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Metaphase II nuclei generated by germinal vesicle transfer in mouse oocytes support embryonic development to term

Hui Liu, Hung Chi Chang, John Zhang, Jamie Grifo and Lewis C.Krey[1]

Program for In vitro Fertilization, Reproductive Surgery and Infertility, New York University School of Medicine, New York, NY 10016, USA

[1]To whom correspondence should be addressed at: 660 First Avenue, Fifth Floor, New York, NY 10016, USA. E-mail: kreyivf@yahoo.com

BACKGROUND: Cytoplasmic defects are thought to cause aneuploidies in oocytes and embryos and oocyte 'reconstruction' by germinal vesicle (GV) transfer may circumvent such defects. In mice 'reconstructed' oocytes undergo meiosis and fertilize normally, but early embryonic development is compromised if their ooplasm matured *in vitro*. This study employs sequential MII spindle and/or pronucleus (PN) transfer to assess the embryonic potential of MII nuclei that form following GV transfer. METHODS AND RESULTS: Mouse embryos generated by these procedures were transferred to the oviducts of pseudopregnant mice to monitor pregnancy outcome. Following GV transfer, the resultant metaphase II (MII) nuclei were activated either *in situ* or transferred and activated in ooplasts from in-vivo matured oocytes. When exchanged with the female PN of a fertilized zygote, only the PNs that developed in in-vivo matured ooplasts generated live offspring. Viable offspring also resulted when MII nuclei were transferred to in-vivo matured ooplasts and fertilized by insemination with sperm or by artificial activation and male PN transfer. Significantly, the offspring displayed normal fertility as adults. CONCLUSION: This report of live births following GV transfer in mice illustrates the importance of the maturational history of the ooplasm at PN formation for normal embryonic and fetal development.

Key words: germinal vesicle/in-vitro maturation/metaphase II nucleus/nuclear transfer/ooplasm

Introduction

Germinal vesicle (GV) transfer, a procedure that allows the GV of one oocyte to mature and complete the first meiotic division in the ooplasm of another oocyte, has been proposed as a means to reduce the incidence of aneuploidy (Zhang *et al*., 1999). Aneuploidy (an abnormal number of chromosomes) is currently considered to be primarily responsible for the high incidence of embryonic lethality observed in women of advanced maternal age. Recent studies suggest that many aneuploidies originate during the first meiotic division in oocytes (Hassold and Hunt, 2001). In turn, this anomalous segregation of chromosomes is believed to be associated with a dysfunctional cytoplasm. Preliminary cytogenetic analyses suggest that the GV transfer procedure itself does not increase the incidence of aneuploidy, and that the transfer of a GV from the oocyte of a woman of advanced maternal age often undergoes a normal meiotic division when transferred into an enucleated oocyte from a young woman. Conversely, aged ooplasts tend to induce an abnormal segregation of meiotic chromosomes (Zhang *et al*., 1999; Takeuchi *et al*., 2001; Palermo *et al*., 2002].

Euploidy in a mature oocyte is required for embryonic viability, but does not guarantee normal embryogenesis. In recent studies with human gametes, GV transferred oocytes that matured *in vitro* underwent fertilization and division but most arrested early in embryogenesis (Takeuchi *et al*., 2001). Because of the micromanipulation steps required, oocytes that receive a transferred GV are denuded and must undergo the final stages of maturation without contacts with their cumulus cells. Significantly, denuded, in-vitro matured oocytes are much less likely to complete embryonic development than in-vivo matured oocytes or cumulus-enclosed oocytes (Nogueira *et al*., 2000). In previous studies with a mouse model of GV transfer, we reported similar consequences and suggested that such poor embryonic development is the result of pronucleus (PN) formation in an incompetent recipient ooplasm (Liu *et al*., 2000).

