Somatic and embryonic cell nucleus transfer into intact and enucleated immature mouse oocytes

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BACKGROUND: The aim of our study was to evaluate the possibility of embryonic or somatic cell haploidization after fusion with intact or enucleated immature oocytes which were subsequently cultured in vitro. Embryonic or somatic cell nuclei do not undergo premature chromosome condensation when fused to intact or enucleated immature oocytes whose maturation is prevented by dibutyryl cyclic AMP (dbcAMP). The presence of dbcAMP permits, however, the completion of DNA replication in somatic cell nuclei. METHODS AND RESULTS: The chromosomes condensed when the reconstructed cells were released from the dbcAMP block. When somatic or embryonic nuclei were introduced into intact immature meiotically competent oocytes and subsequently cultured their chromosomes assembled on a common spindle with meiotic chromosomes and proceeded through the meiotic-like division, judged according to the presence of the first polar body extruded. When embryonic cell nuclei were introduced into cytoplasts obtained from immature meiotically competent oocytes, polar bodies were extruded in about 75% of reconstructed cells but the metaphase plates were abnormal in almost all cases. When somatic cell nuclei were inserted into the above cytoplasts, polar bodies were extruded only very exceptionally and in these cells chromosomes were arranged in abortive metaphase plates. CONCLUSIONS: Our results suggest that somatic cell nuclei are unable to proceed through the reduction division (haploidization) when introduced into an immature oocyte meiotic cytoplasm.

Key words: haploidization/mouse/nuclear transfer/nucleus/oocyte

Introduction

It is proposed that the haploidization of patient somatic cell diploid chromosome complement, within enucleated donor oocytes, may result in the production of cells with half the number of chromosomes which could then be used as gametes with their own genetic identity (male, female) for the treatment of certain forms of infertility (Tsai et al., 2000; Tesarik et al., 2001; Trounson, 2001). This assumption is based on previous observations studying the nucleocytoplasmic interactions in fused oocytes and also the behaviour of embryonic or somatic cell nuclei introduced into a meiotic cytoplasm. It was shown that nuclei always respond to the given cytoplasmic chromosome condensation signal; however, for the correct cell cycle transition the corresponding nucleus cell cycle stage is very important (Fulka et al., 1993, 1995a). When meiotic cell nuclei, which are still diploid, are introduced into immature or maturing oocytes, the cytoplasm induces the reduction division of their chromosome complement. The cells with a haploid chromosome number are able to support the complete embryonic development. It was demonstrated that somatic cell nuclei introduced into immature mouse oocytes underwent the meiotic-like division, albeit without the expulsion of the first polar body (Kubelka and Moor, 1997). Thus in some cells two groups with a half number of chromosomes were observed. Basically two experimental schemes may result in the production of haploidized somatic cells. First, the somatic cell G2 stage nuclei will be introduced into an immature oocyte cytoplasm and subsequently cultured in vitro up to the second metaphase. Following the oocyte activation a haploid pronucleus could theoretically only be formed when the fully replicated originally paternally and maternally derived homologous chromosomes from the diploid nucleus separated faithfully with unseparated sister chromatids to opposite spindle poles in the absence of a physical attachment by chiasmata. The second scheme proposed the introduction of G1 stage nuclei into a mature oocyte (MII) cytoplasm. After oocyte activation the second polar body will be extruded and the oocyte cytoplasm may contain a haploid chromosome set (Kubiak and Johnson, 2001), provided the unreplicated and unattached two homologous chromatids that were originally derived from the parents segregate from each other. It must be assumed, however, that meiotic and mitotic divisions are completely...
different. During the early meiotic stage (zygotene), homologous chromosomes pair through the synaptonemal complex and undergo recombination (pachytene). The crossing-over, which can be seen as chiasmata, then holds the paired homologues together (Simchen and Hugerat, 1993). In meiosis, during the first division, homologues move to opposite spindle poles (reductive segregation) and, at the second division, sister chromatids move to opposite poles as during mitosis (Kleckner, 1996). Also the attachment of meiotic or mitotic chromosomes to the spindle differs. In the first meiotic division sister chromatid kinetochores lie side by side and thus they attach to microtubules from the same pole. In the second meiotic division kinetochores lie back to back and they attach to microtubules from opposite poles, similarly to mitotic chromosomes (Paliulis and Nicklas, 2000). Thus the physical connection and the chromosome position on the spindle seem to be crucial for the successful progress through the final stages of oocyte meiotic maturation. In our experiments, we have studied the first suggested scheme in the mouse model. Our results show that the haploidization through this approach was unsuccessful.

