Developmental incompetency of denuded mouse oocytes undergoing maturation in vitro is ooplasmic in nature and is associated with aberrant Oct-4 expression

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BACKGROUND: Germinal vesicle (GV) oocytes constitute a potential resource but their developmental competence is questionable especially when surrounding cumulus cells are removed. The intercellular factors/mechanisms underlying such poor embryonic competence may originate at a nuclear and/or ooplasmic level. METHODS: Immature or mature oocytes were obtained from three mouse strains following pregnant mare serum gonadotropin (PMSG) or PMSG + human chorionic gonadotropin (hCG) treatment. Immature oocytes were denuded of cumulus cells prior to in vitro maturation. Pronuclear (PN) transfer was used to examine nuclear–ooplasmic interplay on resultant embryonic development and Oct-4 immuno-staining patterns. RESULTS: Embryos arising from ooplasts of in vivo matured oocytes displayed significant increases in blastocyst formation rates and total blastomere numbers when compared to those created from ooplasts of denuded oocytes. Oct-4 staining was more pronounced and restricted to the inner cell mass (ICM) in blastocysts arising from the ooplasm of in vivo matured zygotes than in those created from denuded oocytes. CONCLUSIONS: Developmental defect(s) appear to develop primarily in the ooplasm of oocytes that are denuded of their cumulus cells prior to in vitro maturation. Such oocytes result in embryos with poor developmental competence. These defects result in anomalies in cell number and Oct-4 expression during the morula–blastocyst developmental transition.

Key words: denuded oocyte/in vitro maturation/Oct-4/ootplasm/pronuclear transfer

Introduction

Pregnant mare serum gonadotropin (PMSG) priming of mice has been extensively used to obtain >80 μm diameter oocytes for research study. Although immature, i.e., germinal vesicle (GV) stage, some oocytes lack cumulus cells, whereas others are fully enclosed by cumulus and corona cells. Cumulus-enclosed oocytes (CEO's) have been preferentially used in most studies (O'Brien et al., 1993). When placed in culture these immature oocytes initiate and complete the first meiotic division within 14–16 h and then arrest in metaphase II (MII) until fertilization occurs (Wasserman et al., 1994). During these final stages of oocyte maturation, a series of molecular events take place that are vital for normal fertilization and embryogenesis.

In mouse (Anderiesz et al., 1995; Fulka et al., 1998), rat (Vanderhyden et al., 1989), bovine (Zhang et al., 1995), and human (Goud et al., 1998), the capacity of denuded immature oocytes to undergo maturation and support pronuclear formation and preimplantation development is compromised when compared to that of intact CEO's. The presence of cumulus cells and corona cells during the final stages of oocyte maturation has been proposed to have profound influence(s) on the developmental competence of the genome and/or the ooplasm (Goud et al., 1998; Moor et al., 1998; Downs et al., 2002).

GV transfer is a micromanipulation procedure that can investigate nuclear–ooplasmic interrelationships in oocytes (Liu et al., 1999; Takeuchi et al., 1999; Zhang et al., 1999; Liu et al., 2000; Krey et al., 2001). However, to perform this procedure, one must mechanically strip the surrounding cumulus cells from the oocytes. Prior studies from our laboratory indicate that this ‘denuding’ process has an adverse impact on the ability of the ooplasm to support embryonic development to term when the oocytes are ‘fertilized’ by artificial activation and pronuclear transfer (Liu et al., 2001).

The POU domain transcription factor Oct-4 is a key factor for the first differentiation step in embryogenesis (Schorler et al., 1990). Oct-4 encodes a master transcriptional regulator that influences several genes expressed during early development and its expression is restricted to pluripotent and totipotent lineages (Pesce and Scholer, 2001). Although mouse embryos homozygous for a targeted deletion of Oct-4 can...
develop to blastocyst stage, they die following implantation due to absence of inner cell mass (ICM), (Nichols et al., 1998). Oct-4 expression during early embryogenesis in normal mouse embryos and mouse clones has been characterized by RT–PCR, in situ hybridization and immuno-staining procedures (Hansis et al., 2001; Boiani et al., 2002; Mitalipov et al., 2003).

In this report we investigate whether different gonadotropin priming treatments improve the ability of denuded oocytes to complete maturation, fertilization and preimplantation development normally in three different mouse strains. In every study the oocytes were inseminated normally by exposure to sperm. In addition, we used pronuclear (PN) transfer to create embryo ‘models’ to determine whether nuclear or ooplasmic defects occurred after premature cumulus cell removal. Finally, we characterized cell number and the temporal and spatial distributions of Oct-4 immuno-staining during the morula–blastocyst transition in these embryo ‘models’.

Materials and methods

Mice strains and culture media

CB6F1 mice (BALB/cAnNCrlBR × C57BL/6NCrlBR, 6–8 weeks of age, Charles River Laboratories, Wilmington, MA) generated the oocytes and sperm in the majority of our study. To ensure good embryogenesis, HEPES-buffered modified human tubal fluid (HTF) (Irvine Scientific, Irvine, CA), HTF supplemented with 5% fetal bovine serum (HTF-FBS; HyClone, Logan, UT), G1 and G2 (Scandinavian IVF Science, Gothenburg, Sweden) were used as media for embryo harvesting, in vitro maturation and sequential embryonic culture, respectively (Liu et al., 2000).

Additional studies were conducted using oocytes from B6D2F1 (C57BL/6 × DBA/2, 6–8 weeks of age; Charles River Laboratories, Wilmington, MA) and B6SІFLF1 mice (C57BL/6J × SJL/J, 6–8 weeks of age; Jackson Laboratory, Bar Harbor, ME). These three different strains of mice belonged to similar Ped-fast background (Warner et al., 1998). Their oocytes were also allowed to mature in HTF-FBS and the resultant embryos cultured in G1 and G2 media.