Recently, we described sequential nuclear transfer procedures that improved the embryonic developmental potential of mouse oocytes that matured *in vitro*. These procedures included the following steps—MII spindle transfer into an ooplast that had matured *in vivo* and/or PN transfer into cytoplasts from enucleated, in-vivo fertilized oocytes (Liu *et al*., 2000; 2001). Using these procedures embryonic development improved significantly as judged by development to morphologically normal blastocysts. The present study was

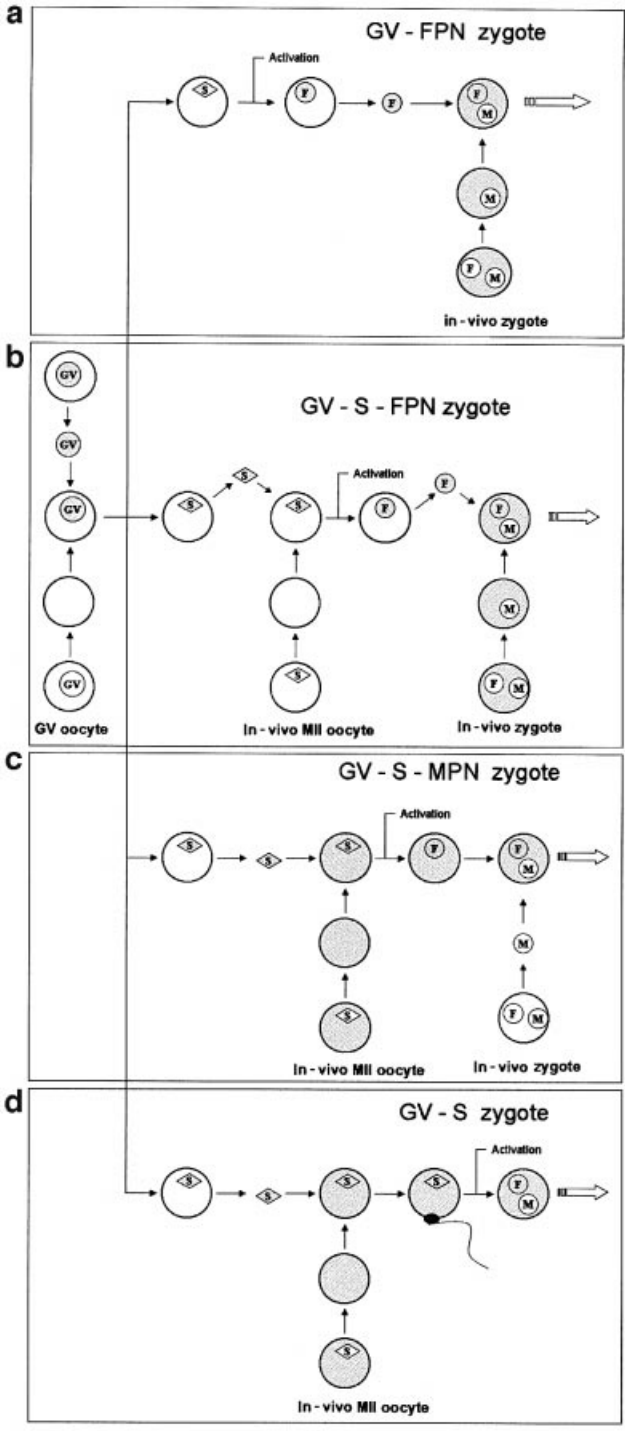
designed to examine whether we could couple these procedures with GV transfer to generate live-born offspring.

Materials and methods

Recovery of GV oocytes and in-vivo matured oocytes, and fertilized oocytes

Female CB6F1 mice (6–8 weeks old; Charles River Laboratories, Wilmington, MA, USA) received i.p. injections of 5 IU pregnant mare's serum gonadotrophin (PMSG; Sigma, St Louis, MO, USA) and 5 IU hCG (Sigma) 48 h later. Some mice were sacrificed after 1 h to collect the GV-stage oocytes by needle puncture. Cumulus cells attached to the oocytes were removed by repeated aspiration through a 200 µl pipette tip. The denuded oocytes were cultured in human tubal fluid medium (hTF, Irvine Scientific, Irvine, CA, USA) supplemented with 10% fetal calf serum (FCS, HyClone, Logan, UT, USA) and 50 µg/ml 3-isobutyl-1-methylxanthine (IBMX, Sigma) for 4–6 h before selecting those oocytes that grew to 80 µm and were characterized by a visible perivitelline space for micromanipulation.