Materials and methods

Mouse oocytes were released into M2 medium containing dibutyryl cyclic AMP (dbcAMP) (150 µg/ml) from large antral follicles of ICR females stimulated previously with 5 IU of pregnant mare serum gonadotrophin (PMSG). Their cumulus cells were removed by pipetting and only those oocytes containing distinct germinal vesicles (GV) were used. Zonae pellucidae were removed by pronase treatment (0.5%). For enucleation, oocytes were incubated in M2 supplemented with dbcAMP, cytochalasin D (5 µg/ml) and nocodazole (3 µg/ml) for 30 min. Thereafter they were elongated in a very narrow pipette and the oocyte part containing GV was cut off with a glass needle (Karnikova et al., 1998). Nuclei (karyoplasts) or whole somatic cells (diaffected cells (trypsin-EDTA) from cumulus cell cultures) were agglutinated with either intact or enucleated oocytes in phosphate buffered saline (PBS) supplemented with phytohaemagglutinin (200 µg/ml), washed in M2 and transferred into polyethylene glycol solution (PEG, relative molecular weight 1000) for 50 s (Fulka et al., 1995b). Then they were washed several times in M2 and cultured in medium M199 containing bovine serum albumin (BSA) (4 mg/ml), Na-pyruvate (0.2 mmol/l) and gentamicin (50 µg/ml) in an atmosphere 5% CO₂ in air and 37°C for up to 14–16 h. The efficiency of fusion was evaluated 30 min post-induction and those cells which did not fuse were discarded. At the end of culture the fusion products were evaluated under the inverted microscope, then fixed in acetalcohol and stained with aceto-orcein. In order to assess if somatic cell replicate DNA when fused to immature oocytes bromodeoxyuridine was added to the culture medium (Ouhibi et al., 1994). Oocytes were then fixed in methanol, labelled with anti BrDU antibody (Dako, Glostrup, Denmark) and examined under the fluorescence microscope. Unless otherwise stated all chemicals were purchased from Sigma.

Results

In total 127 (efficiency 127/165; 77%) somatic cells were fused to intact GV staged oocytes, 132 somatic cells to 195 enucleated oocytes (68%), 27 embryonic karyoplasts to 37 intact GV staged oocytes (72%) and 30 embryonic karyoplasts to 42 enucleated oocytes (71%; Table I). When somatic cells are fused to intact or enucleated oocytes and the fusion products are kept in medium with dbcAMP the introduced nuclei remained intact (Figure 1). This situation persists as long as dbcAMP is present in the medium but nuclei slightly increase their diameter (Figure 2).

We have assumed that for successful haploidization the nuclei introduced into oocytes must be at G2 phase. First we labelled somatic cell cultures with BrDU to assess the percentage of cells in S-phase. After evaluation we found 51% of cells with positive labelling (51/100). Thus we assumed that only a minimum of cell nuclei will be in G2 phase when randomly selected. For this reason another strategy was chosen. The randomly selected somatic cells were fused to intact or enucleated GV staged oocytes and cultured for 24 h in the medium with dbcAMP and BrDU and thereafter processed for fluorescence microscopy. When evaluated, 48% of fused cells showed positive labelling (27/56). However, when BrDU was added after 24 h of culture only two cells from 52 showed positive signal. These results indicate that the immature oocyte cytoplasm does not prevent the replication of DNA and within a 24 h lasting culture somatic cell nuclei can be synchronized in G2 phase. This has been further confirmed according to the metaphase chromosome morphology as already described (Rao et al., 1977).

When released from dbcAMP block the meiotic cell cycle progression is under the control of the oocyte cytoplasm. This means that the nuclear envelope breakdown and chromosome condensation is typically detected within 1 h of culture in dbcAMP free medium in both intact and enucleated fused cells. The first meiotic spindle could be clearly detected after 6 h of culture. Typically, only one spindle was detected in cells produced by fusion of a somatic cell to an intact oocyte. This spindle contained both the meiotic and mitotic chromosomes resulting from the unification of both groups.

