Zygote **13** (February), pp. 35–38. © 2005 Cambridge University Press
doi:10.1017/S0967199405003084 Printed in the United Kingdom

Transplantation of male pronucleus derived from *in vitro* fertilization of enucleated oocyte into parthenogenetically activated oocyte results in live offspring in mouse

Feng-Yun Kong[1,2,*], *Gang Zhang*[1,2,*], *Zhi-Sheng Zhong*[1], *Yun-Long Li*[2], *Qing-Yuan Sun*[1] *and Da-Yuan Chen*[1]

State Key Laboratory of Reproductive Biology, Institute of Zoology, The Chinese Academy of Sciences, Beijing, and Key Laboratory of Animal Resistance, School of Life Science, Shandong Normal University, Jinan, China

Date submitted: 15.12.04. Date accepted: 25.01.05

Summary

In this study, inter-strain reconstructed embryos were produced by combining the female pronucleus of Kunming mouse (white) with male pronucleus of C57BL/6 strain (black). Metaphase II (MII) oocytes of Kunming mouse were enucleated and the zona pellucida was removed. Then, the enucleated oocytes were inseminated by capacitated sperm of C57BL/6 mouse *in vitro*. At the same time, MII oocytes of Kunming mouse were artificially activated using strontium chloride solution, which did not contain cytochalasin B. Finally, we removed the male pronucleus derived from C57BL/6 sperm and injected it into a parthenogenetically activated one-pronucleus oocyte by micromanipulation. The reconstructed 2-cell embryos were transplanted into the oviducts of 22 foster mother mice, each receiving about 20 embryos. In the end, seven healthy and live pups were born from one recipient.

Keywords: Fertilization, Ovum, Reproductive biotechnology

Introduction

The first pronuclear transplantation procedure for the mouse egg was developed in 1983 (McGrath & Solter, 1983). Their procedure included removal of the pronuclei by a sharp enucleation pipette without penetration of the plasma membrane. The fusion of the isolated pronuclei surrounded by cytoplasm/plasma membrane with a previously enucleated egg was induced by Sendai virus (HVJ) (Tsunoda *et al*., 1985*b*). This procedure may aid in further defining the possible roles of nucleus and cytoplasm during mammalian embryogenesis. In addition, reciprocal pronuclear transplantations between genetically distinct 1-cell embryos may be used to define the roles of male and female genetic materials in phenotype determination. Tsunoda *et al*. (1986) have examined the effect of cutting the zona pellucida of eggs on pronuclear transplantation. By making a slit in the zona of an egg, the time for pipetting and exchange of pronuclei between eggs was shortened. Although the proportions of eggs which developed to blastocysts *in vitro* and to term after embryo transfer were relatively high (Surani *et al*., 1984; Tsunoda *et al*., 1985*a*), there are still technical difficulties, in particular that of distinguishing male from female pronuclei.

In the present study, on the basis of previous pronuclear transfer experiments a new and reliable pronuclear transplantation procedure for the mouse egg has been developed. The male pronucleus from *in vitro* fertilization of an enucleated oocyte was combined with a genetically distinct female pronucleus derived from parthenogenetic activation. Normal offspring were obtained after embryo transfer.

All correspondence to: Da-Yuan Chen, State Key Laboratory of Reproductive Biology, Institute of Zoology, The Chinese Academy of Sciences, Beijing, 100080, China. Tel: +86 10 62560528. Fax: +86 10 62565689. e-mail: chendy@panda.ioz.ac.cn

[1] State Key Laboratory of Reproductive Biology, Institute of Zoology, The Chinese Academy of Sciences, Beijing, 100080, China.

[2] Key Laboratory of Animal Resistance, School of Life Science, Shandong Normal University, Jinan, 250014, China.

[*] These authors contributed equally to this work.

Materials and methods

Chemicals

All the chemicals used in this experiment were purchased from Sigma Chemical (St Louis, MO) except for those specifically noted.