A second group of mice were killed 16–18 h after hCG to harvest metaphase II (MII) stage oocytes. A final group was mated immediately after hCG injection and killed 22 h later to collect fertilized zygotes. MII oocytes and zygotes were harvested from the ampullae of excised Fallopian tubes in hTF medium supplied with HEPES-buffered hTF medium (MhTF, Irvine Scientific). Cumulus cells were removed by briefly exposing MII oocytes and zygotes to serum-free, MhTF medium containing 300 IU/ml hyaluronidase (Sigma). Collected oocytes were rinsed in three washes of MhTF prior to experimentation.



Nuclear transfer

The micromanipulation procedures for GV, MII nucleus and pronuclear transfer have been detailed elsewhere (Liu *et al*., 1999; 2000). Briefly, oocytes at GV, MII and PN stages are placed in a drop of MhTF containing 10% FCS and cytochalasin B (7.5 µg/ml; Sigma) for 15 min. The zona pellucida adjacent to the nucleus is slit with a sharp needle to facilitate the insertion of a transfer pipette to aspirate the nucleus and surrounding cytoplasm gently and slowly without rupturing the oolemma. The karyoplast is then transferred to the perivitelline space of an enucleated recipient oocyte. Fusion between the karyoplast and cytoplast takes place in a fusion chamber filled with medium (0.3 mol/l mannitol, 0.1 mmol/l $CaCI_2$ and 0.05 mmol/l $MgSO_4$ in H_2O) and is initiated by a single pulse of direct current (1.8–2.5 kV/cm DC for 50 µs). Fused GV stage oocytes are placed in hTF drops for 16–18 h in order to complete the first meiotic division. Fused MII stage oocytes are transferred to new HTF drops for 30 min prior to artificial activation or insemination. Fused reconstructed zygotes are removed to drops of G1 medium (IVF Science, Vitrolife AB, Gothenburg, Sweden) for overnight culture.

Figure 1. Reconstruction strategies for GV-FPN zygotes (**a**), GV-S-FPN zygotes (**b**), GV-S-MPN zygotes (**c**) and GV-S zygotes (**d**). Each zygote group starts from a common origin—the MII nucleus that matures after GV transfer. (**a**) For the GV-FPN zygote, the MII nucleus is activated artificially to form a pronucleus (PN) which is then transferred into the cytoplasm of an in-vivo fertilized oocyte from which the female PN has been removed. (**b**) For the GV-S-FPN zygote, the MII nucleus is transferred into an enucleated oocyte that matured *in vivo*, is activated artificially to form a PN which is then transferred into ooplasm of an in-vivo fertilized oocyte from which the female PN has been removed. (**c**) For the GV-S-MPN zygote, the MII nucleus is transferred into enucleated oocyte that matured *in vivo*; after artificial activation, the reconstructed oocytes received a male PN from in-vivo zygote via transfer. (**d**) For the GV-S zygote, the MII nucleus is subjected to MII spindle removal and transfer to an enucleated oocyte that has matured *in vivo*; this reconstructed oocyte is then inseminated by mixing with epididymal sperm. Circle in shadow represents contribution origin of female nucleus and cytoplasm for embryonic development. GV = germinal vesicle; S = spindle; F = female PN; M = male PN.

Table I. Reconstruction and development of zygotes created by sequential nuclear transfer. Values given are numbers with percentage successful results in parentheses

Type of zygote	MII	S-fusion	Fertilization	Activation	PN-fusion	2 cell	Live birth
GV-FPN	98	–	–	92 (94)	88 (96)	86 (98)	0
GV-S-FPN	90	82 (91)	–	76 (93)	72 (95)	68 (94)	8 (12)
GV-S-MPN	60	52 (87)	–	49 (94)	49 (100)	48 (98)	6 (13)
GV-S	63	57 (90)	56 (98)	–	–	37 (66)	6 (16)

GV = germinal vesicle; MII = metaphase II nucleus; FPN = female pronucleus; MPN = male pronucleus.
See text for details of GV-FPN, GV-S-FPN, GV-S-MPN and GV-S.
S-fusion: karyoplast of MII spindle nucleus fuse into cytoplasm of in-vivo mature oocytes enucleated.
PN-fusion: karyoplast of female pronucleus fuse into cytoplasm of semi-male zygote.