When evaluated after 14–16 h of culture in dbcAMP free medium 31% of reconstructed oocytes (somatic cell×intact oocyte) extruded the first polar body (40/127; Table I); however, the evaluation of the second metaphase plates was extremely difficult as they contained both the meiotic and mitotic chromosomes. We may however assume that they were mostly abnormal. This assumption came from the evaluation of those oocytes which did not extrude the first polar bodies. While the organization of meiotic chromosomes was almost exclusively

<table>
<thead>
<tr>
<th>Type of fusion</th>
<th>Total no. cells fused</th>
<th>No. of oocytes with polar bodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sc×intact GV oocyte</td>
<td>127/165 (77%)</td>
<td>40/127 (31%)</td>
</tr>
<tr>
<td>Sc×enucleated GV oocyte</td>
<td>132/195 (68%)</td>
<td>2/132 (1%)</td>
</tr>
<tr>
<td>Bn×intact GV oocyte</td>
<td>27/37 (72%)</td>
<td>20/27 (74%)</td>
</tr>
<tr>
<td>Bn×enucleated GV oocyte</td>
<td>30/42 (71%)</td>
<td>23/30 (77%)</td>
</tr>
</tbody>
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GV = germinal vesicle; Sc = somatic cell; Bn = blastomere nucleus.
Figure 1. Somatic cell nucleus introduced into an enucleated germinal vesicle (GV) stage mouse oocyte. Fixed 1 h post-induction of fusion. Phase contrast, ×600.

Figure 2. Somatic cell nucleus introduced into an enucleated GV stage mouse oocyte. Fixed 20 h post-induction of fusion. Note the nucleus enlargement and a well visible nucleolus. Phase contrast, ×600.

Figure 3. Mouse oocyte metaphase I spindle with an equatorial arrangement of meiotic chromosomes while the mitotic chromosomes are located on spindle poles. Phase contrast, ×600.

Figure 4. Mouse anaphase–telophase I oocyte with somatic cell chromosomes located outside the spindle (arrow). Phase contrast, ×600.

Figure 5. Enucleated mouse oocyte fused to somatic cell and matured for 16 h. Note that in this case the first polar body was extruded but the metaphase II plate is evidently abnormal (arrow). Phase contrast, ×600.

Figure 6. Enucleated mouse oocytes fused to somatic cells and thereafter matured in vitro did not extrude the first polar body and are arrested in metaphase I-like stage with chromosomes dispersed chaotically on the spindle. Phase contrast, ×600.
normal, the somatic chromosomes were allocated, in most cases, outside the spindle (Figures 3, 4). The incompatibility of a meiotic spindle and mitotic chromosomes was even more evident when somatic cells were fused to enucleated oocytes. Here only two oocytes (2/132) extruded the first polar bodies (Table I). When examined after staining, the chromosomes remaining in the cytoplasm were rather abnormal and formed a cluster of chromatin (Figure 5). The oocytes without polar bodies were also stained and evaluated. Figure 6 shows the most typical configuration of chromatin where chromosomes are randomly allocated on the spindle.

Next the behaviour of early embryo chromosomes in a meiotic cytoplasm were evaluated. Nuclei were isolated from two-cell stage embryos on the next day after the detection of a vaginal plug because it is known that they are G2-phase staged. This experiment was designed to exclude the possibility that our somatic cell cultures have an adverse effect on cultured cells. Fused cells were cultured with dbcAMP for 1 h and thereafter in an inhibitor free medium for 14–16 h. In general, the frequency of oocytes with polar bodies was evidently higher compared with somatic cell fusion—in both groups >70% of reconstructed cells exhibited the polar body—intact oocyte×embryonic karyoplast (20/27); enucleated oocyte×embryonic karyoplast (23/30; Table I). These polar bodies were only slightly smaller than the oocyte cytoplasm. When these cells were evaluated after staining, again the configuration of mitotic chromosomes showed gross abnormalities which were typically seen as a cluster or patches of chromatin. In conclusion these results show rather the inability of the mitotic cell nucleus (chromosomes) to undergo haploidization in a meiotic cytoplasm. This resulted typically in an abnormal allocation of the chromatin on the meiotic spindle and thus the inability to secure the proper separation of mitotic chromosomes.

Discussion

The possibility of somatic cell nucleus haploidization in the meiotic cytoplasm has been suggested in several articles. Our results, however, showed that under the experimental scheme and conditions used, the expected haploidization was not possible. The abnormal organization of chromosomes on the first meiotic spindle was the main problem when somatic cells were transferred into immature cytoplasts. The chromosomes only exceptionally formed the regular metaphase plate, instead they were typically arranged along the whole spindle. This abnormal organization prevented the anaphase to telophase I transition. Only in two cases were the second metaphases detected. The reason for the improper chromosome organization is not known. It is, however, interesting that the same phenomenon was already observed when early metaphase I oocytes were fused to anaphase-telophase I oocytes. Here, too, the anaphase–telophase chromosomes were dispersed on their original spindle. Similar abortive organization or dispersion was documented in some cases (Tarkowski and Balakier, 1980; Grabarek and Zernicka-Goetz, 2000) when maturing mouse oocytes were fused to G2 phase blastomeres or to follicular cells, and some pictures from the article published by Kubelka and Moor also resemble the situation commonly observed in our fused cells (Kubelka and Moor, 1997).