Recovery of male pronucleus derived from C57BL/6 mouse

Female Kunming mice (white) 4–6 weeks old were injected with 10 IU pregnant mare's serum gonadotropin (PMSG) and 10 IU hCG 48 h later. The mice were killed by cervical dislocation 16 h after hCG injection. Cumulus-cell-enclosed, metaphase II (MII)-arrested eggs were obtained. The cumulus cell masses surrounding the eggs were removed by brief exposure to 300 IU/ml hyaluronidase in M2 (Sigma) medium. The micromanipulation procedure has been detailed elsewhere (Heindryckx *et al*., 2001, Liu *et al*., 1999, 2000, 2001). Briefly, MII oocytes were placed in a micromanipulation droplet containing 7.5 μg/ml of cytochalasin B (Sigma) and 3% sucrose overlaid with oil. A slit was made with a sharp needle in the zona pellucida adjacent to the spindle to facilitate the insertion of a pipette to aspirate the nucleus and surrounding cytoplasm gently and slowly without rupturing the oolemma. Then, male C57BL/6 mice (black) were also killed by cervical dislocation. The cauda epididymides were dissected away and minced slightly into pre-warmed M16 medium at 37 °C for sperm capacitation. The zona pellucida of enucleated oocytes was removed in a solution of acidified M2 (pH 2.5). After this, the oocytes were inseminated in a 50 μl drop of M16 medium with capacitated spermatozoa (1×10^6 cells/ml). The extrusion of the second polar body and the formation of the male pronuclei were observed with an inverted microscope (Hunter, 1974; Rho *et al*., 1998).

Artificial activation of MII oocytes

MII oocytes were activated as previously described (Barton *et al*., 1987; Hagemann *et al*., 1995; Wang *et al*., 1999; Liu *et al*., 2000). After collection of MII oocytes from Kunming mice, the oocytes were rinsed and cultured for 5–6 h in CZB–strontium chloride ($SrCl_2$) solution without cytochalasin B. The oocytes were then monitored under a dissection microscope for polar body extrusion and female pronucleus formation. Oocytes that displayed a second polar body and a single female pronucleus were selected.

Reconstruction of zygotes

Activated eggs and *in vitro*-fertilized eggs were placed in a micromanipulation droplet overlaid with oil. First, the zona pellucida of artificially activated eggs was pierced with a sharp glass needle near the second polar body. Then, one male pronucleus and its surrounding cytoplasm of an *in vitro*-fertilized egg were aspirated with a pipette gently and slowly, and inserted into the perivitelline space of the activated recipient egg. The procedure for electrofusion of the recipient egg and the transferred male pronucleus has been described (Liu *et al*., 1999; Takeuchi *et al*., 1999).

Following fusion, reconstructed zygotes were cultured in CZB at 37 °C with 5% CO_2 overnight until embryo transfer. Some reconstructed zygotes were used for *in vitro* culture to observe their development.

Confocal microscopy

After removal of the zona pellucida in acidified Tyrode's solution (pH 2.5), activated eggs with a single female pronucleus and enucleated, *in vitro*-fertilized eggs with a male pronucleus were fixed in 4% paraformaldehyde in PBS for 30 min and then incubated in incubation buffer (0.5% Triton X-100 in 20 mM Hepes, pH 7.4, 3 mM $MgCl_2$, 50 mM NaCl, 300 mM sucrose, 0.02% NaN_3) for 30 min. After being placed in methanol for 5 min at –20 °C, the eggs were washed in phosphate-buffered saline containing 0.1% Tween 20 and 0.01% Triton X-100 three times and then stained with 10 mg/ml propidium iodide (PI) for 10 min. Finally, the eggs were mounted on glass slides and examined using a TCS-4D laser scanning confocal microscope (Leica Microsystems, Bensheim, Germany) (Gard *et al*., 1995; Lee *et al*., 2000; Meng *et al*., 2004).

Embryo transfer

Reconstructed embryos were surgically transferred into oviducts of Kunming foster mothers mated with vasectomized males on day 1 of pseudopregnancy (Hogan *et al*., 1994). The recipients were allowed to develop to term.

Results

When allowed cultured *in vitro* for 6–8 h following insemination with C57BL/6 capacitated spermatozoa, 510 of 845 enucleated oocytes (61%) formed at least one male pronucleus. After exposure to $SrCl_2$ for 6–8 h, 660 of 824 MII oocytes (80%) extruded a second polar body and formed a single female pronucleus. After staining with PI for 10 min, a single female pronucleus was observed with an obvious red colour in activated oocytes, and one or two male pronuclei were visualized in *in vitro*-fertilized eggs (data not shown). The results proved the success of artificial activation and *in vitro* fertilization after enucleation.