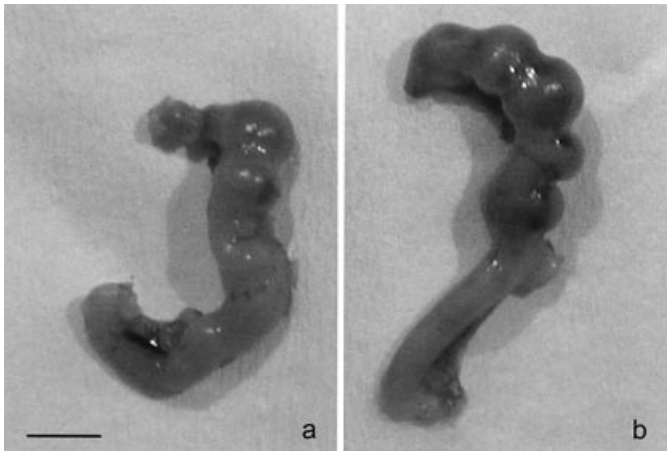


Figure 2. Day 11 uterus after embryo transfer. The two fetuses that originated from GV-FPN zygotes (**a**) are comparable in size as a control fetus that originated from an in-vivo fertilized zygote (**b**). However, there were three smaller GV-FPN fetuses that were in the process of being reabsorbed. Bar = 0.5 cm.

Artificial activation

MII stage oocytes were placed in PBS containing 3 μm ionophore A23187 (Sigma) for 5 min at 22°C, washed twice with MhTF, and then transferred to hTF supplemented with 10% FCS and cycloheximide (5 μg/ml; Sigma) for 4–5 h. Activated oocytes displayed a pronucleus (PN) and extruded second polar body at this time (Liu *et al* 2000).

Embryo transfer

Reconstructed zygotes were cultured in G1 medium for 18–20 h *in vitro*. Those embryos that developed to the 2-cell stage, were surgically transferred into oviduct of day 1 pseudopregnant CD-1 foster mice as described previously (Liu *et al*., 2001). Pregnancy was checked by palpation at 11 days and thereafter. Whenever the foster mother carried a single fetus by palpation, Caesarean section was performed to deliver that pup at 20 days post coitus.

Experimental design

Four groups of zygotes were reconstructed to evaluate the development potential of a MII nucleus that had derived from a transferred GV. For the first group—GV-FPN zygote—the MII nucleus was activated artificially and the resultant PN transferred to the cytoplasm of an in-vivo fertilized oocyte from which the female PN had been removed (Figure 1a). In the second group—GV-S-FPN zygote—the MII nucleus was transferred into an enucleated oocyte that had matured *in vivo*. After artificial activation, the resultant PN was transferred into cytoplasm of an in-vivo fertilized oocyte from which the female PN had been removed (Figure 1b). In the third group—GV-S-MPN zygote—the MII nucleus was transferred into an enucleated oocyte that had matured *in vivo*. After artificial activation, the reconstructed oocyte was 'fertilized' by fusing a male PN from an in-vivo zygote (Figure 1c). For the fourth group—GV-S zygote—the MII spindle was removed and transferred to an enucleated oocyte that had matured *in vivo*. This reconstructed oocyte was inseminated during a 6 h incubation in a droplet containing sperm harvested from the epididymis (Figure 1d).

Results

After GV transfer and in-vitro culture for 16–18 h, 311 of 483 reconstructed oocytes extruded a polar body and formed an MII spindle. The embryonic viability of these MII nuclei was evaluated by sequential nuclear transfer, e.g. spindle and PN transfer.

The efficiency for each step in zygote reconstruction for the GV-FPN, GV-S-FPN, GV-S-MPN and GV-S groups are presented in Table I. No significant procedural variation was noted between groups and a ⩾90% success rate was noted at each stage.

