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體細胞核轉移胚胎及幹細胞 DNA 甲基化及基因表現之研究

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中文摘要

將體細胞的核轉植至去核之卵子(複製)，卵子之細胞質具有將核重新設置的能力，並支持胚胎細胞之繼續分裂與成長，但其效率並不高。我們將老鼠體細胞注射於老鼠未去核成熟卵子內(半複製)，發現會形成紡綞體，並進行類似染色體減數分離，但染色體分離之情形並不正常，另外其基因上修飾可能異常，而形成之胚胎發育能力差，因此半複製之胚胎於體外及體內之成長皆受此兩大因素的影響。
關鍵詞：核轉植，未去核成熟卵子，紡綞體，染色體。

Abstract

We investigated haploidization of somatic cell nuclei in non-enucleated mature oocytes regarding spindle formation, chromosomes, and developmental potential. Mouse cumulus cells were injected into metaphase II oocytes. Some injected oocytes were examined for morphological changes of chromosomes and spindle immediately, at 30 min, 1h, or 2 h after the injections. The remaining oocytes were activated by Sr^{2+} after various incubation periods and observed for formation of second polar body and pseudo-polar body. Cytogenetic analysis was performed for some of the resulting zygotes. The progress to blastocysts in vitro and the possibility of conception in vivo were assessed. Immediately after injection, the cumulus cell nucleus was still in interphase without spindle formation. The presence of premature chromosome condensation (PCC) and spindle formation increased when the incubation time increased. The percentages of activated oocytes increased with the incubation time after nuclear transfer, but the difference was not significant between 1h (58%) and 2 h (62%). The incidence of chromosomal aberrations was high for the derived embryos. Development in vitro was poor, and no procreation of pups occurred after transfer of the 324 embryos. The PCC and spindle formation induced by cumulus cell nuclei in mature oocytes was time-dependent, as was the chance for successful activation. The chromosomal abnormalities from segregation errors presented one obvious cause apart from the potential epigenetic defects of developmental failure for the semi-cloned embryos.

Introduction and purpose

The possibility to create offspring using haploidization of somatic cell nuclei in mature oocytes (reproductive semi-cloning) has remained elusive (Tesarik *et al.*, 2001; Lacham-Kaplan *et*

al., 2001; Tateno *et al.*, 2003). Tesarik *et al.* (2001) proposed that the cytoplasm of metaphase II (MII) oocytes was able to drive G0/ G1 somatic cell nuclei to premature M-phase without previous S-phase. They claimed that after activation, segregation of the two sets of parental homologous chromosomes and production of pseudo-second polar body could occur in the absence of recombination. They performed fluorescence in-situ hybridization (FISH) for two pseudo-second polar bodies extruded after injection of cumulus cells to mature human oocytes. The results revealed normal haploid chromosome complements for 13, 18, 21, X, and Y.

Using a confocal microscope, Lacham-Kaplan *et al*. (2001) analyzed seven mouse oocytes activated with cumulus cells and showed separation of cumulus cell chromosomes into two sets. One of these sets extruded into a pseudo-second polar body. They transferred 20 semi-cloned embryos to foster females; however, none of them developed to live pups. The process of haploidization, accuracy of chromosomal segregation, and developmental potential of the resulting embryos needs further investigation.

Microtubules are crucial for the events following activation of oocytes as completion of meiosis and second polar body formation. Defects in meiotic spindle formation may be one of the reasons causing failure of normal chromosomal division and termination of subsequent development. In this study, we used a mouse model to evaluate changes of microtubular spindle and chromosomes of somatic cell nuclei after injection into non-enucleated mature oocytes. The relationship between the timing of activation and activation outcome was investigated. Cytogenetic analysis of the resulting embryos was performed. We explored the development of semi-cloned embryos in vitro and their pregnancy potential in vivo.

Materials and methods

Preparation of oocytes

Each female C57BL/6 x DBA/2 F1 hybrid mouse (8 to 12 weeks old) was injected intraperitoneally with 5 IU of pregnant mare serum gonadotrophin. Forty-eight h later, 5 IU of human chorionic gonadotrophin was administered to induce ovulation. Fourteen h later, oocytes were collected in CZB medium. Cumulus cells were dispersed by medium containing 80 IU/ ml hyaluronidase. Mature oocytes with first polar body were picked up for experiments.

Preparation of cumulus cells

Cumulus cells were washed in CZB medium using centrifugation at 300 g for 5 min, and the pellet was kept and suspended in 0.1 ml medium. One µl of cell suspension was mixed with 3 µl of 12% polyvinylpyrrolidone (PVP) in Hepes-buffered CZB medium. Cumulus cells from one mouse were used for injection of oocytes from another

mouse.