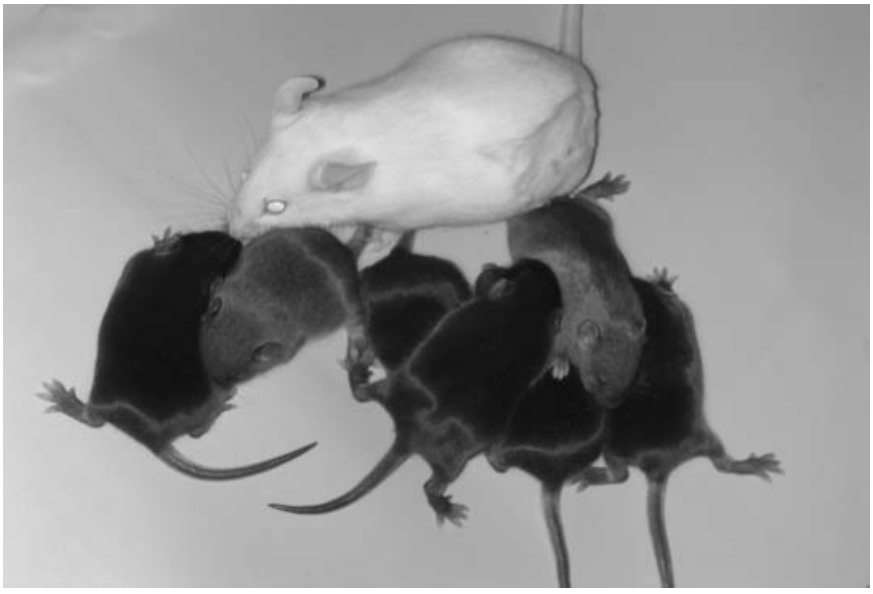

Figure 1 After transfer, one foster mother became pregnant, bearing seven normal, live pups, of which 5 were black and 2 were grey.

A total of 487 reconstructed zygotes were used for *in vitro* culture. About 80% (390/487) developed to the 2-cell stage after cultured for 1 day and 122 of 485 eggs (25%) developed to the morula stage after culture for 3 days.

About 20 2-cell embryos were transferred into the oviduct of each pseudopregnant mouse. A total of 22 recipients were used for embryo transfer and one became pregnant, giving birth to seven normal, live pups 20 days after transfer. Among the seven pups, 5 were black and 2 were grey (Fig. 1).

Discussion

Pronuclear transplantation in the mouse was first achieved by using a method that combines microsurgical removal of the zygote pronucleus with the introduction of a donor nucleus by a virus-mediated cell fusion technique (McGrath & Solter, 1983). Survival of embryos was greater than 90% and the embryos developed to term at a frequency not significantly different from that of non-manipulated control embryos.

The present study was different from previous reports in experimental design and techniques. In previous related studies, male and female pronuclei were from different fertilized eggs of the same strain, while in our study the male and female pronuclei were from genetically distinct mouse strains. In addition, the female pronucleus was from *in vitro* fertilization of an enucleated egg and the female pronucleus was from a parthenogenetic egg. Thus, in the reconstructed zygotes there were two genetically distinct pronuclei. In this way, we can distinguish the role of male and female pronuclei in the course of embryonic development. For a long time, our understanding and knowledge about male and female pronuclei was obtained only from their morphology and position (van de Sandt *et al*., 1990; Eppig *et al*., 1992; Ono *et al*., 2001). It is normally believed that in the early period of male pronuclear formation the remaining sperm tail is still observed, which becomes an important criterion for distinguishing male pronucleus from female pronucleus (Cross & Brinster, 1970). With the development and expansion of the pronuclei, the male pronucleus becomes larger than the female pronucleus, but this is not an accurate criterion for distinguishing them (Liu *et al*., 2000). The female pronucleus is closer to the second polar body, while the polar body is often translocated in the perivitelline space (Wang & Keefe, 2002). To overcome the difficulty in distinguishing male and female pronuclei, in the present study we obtained male and female pronuclei separately for transplantation. After transfer of reconstructed zygotes to the oviducts of 22 pseudopregnant mice, one became pregnant. Among the 7 pups born, 5 were black and 2 were grey. Because the transferred male pronucleus was derived from a C57BL/6 mouse (black), while the female pronucleus was derived from a Kunming strain mouse (white), our results show that the male pronucleus plays a dominant role in the determination of hair colour. The female pronucleus to some extent also has effects on progeny hair colour, which is proved by the production of 2 pups with grey hair.

Acknowledgement

Grant sponsor: the National Natural Science foundation of China (39830280); Major State Basic Research Project (G20000161).

References

Barton, S.C., Norris, M.L. & Surani, M.A. (1987). Nuclear transplantation in fertilized and parthenogenetically activated eggs. In *Mammalian Development: A Practical Approach* (ed. M. Monk), pp. 235–53. Oxford: IRL Press.

Cross, P.C. & Brinster, R.L. (1970). *In vitro* development of mouse oocytes. *Biol. Reprod*. **3**, 298–307.

Eppig, J.J., Wigglesworth, K. & O'Brien, M.J. (1992). Comparison of embryonic developmental competence of mouse oocytes grown with and without serum. *Mol. Reprod. Dev*. **32**, 33–40.

Gard, D.L., Cha, B.J. & Schroeder, M.M. (1995). Confocal immunofluorescence microscopy of microtubules, microtubule-associated proteins, and microtubule-organizing centers during amphibian oogenesis and early development. *Curr. Top. Dev. Biol*. **31**, 383–431.

