in acetic acid/ethanol and stained with 1% orcein to confirm enucleation, producing a cytoplast, or not, producing a demi-oocyte. Hybrids were constructed by fusing a blastomere, placed in the perivitelline space beneath the zona pellucida to an oocyte, cytoplast or demi-oocyte.

The pair to be fused was placed in a 200 μl droplet of fusion medium (0.275 M mannitol and 0.1 mM CaCl_2) between a pair of stainless steel wire electrodes 575 μm apart. The zone of membrane contact was aligned parallel to the electrodes by application of a 6 V, 600 kHz AC field for 5 s followed immediately by the activation/fusion stimulus, a 180 μs DC pulse of 1.75 kV/cm using a pulse generator (Electronics Lab, Agriculture and Agri-Food Canada, Ottawa). This pulse was found to stimulate fusion and also activation in > 85% of control oocytes of the age of 20 and 24 h post-hCG administration. The cells were transferred 1 min after stimulation into 100 μl droplets of CZB in 35 mm culture dishes, overlaid with paraffin oil, and incubated at 37°C in an atmosphere of 5% CO_2 in air for 2 h. After the culture period, the hybrids were fixed in acetic acid/ethanol 1:3, stained with 1% orcein in 45%

acetic acid, and the nuclear configuration determined by examination by phase contrast microscopy at 400 x. Experiments were done in 3 replicates.

The following types of hybrids were constructed (times are hours after hCG administration to oocyte donors): *Group 1*: blastomeres were fused with oocytes, demi-oocytes and cytoplasts at 20 h; and *Group 2*: blastomeres were fused with intact oocytes at 20 or 24 h. Spontaneous activation of non-manipulated and sham-manipulated oocytes was assessed with the aim of excluding its effect on nuclear behaviour in this experiment. The non-manipulated oocytes were fixed at 20 and 24 h post-hCG administration. In sham-manipulated oocytes, zona pellucida was opened and the oocytes exposed to fusion medium for 1 min. They were then cultured for 2 h and fixed.

RESULTS

Experimentally constructed hybrids of *Groups 1* and *2* are shown in figure 1 and the behaviour of their nuclei summarized in tables I and II. There was no difference between whole oocytes and demi-oocytes in their effect on introduced nuclei. This indicates that reduction in cytoplasmic volume does not affect nuclear behaviour.

Among the cytoplast-derived hybrids of *Group 1*, the vast majority (83.3%) of the introduced nuclei was found in interphase configuration (table I; fig 1b). The remain-

Table I. Configuration of blastomere nucleus in hybrids prepared by simultaneous fusion and activation at 20 h post-hCG administration.

Recipient cell	Number of hybrids	Metaphase (%)	Telophase (%)	Interphase (%)
Oocyte	42	7 (16.6)	23 ^a (54.8)	12 ^a (28.6)
Demi-oocyte	36	7 (19.4)	21 ^a (58.3)	8 ^a (22.2)
Cytoplast	37	6 (16.2)	0 ^b	31 ^b (83.3)

Values with different superscripts within the column are different ($P < 0.001$, χ^2 analysis).

ing hybrids contained a single mitotic spindle in their cytoplasm (fig 1a). The blastomere nuclei in 55–58% of the oocyte and demi-oocyte derived hybrids (*Group 1*) underwent nuclear membrane breakdown (NMBD) and proceeded to telophase concomitantly with resident meiotic chromosomes (fig 1c). Some hybrids contained both meiotic and mitotic spindles in metaphase

configuration, suggesting that the oocyte cytoplasm was not activated. The remaining hybrids contained the blastomere nucleus in interphase, always accompanied by meiotic chromosomes in telophase (fig 1d).

When the influence of the oocyte age on behaviour of introduced nuclei was assessed by constructing hybrids by simul-