Injection of cumulus cells into oocytes

The needle holder for injection was attached to a piezo electric actuator that was driven by a controller. Because mouse oocytes were vulnerable to injection at 37 ^{o}C, we performed this procedure at room temperature (Wakayama *et al.*, 1998). The cumulus cells were injected one by one into oocytes with minimal accompanying medium. During the same period, control oocytes were also placed on the dish. They were then transferred back to CZB culture medium.

Fluorescent staining of chromosomes and spindles

In the first experiment, the morphologically surviving oocytes were examined for chromosomes and spindles immediately, at 30 min, 1h, or 2 h after injection. Some injected oocytes were incubated for 2 h and then activated. At 3 h of activation, they were examined for the status of chromosomes and microtubules. Fixation and staining has been described in detail previously (Chen *et al.*, 2000).

Activation of oocytes with Sr^{+2}

In the second experiment, oocytes were activated immediately, at 30 min, 1h, or 2 h after injection using Ca^{2+} and Mg^{2+}-free CZB medium containing 10 mM Sr^{+2} for 6 h. Some mature oocytes without injection were exposed to the above medium with 5 μg/ ml cytochalasin B as parthenogenetic controls. After activation, they were washed and transferred to CZB medium.

Definition of normal activation

The presence of two pronuclei with a second polar body and a pseudo-second polar body in oocytes was defined as normal activation. Those with one or three pronuclei as well as two pronuclei with one second polar body were thought to be abnormal. For the parthenogenetic control specimens, the oocytes with two pronuclei without extrusion of second polar body were selected for further culture.

Development in vitro

We observed the normally activated oocytes and parthenogenetic controls daily for 5 days. Development from the 2-cell stage to blastocysts was recorded. Blastocysts were fixed on slides individually. After staining with Hoechst 33258, the number of nuclei was calculated as the total cell counts under the fluorescent microscope.

Transfer to pseudopregnant female mice

In the third experiment, oocytes were activated at 2 h after injection. Embryos at the 2-cell stage from normally activated oocytes were transferred to oviducts of ICR females which had been mated with vasectomized ICR males 1 day previously. Recipient females were cesarean sectioned on the 20th day post-coitum and their uteri were examined for implantation sites or fetuses.

Cytogenetic analysis of embryos

For the fourth experiment, some normally activated oocytes were incubated in CZB medium with 1 µg/ ml colchicines for 13 to 14 h to enforce arrest at the metaphase of the first mitotic division. Each oocyte was fixed on a slide. Some zygotes from natural conception or parthenogenesis were treated as described for controls. Chromosome numbers were counted after the Wright's stain using the microscope with a magnification of 1000X.

Micronuclei assessment

A part of the normally activated oocytes were cultured in CZB medium to the 2-cell stage, and then transferred to medium containing Hoechst 33258 (20 µg/ ml) for 1 h. They were observed for the presence of or absence of micronuclei under the fluorescent microscope. Some two-cell embryos from natural conception or parthenogenesis were examined as the control specimens.

Results

In the first experiment, 356 (85%) of 419 oocytes survived from injection of cumulus cells. Immediately after injection, the cumulus cell nucleus was still in interphase without spindle formation. The percentage of PCC with spindle at 1 h after injection was significantly greater than at 30 min after injection. There was no difference in rate of PCC between 1h and 2 h after injection. The prematurely condensed chromosomes with spindle of all 176 oocytes observed at various incubation time displayed disorderly arrangements. At 3 h during activation, both of the nuclei of an oocyte and a cumulus cell were in the process of division in the presence of microtubules in 24 of 40 injected oocytes. Forty-eight mature oocytes were activated only after 3 h. Although we injected cumulus cells into these oocytes, the interphase nuclei of cumulus cells remained unchanged without spindle formation (0/ 48) 2 h after injection.

In the second experiment, we injected 1094 oocytes of which 952 (87%) survived. The percentage of normal activation was significantly increased for those injected oocytes activated after 1-2 h of incubation compared to those activated immediately or at 30 min. The differences were not significant for the former two groups. The cleaving capability of semi-cloned embryos to the 2-cell stage was not different from parthenogenetic control specimens. However, development to 4-cell, 8-cell to morula, and blastocyst stages was significantly impaired for semi-cloned embryos, compared with the parthenogenetic control specimens. Cleavage of semi-cloned embryos was not different among the four activated groups. The mean number of nuclei of blastocysts was not different among the 30 min (47 ± 11, n= 6), 1 h (49 ± 10, n= 22), and 2 h (50 ± 12, n= 24) groups, but that was significantly lower than control specimens (104 ± 12, n= 55).

In the third experiment, we injected 642 oocytes and activated them after 2 h incubation. In the 578 (90%) surviving oocytes, 341 (59%) reached normal activation and 324 (95%) cleaved. After transferring the 2-cell semi-cloned embryos to foster ICR mothers, no implantations or live births were achieved.