Hagemann, L.J., Hillery-Weinhold, F.L., Leibfried Rutledge, M.L. & First, N.L. (1995). Activation of murine oocytes with Ca^{2+} ionophore and cycloheximide. *J. Exp. Zool*. **271**, 57–61.

Heindryckx, B., Rybouchkin, A., Van Der Elst, J. & Dhont, M. (2001). Effect of culture media on *in vitro* development of cloned mouse embryos. *Cloning* **3**, 41–50.

Hogan, B.L., Beddington, T., Costantini, F. & Lacy, E. (1994). *Manipulating the Mouse Embryo: A Laboratory Manual*, 2nd edn, pp. 170–184. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Hunter, R.H. (1974). Chronological and cytological details of fertilization and early embryonic development in the domestic pig, *Sus scrofa. Anat. Rec*. **178**, 169–85.

Lee, J., Miyano, T. & Moor, R.M. (2000). Spindle formation and dynamics of gamma-tubulin and nuclear mitotic apparatus protein distribution during meiosis in pig and mouse oocytes. *Biol. Reprod*. **62**, 1184–92.

Liu, H., Krey, L.C., Zhang, J. & Grifo, J.A. (2001). Ooplasmic influence on nuclear function during the metaphase II–interphase transition in mouse oocytes. *Biol. Reprod*. **65**, 1794–9.

Liu, H., Wang, C.W., Grifo, J.A., Krey, L.C. & Zhang, J. (1999). Reconstruction of mouse oocytes by germinal vesicle transfer: maturity of host oocyte cytoplasm determines meiosis. *Hum. Reprod*. **14**, 2357–61.

Liu, H., Zhang, J., Krey, L.C. & Grifo, J.A. (2000). *In vitro* development of mouse zygotes following reconstruction by sequential transfer of germinal vesicles and haploid pronuclei. *Hum. Reprod*. **15**, 1997–2002.

McGrath, J. & Solter, D. (1983). Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. *Science* **220**, 1300–2.

Meng, X.Q., Fan, H.Y., Zhong, Z.S., Zhang, G., Li, Y.L., Chen, D.Y. & Sun, Q.Y. (2004). Localization of gamma-tubulin in mouse eggs during meiotic maturation, fertilization, and early embryonic development. *J. Reprod. Dev*. **50**, 97–105

Ono, Y., Shimozawa, N., Ito, M. & Kono, T. (2001). Cloned mice from fetal fibroblast cells arrested at metaphase by a serial nuclear transfer. *Biol. Reprod*. **64**, 44–50.

Rho, G.J., Kawarsky, S., Johnson, W.H., Kochhar, K. & Betteridge, K.J. (1998). Sperm and oocyte treatments to improve the formation of male and female pronuclei and subsequent development following intracytoplasmic sperm injection into bovine oocytes. *Biol. Reprod*. **59**, 918–24.

Surani, M.A., Barton, S.C. & Norris, M.L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature* **308**, 548–50.

Takeuchi, T., Ergun, B., Huang, T.H., Rosenwaks, Z. & Palermo, G.D. (1999). A reliable technique of nuclear transplantation for immature mammalian oocytes. *Hum. Reprod*. **14**, 1312–17.

Tsunoda, Y., Wakasu, M. & Sugie, T. (1985*a*). Viability of blastocysts and morulae cultured from 1-cell stage and transferred to recipients in the mouse. *Jpn. J. Zootech. Sci*. **56**, 417–19.

Tsunoda, Y., Yasui, T., Tokunaga, T., Uchida, T. & Sugie, T. (1985*b*). Pronuclear transplantation in the mouse. *Jpn. J. Anim. Reprod*. **31**, 130–4.

Tsunoda, Y., Yasui, T., Nakamura, K., Uchida, T. & Sugie, T. (1986). Effect of cutting the zona pellucida on the pronuclear transplantation in the mouse. *J. Exp. Zool*. **240**, 119–25.

van de Sandt, J.J., Schroeder, A.C. & Eppig, J.J. (1990). Culture media for mouse oocyte maturation affect subsequent embryonic development. *Mol. Reprod. Dev*. **25**, 164–71.

Wang, W.H. & Keefe, D.L. (2002). Spindle observation in living mammalian oocytes with the polarization microscope and its practical use. *Cloning Stem Cells* **4**, 269–76.

Wang, W.H., Machaty, Z., Ruddock, N., Abeydeera, L.R., Boquest, A.C., Prather, R.S. & Day, B.N. (1999). Activation of porcine oocytes with calcium ionophore: effects of extracellular calcium. *Mol. Reprod. Dev*. **53**, 99–107.