Of the 88 GV-FPN zygotes created, 98% divided after overnight culture and were then transferred into the oviducts of pseudopregnant foster mice. Six mice became pregnant as detected by palpation at 11 days post-transfer. When one mouse was laparotomized there were five fetuses in the uterine horn which received the transferred embryos. Two fetuses grew to the normal size (Figure 2), the rest were smaller suggesting that they were being absorbed. The other five pregnant mice survived to term, but none delivered a live-born pup.

Of the 82 GV-S-FPN zygotes created, 94% divided to two cells after overnight culture and were then transplanted into the oviducts of foster mice. Eight continued to term and delivered their offspring normally. This result suggests that the maternal genome was fully functional despite several transfer steps.

Of the 49 GV-S-MPN zygotes created, 98% cleaved after overnight culture and were then transplanted to oviducts of foster mice. Three mice became pregnant and delivered six live offspring.

Of the 57 GV-S zygotes created, 66% were two cells after overnight culture and were then transplanted to oviducts of

Figure 3. When mated with a CB6F1 male, the female offspring from a GV-S zygote gave birth herself to 10 pups. Subsequent studies reveal that these second generation post-GV transfer offspring are themselves fertile.

foster mice. Four mice became pregnant and delivered six live offspring.

After GV transfer and sequential nuclear transfer, 20 live offspring were born with a weight range from 1.9–2.1 g. Two pups were cannibalized by their foster mothers and two others died due to lack of breastfeeding. The remaining 16 pups grew normally to adulthood. The six male mice weighed 22–25 g at 35 days of age; the 10 female mice weighed 19–23 g. We monitored reproductive function in the six adult mice that originated from GV-S zygotes. All six (two male and four female) were fertile (Figure 3) and their offspring, in turn, also displayed normal fertility.

Discussion

In the present study, sequential nuclear transfer at the MII spindle and/or pronuclear stage was used to assess the embryonic potential of MII nuclei that mature following GV transfer. Using CB6F1 mouse oocytes, we previously reported that the MII nuclei that form following GV transfer are functionally competent to support embryonic development to the blastocyst stage (Liu *et al*., 2000). In the present study we report that, when transferred to the uterus of a pseudopregnant foster mouse at the 2-cell stage, embryos that develop from MII nuclei post GV-transfer do continue to develop and result in the birth of live offspring. Moreover, the live birth rate observed (12–16%) for these embryos, approaches that noted previously when non-GV transferred oocytes are denuded, fertilized and transferred as 2-cell embryos to the uteri of foster mice (18%; Liu *et al*., 2001). Such findings suggest that the microsurgical transfer of the GV from one immature oocyte to another does not have any adverse effect on the embryonic competence of the maternal genome that develops following the first meiotic division. Moreover, considering that secondary transfers of MII spindles and female PN were also performed prior to the uterine transfer, it would appear that a microsurgical transfer of the maternal nucleus between mature oocytes or zygotes, even when performed multiple times, does not compromise its ability to support embryonic development to term.

Live-birth outcomes have been reported previously following GV transfer in mice (Kono *et al*., 1996; Bao *et al*., 2000). Significantly, these studies also employed sequential transfer following GV transfer; following blastocyst transfer a 30% live birth rate was observed. Live births have also been noted following GV transfer in rabbits (Li *et al*., 2001) and cows (Kuwayama, 2002); however, in these species the oocytes reconstructed by GV transfer were fertilized directly and the embryos transferred to recipient uteri. The resultant live birth rate in the rabbit was extremely low, <3% of the embryos resulting in a live birth; unfortunately the birth rate for the cow was not reported.

In previous studies with CB6F1 mouse oocytes, we reported that GV transfer resulted in reconstructed oocytes that, following artificial activation and male PN transfer, generated poor quality embryos that failed to generate a live birth (Liu *et al*., 2000). However, in order to perform GV transfer, the immature oocytes were denuded of their surrounding cumulus cells and subsequent studies in denuded oocytes not subjected to GV transfer produced similar findings (Liu *et al*., 2001). Further studies using MII spindle transfer in denuded oocytes suggested that the source of the developmental incompetence originated in the cytoplasm and not the nucleus of oocytes reconstructed by GV transfer (Liu *et al*., 2001). The present results in the GV-S-MPN group provide the most convincing evidence to support this view. Live births were only observed with embryos that developed with a maternal PN that formed following MII spindle transfer to in-vivo matured ooplasm. Significantly, similar birth rates were observed when fertilization was achieved by transfer of the female or male PN or by normal fertilization.