For the fourth experiment, 467 oocytes were injected and activated after 2 h of incubation. A total of 280 normal zygotes were attained. One hundred and seventy-two were examined for chromosomes at the metaphase of first mitosis. One hundred and fifty zygotes from natural conception and 179 from parthenogenesis were used as the control specimens. Among the embryos examined, 112 (65%), 105 (70%), and 118 (66%) of each group were analyzed successfully. The percentage of semi-cloned embryos with normal number of chromosomes was significantly lower than for embryos from natural conception and parthenogenesis. There were no distinct differences between the latter two groups. Chromosome breakage was found in 10% of semi-cloned embryos, but the breakage was not detected in embryos from natural conception or parthenogenesis.

One hundred and two embryos at interphase of the 2-cell stage, which developed from 108 normally activated oocytes, were examined for micronucleation. The percentage of embryos with micronuclei in semi-cloned embryos (15%, 15/102) was significantly higher than embryos from natural conception (0%, 0/98) and parthenogenesis (0%, 0/97).

Discussion

The results of our study demonstrated that the cytoplasm of mature oocytes triggered PCC and construction of microtubular spindle for injected somatic cell nuclei in a time-dependent way. The high level of maturation promoting factor (MPF), a cell-cycle regulator maintaining mature oocytes at MII, may be responsible for these events. After activation, elevation of intracellular calcium induces a decline in the activity of MPF. We saw that both the somatic cell nuclei and maternal genome underwent reduction segregation through the action of their spindles. When cumulus cells were injected into activated oocytes, their interphase nuclei remained unchanged. The ooplasm with low MPF did not force PCC or spindle formation for somatic cell nuclei.

The percentages of normal activation for injection of cumulus cells into mature oocytes increased with the incubation period before activation. Immediately after injection, the cumulus cell nuclei were at interphase and there was no spindle formation. Activation in this situation failed to induce haploidization of somatic cell nuclei. The percentages of PCC with spindle of cumulus cell nuclei and normal

activation were significantly greater for injected oocytes with 1 h of incubation than those with 30 min of incubation. The percentages were not different between those activated 1h and 2 h after incubation. However, the prematurely condensed chromosomes with spindle derived by injected cumulus cell nuclei exhibited a chaotic array. The gross morphology of normal activation for these oocytes was not equal to normal complements of chromosomes.

We examined the chromosomes of semi-cloned embryos before the first mitotic division that reflected the status after haploidization of somatic cell nuclei and the second meiosis of oocytes. Judging from the numerical data alone the incidence of chromosomal aberrations was high. As FISH has not been used, it is possible that not a single case of haploidization was successful.

In this study, some chromosome breakage and micronucleation were observed for embryos derived from artificial haploidization of somatic cell nuclei in non-enucleated mature oocytes. Micronuclei of blastomeres may represent chromosome fragments or chromosomes that were not incorporated into daughter nuclei during mitosis (Kamiguchi *et al.*, 1991). Chromosome breakage was also reported for the reduction division of primary spermatocyte nuclei in the non-enucleated mature oocytes (Kimura *et al.*, 1998) and of the cumulus cell nuclei in enucleated immature oocytes (Palermo *et al.*, 2002). The mechanisms were unclear, but may be due to injection of S-phase cells. Although a majority of cumulus cells surrounding recently ovulated oocytes were in G0/G1 phase of the cell cycle, some cells of S-phase or G2/M phase might exist. If the injected nucleus was in the S-phase and was driven into premature M-phase by the MPF of oocytes, it could result in broken chromosomes after reduction segregation. This catastrophe was precluded in normal cell cycles by a DNA replication checkpoint mechanism, which ensured that the initiation of M-phase could not occur until the completion of S-phase.

Development of semi-cloned embryos was poor in vitro, and no procreation of pups occurred after transfer of 324 embryos in our study. One of the causes was the serious abnormalities of chromosomes. Correct imprinting during gametogenesis and epigenetic reprogramming during the preimplantation period are essential for normal developmental potential. They involve DNA methylation, modification of histones, and chromatin remodeling that regulates gene expression of early embryos, cell cleavage and cell determination. The epigenetic status of donor nucleus is different from that of the gametes. Therefore, the other major determinant of success would hinge on the degree of epigenetic modification of somatic DNA in the cytoplasm of oocytes (Wakayama *et al.*, 1998). In

conventional cloning, reprogramming led to low efficiency and high rates of embryonic arrest, fetal death, and anomalies. For the semi-cloning, embryonic nuclei were constructed by the genomes of oocytes and somatic cells. The reprogramming of somatic cell nuclei in non-enucleated oocytes deserves further study.

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