The abnormal chromosome organization on the first meiotic spindle is not easy to explain; however, some recent results indicate that the absence of chromosome synopsis plays a crucial role. In mouse oocytes from animals homozygous for a targeted disruption of the DNA mismatch repair gene Mlh1, the absence of MLH1 protein dramatically reduces the meiotic recombination. The chromosomes in maturing oocytes are present as univalents and are unable to establish the correct spindle attachment (Woods et al., 1999). Also the absence of Spo 11p results in the defects of chromosome synopsis and a random segregation at meiosis I (Lichten, 2001). The mouse meiotic mutation mei1 disrupts chromosome synopsis but some oocytes progress to metaphase I; their chromosomes are, however, unpaired and not properly organized on the spindle (Libby et al., 2002). These results indicate that the meiotic recombination ensures the correct attachment and segregation of chromosomes during meiosis and is essential for its progression, but certainly some other factors may play an important role in the chromosome spindle arrangement and subsequent segregation (Bernard et al., 2001; Kaplan et al., 2001). On the other hand, when grasshopper spermatocytes in metaphase I were fused to spermatocytes in metaphase II and a single chromosome was moved from one spindle to the other, chromosomes placed on the spindle of a different meiotic division behaved as they do on their native spindle. Thus metaphase II chromosomes attached to the metaphase I spindle and in anaphase I individual chromatids were separated (Palilulis and Nicklas, 2000). This phenomenon has been observed also in fused metaphase I to metaphase II mouse oocytes (Fulka et al., 1995a). However, in both these cases the chromosomes in fused cells still belong to a category of ‘meiotic chromosomes’. It may be possible that chromosomes in mitotic cells are further modified and thus incompetent to undergo the proper congression and attachment to the spindle.

The frequency of polar bodies extruded was higher when G2 blastomere nuclei were introduced into immature cytoplasts, but the resulting metaphase plates were again abnormal. This higher frequency may be influenced by the absence of cell cycle checkpoint controls (Fulka et al., 2000).

It is not surprising that, after fusion of either somatic or embryonic cells to intact oocytes, polar bodies were frequently observed. It has been shown recently (Fulka et al., 1997; Rieder et al., 1997) that the cell cycle progression in oocytes with two chromosome groups (spindles) is under the control of the more advanced (or normal) spindle. In the mouse both groups of chromosomes form a single common spindle, on the other hand both spindles are separated in fused pig oocytes (Fulka, 1983) and also in bovine oocytes. Thus, Salamone et al. postulated the successful haploidization of somatic cells fused to GV stage bovine oocytes (Salamone et al., 2001, 2002).

It is interesting to note that the behaviour of meiotic cells injected or fused to immature or maturing oocytes is completely different. Normal metaphase plates are frequently formed and the number of chromosomes is reduced (Ogura et al., 1998;
when somatic cells were fused to post-metaphase I oocytes, it was shown that the compatibility between this type of cytoplasm and a somatic cell is much better and newly formed metaphase plates seem to be normally organized (unpublished results). This is supported by earlier studies when G2-phase blastomere nuclei were introduced into chemically enucleated oocytes (post-telophase I). Here the metaphase plates were normal and chromosomes segregated properly into their sister chromatids (equatorial division). Other studies claimed the successful haploidization of somatic cells after the injection of their nuclei into mature oocytes which were subsequently activated (Lacham-Kaplan and Daniels, 2001; Tesarik et al., 2001). It must be stressed that a meiotic division is not simply a condensation or movement of chromosomes. The first meiotic division requires the pairing and separation of homologous chromosomes; during the second meiotic division the equal distribution of corresponding chromosomes was confirmed (Lacham-Kaplan and Daniels, 2001; Tesarik et al., 2001). In order to obtain a haploid somatic cell, however, a second meiotic division was required. This was achieved in two different ways: by injection of nuclei into oocytes from the same species (Sasagawa et al., 1996, 2001; Tesarik et al., 2001). The second meiotic division requires the pairing and separation of homologous chromosomes; during the second meiotic division the equal distribution of corresponding chromatids must be secured. Our observations suggest that the haploidization of somatic cell by their transition through the ‘whole’ meiotic cell cycle was unsuccessful due to intrinsic characteristics of somatic chromosomes.

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References


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