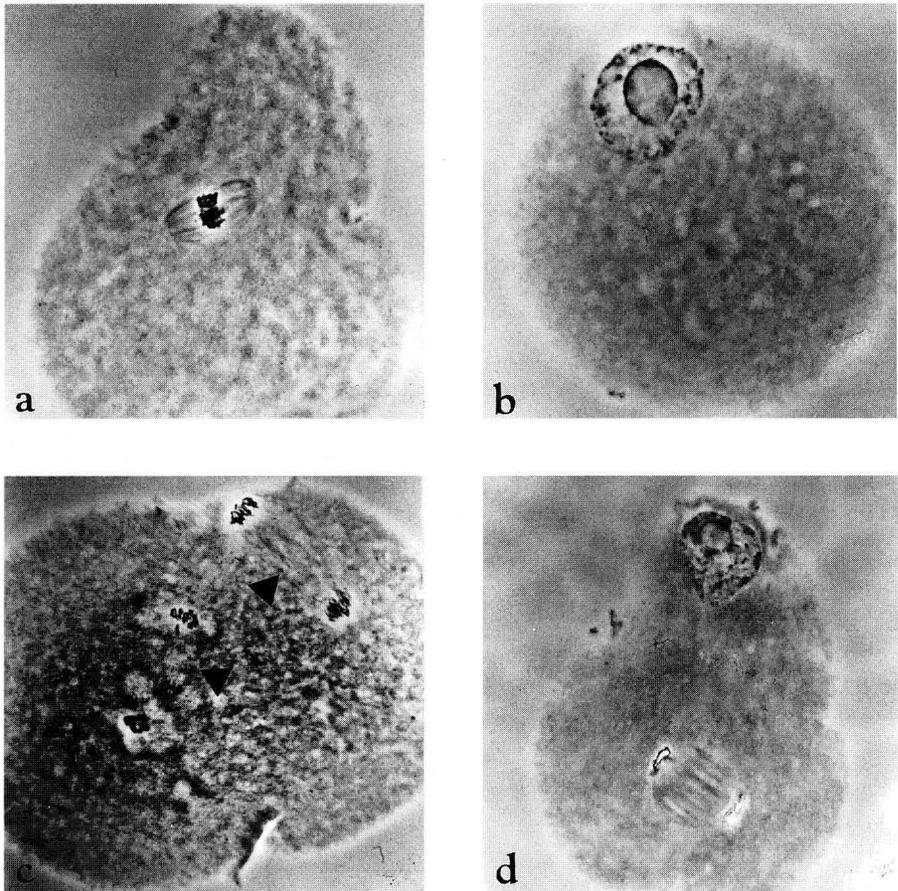


Fig 1. The nuclear configuration of hybrids 2 h after inducing fusion/activation: (a) cytoplasm–blastomere hybrid: donor nucleus in metaphase configuration (the shape of cell was distorted by the escape of cytoplasm through a slit in the zona during mounting); (b) cytoplasm–blastomere hybrid: donor nucleus in interphase configuration; (c) oocyte–blastomere hybrid: host and introduced nucleus in telophase (arrowheads); (d) oocyte–blastomere hybrid: blastomere nucleus in interphase, host nucleus in telophase; x 400.

Table II. Configuration of blastomere nucleus in hybrids prepared by simultaneous fusion and activation at 20 or 24 h post-hCG administration.

<i>Time of fusion and activation (hours post-hCG)</i>	<i>Number of hybrids</i>	<i>Metaphase (%)</i>	<i>Telophase (%)</i>	<i>Interphase (%)</i>
20	54	8 (14.8)	34 (63.0)	10 (12.2)
24	60	6 (10)	17 (28.3)	37 (61.7)

Results at 20 and 24 h differ significantly ($P < 0.001$, χ^2 analysis).

taneous fusion and activation at 20 and 24 h post-hCG administration (*Group 2*; table II), both host and introduced nuclei underwent NMBD and reached telophase in 63% of hybrids constructed at 20 h, but in only 28% of those constructed at 24 h. Activation rates of non-manipulated oocytes isolated at 20 and 24 h post-hCG administration were 9.2% (12/131) and 13.6% (16/118), respectively. Activation rates of sham-manipulated oocytes were 12.8% (5/39) and 18.6% (8/43) respectively, for 20 and 24 h post-hCG oocytes.

DISCUSSION

The present data show that behaviour of nuclei fused to metaphase oocytes simultaneously with activation is greatly affected by the resident meiotic apparatus. Most nuclei introduced into nucleate (oocyte and demi-oocyte) and relatively young (20 h post-hCG) host cells underwent chromosome condensation, formed telophase spindles and regularly extruded half of the chromosomes into an additional polar body. In contrast to the nucleate host cells, fusion of blastomere nuclei to cytoplasts did not lead to the breakdown of nuclear envelope and chromosome condensation. Different processing of the donor nucleus in demi-oocytes and cytoplasts, which were both

subjected to enucleation procedure, clearly shows that the absence of nucleus breakdown in cytoplasm–blastomere hybrids was not due to precocious activation of micro-manipulated oocytes. Our results rather indicate that in cytoplasts MPF became inactivated during the approximately 20 min required for fusion, so that it was unable to induce chromosome condensation. It has been demonstrated in the mouse that the chromosome condensation factors are predominantly localized on meiotic apparatus of the maturing and M II oocytes (Balakier and Masui, 1986; Czolowska *et al*, 1986). Moreover, in somatic cells in mitosis, the bulk of MPF is localized on structures of the mitotic apparatus, mainly on the centrosome (Bailly *et al*, 1989; Riabowol *et al*, 1989). Enucleation may thus deprive the oocytes of a substantial amount of MPF. The remaining MPF, yet further decreased by ongoing activation, is not sufficient to disassemble the nuclear membrane of the donor nucleus. The stoichiometric nature of the reaction of MPF with the nuclear structures and its affect on induction of the nucleus disassembly have been well established (Newport and Spann, 1987).

Allowing the recipient oocytes an additional 4 h of aging *in vivo* prior to fusion/activation resulted in a significant decrease in their ability to induce chromosome condensation in the foreign nuclei. The significant

loss of the condensation activity cannot be explained by spontaneous activation of aged oocytes, as resulted from our experiments. As MPF is responsible for this chromosome condensation activity, it is obvious that its concentration or stability weakens in aging mouse oocytes. A good candidate for the protein responsible for stabilizing MPF activity in matured oocytes is a product of *c-mos* protooncogene, *p39^{c-mos}* (see Masui, 1991, for review). This protein has been identified in maturing and metaphase II arrested *Xenopus* and mouse oocytes (Sagata *et al*, 1988; O'Keefe *et al*, 1989; Paules *et al*, 1989; Nishizawa *et al*, 1992). One of its proposed functions was to prevent cyclin B molecules from degradation *via* ubiquitin-mediated proteolysis. Thus, the activity of MPF would be preserved since cyclin B is a major component of MPF and its degradation is a prerequisite for MPF inactivation (Draetta *et al*, 1989; Murray *et al*, 1989; Gautier *et al*, 1990). Recent results, however, suggest that *p39^{c-mos}* may not be the only molecule involved in stabilization of MPF since its level remains high after completion of second meiotic division in activated mouse and *Xenopus* oocytes (Lorca *et al*, 1991; Watanabe *et al*, 1991; Weber *et al*, 1991). Further experiment are thus needed to elucidate mechanisms disturbed in aging mammalian oocytes.

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