Another group of embryos (GV-FPN) were created by exchanging the PN that formed following GV transfer and artificial activation with the maternal PN of an in-vivo fertilized oocyte. Although this procedure improved embryo quality, no live births were observed following oviduct transfer. These observations point to cytoplasmic-induced changes in long-term nuclear function that normally occur during oocyte activation but do not take place in the ooplasm that matured *in vitro*. As a result the nucleus completes the second meiotic division as a euploid PN incapable of supporting long-term embryonic development even when placed in an in-vivo fertilized oocyte. As above, these effects can be circumvented by MII spindle transfer to ensure that activation and PN formation take place in cytoplasm that had completed maturation *in vivo*. Epigenetic mechanisms such as DNA methylation have been proposed to explain how cytoplasmic factors may influence long term nuclear function and have been demonstrated to take place during fertilization and early embryonic development (Renard *et al*., 1994).

In a series of follow-up studies we determined whether GV, MII spindle and/or PN transfer had any long-term effect on the resultant offspring. Not only were their birth size and growth rate normal, but these offspring, both male and female, displayed normal mating behaviour as adults. These matings resulted in normal offspring who were also fertile after

reaching adulthood. Such a multi-generational follow-up is important considering the long-term safety concerns associated with these procedures and should be required for any future study involving nuclear transfer.

It is important to reiterate at this point that all of our studies were performed exclusively with oocytes from CB6/F1 mice cultured in hTF media supplemented with fetal calf serum. In contrast, Schroeder and Eppig (1984) noted that denuded oocytes of B6SJLF1 mice which had matured *in vitro* and were fertilized in MEM media similarly supplemented were competent to support embryonic growth to term and deliver live births even when matured *in vitro*. Whether this dichotomy is due to strain of mouse or culture system is currently under investigation.

GV transfer has been proposed as a potential procedure to circumvent the age-related increase in aneuploidy observed in the human female (Zhang *et al*., 1999). The concept is that by transferring the GV from an older women's oocyte into a donated ooplast from a young woman may reduce the aneuploidy rate in the first meiotic division. However, as with mouse oocytes, immature human oocytes must be stripped of their cumulus cells in order to perform the microsurgical transfer. Although cumulus-enclosed oocytes that mature *in vitro* are able to fertilize and support embryonic development to term (Chian *et al*., 1999; Cha *et al*., 2000; Child *et al*., 2002), significantly lower rates of maturation and early embryonic development have been noted when immature, denuded, human oocytes are compared with cumulus-enclosed oocytes (Hwang *et al*., 2000; Kim *et al*., 2000; Nogueira *et al*., 2000). Significantly, no births have been reported when embryos developing from denuded oocytes were transferred to the uterus. Thus, the impact of cumulus–oocyte interactions on ooplasmic development may occur in the human as well as in the mouse. Several approaches may be taken to overcome this cytoplasmic effect. As reported here, sequential nuclear transfer could be used. However, this approach would require two different types of donated oocytes, immature oocytes for GV transfer and mature oocytes for MII spindle transfer—a clinically impractical arrangement. Another option would be to harvest immature oocytes immediately prior to GV breakdown; however, we must first learn when the important changes in cytoplasmic make-up take place during the final stages of maturation. A final possible option is to supplement the ooplasm of the reconstructed oocyte following GV transfer with the injection of ooplasm from an oocyte that matured normally *in vivo*. Such injections have been successful in treating women whose oocytes result in extensively fragmented embryos (Cohen *et al*., 1998; Lanzendorf *et al*., 1999). Although each of these options presents major problems including multiple oocyte donors, future work may uncover a more practical solution to this problem. Nonetheless, GV transfer, like all nuclear transfer procedures, is an important research tool to investigate the relationships between the ooplasm and maternal genome that are necessary for normal maturation, fertilization and embryonic and fetal development. Such analyses can then be used to develop models for molecular approaches that will identify the cellular factors underlying these relationships.

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