Hormonal priming

Three different protocols of hormonal treatments were tested:

(a) no gonadotropin treatment prior to oocyte retrieval
(b) 5 IU PMSG (Sigma) injection i.p. 48 h prior to sacrifice
(c) 5 IU of PMSG injection for 48 h followed by 5 IU of human chorionic gonadotropin (hCG; Sigma) at 1.5 h prior to sacrifice.

Preliminary experiments indicated that hCG treatments at 2 h prior to sacrifice resulted in the collection of oocytes that had already initiated GV breakdown.

Additional CB6F1 mice were injected with 5 IU of PMSG for 48 h before sacrifice to obtain CEOs. In vivo matured oocytes were retrieved after injection with 5 IU of PMSG for 48 h and then 5 IU of hCG for 15 h before sacrifice. Female B6D2F1 and B6SIJLF1 mice were also injected i.p. with 5 IU PMSG (Sigma, St. Louis, MO) 48 h prior to sacrifice. Mature oocytes from each of these mouse strains were fertilized in vitro with sperm from their own strain.

Recovery of in vitro matured oocytes and in vivo matured oocytes

Oocytes were retrieved by ovarian puncture with a fine needle. Only CEOs with intact cumulus layers were selected for study. Cumulus cells were removed mechanically by pipetting vigorously in HEPES-buffered HTF + hyaluronidase (300 IU/ml, Sigma). Once completely denuded, the GV oocytes, >80 μm in diameter, were washed three × in HEPES-buffered HTF and placed into microdrops of HTF-FBS and covered with mineral oil (Sigma). Additional CEOs were not denuded and were allowed to mature spontaneously in vitro in HTF-FBS. Maturation status was evaluated, after 14–15 h of in vitro culture at 37°C in a 5% CO2 incubator. Oocytes with a polar body were defined as mature MII oocytes.

In vivo matured oocytes were recovered from oviductal ampullae into HEPES-buffered HTF. Mature oocytes with well-expanded cumulus cells surrounding a healthy-looking MII oocyte were transferred into 150 μL microdrops containing HTF supplemented with 30 mg/ml bovine serum albumin (HTF-BSA; Sigma, St. Louis, MO) under oil for fertilization.

In vitro fertilization (IVF) and in vitro culture

IVF procedures were modified from those of Hogan and co-workers (1994). Male mice were sacrificed by cervical dislocation. The vas deferens was cut close to the cauda epididymis and the sperm was extruded using watchmaker’s forceps into a dish containing HTF-BSA. The dish was then placed in a 37°C incubator for more than 1 h to allow the sperm to swim out and capacitate.

Mature oocytes (n = 10–20) were placed into separate 150 μL microdrops containing HTF-BSA under mineral oil. Sperm mixture (10–20 μJ) was added to each microdrop to obtain a concentration of 1–2 × 10⁶ sperms/μl. After co-incubation for 4 h at 37°C, the oocytes were removed and washed in 2 ml HEPES-buffered HTF and transferred to microdrops of HTF-FBS and placed in a 5% CO2 incubator at 37°C. At 6–8 h post-insemination, embryos with two distinct pronuclei and a second polar body were classified as PN stage and transferred into G1 microdrops. Only those zygotes presenting distinct male and female PN at this time were cultured further. The embryos were observed every day. At 48 h, 4-cell embryos were transferred into G2 microdrops and monitored to blastocyst stage.

Nuclear transfer and electrofusion

Four types of reconstructed zygotes were generated (Figure 1):

Group I in vivo PN—in vitro ooplasm: PN of zygote from in vivo matured CEO was transferred into enucleated cytoplast of zygote from demuded, in vitro matured oocyte.

Group II in vitro PN—in vitro ooplasm: PN of zygote from in vivo matured CEO was transferred into enucleated cytoplast of zygote from demuded, in vitro matured oocyte.

Group III in vitro PN—in vitro ooplasm: PN of zygote from in vivo matured oocyte was transferred into enucleated cytoplast from an in vitro matured oocyte.

Group IV in vivo PN—in vivo ooplasm: PN of zygote from in vivo matured oocytes was transferred into enucleated cytoplast of in vivo matured oocytes.

PN transfer was accomplished as previously described (Liu et al., 2000). After preincubation in a microdrop of HEPES-HTF supplemented with 7.5 μg/ml cytochalasin B (Sigma) for 15 min at 37°C, zygotes were subjected to micromanipulation. First, the zona pellucida was pierced with a sharp glass needle near the second polar body. A transfer pipette (internal diameter: 20 μm) was passed through the slit and the PNs aspirated. The PNs were then inserted into the peri-vitelline space of a enucleated recipient zygote. Electrofusion of the reconstructed PN—cytoplast complex was performed in a chamber (microslide 453, BTX, San Diego, CA) containing...
In vivo maturation and preimplantation development of denuded oocytes

When denuded after retrieval, the majority of denuded oocytes (85%) from unprimed mice underwent spontaneous meiotic maturation in vitro with polar body extrusion. Gonadotropin priming, either PMSG or PMSG + hCG, did not improve the meiotic maturation rate (Table I). The nuclear maturation rate was better when cumulus cells remained intact than removed prior to maturation in vitro (95% versus 85%, P < 0.05).

The fertilization rate for in vivo matured oocytes was significantly higher than that of any in vitro matured group (P < 0.001; Table I). Moreover, the fertilization rate of...
Preimplantation development of zygotes following reciprocal PN transfer between in vivo matured oocytes and in vitro matured, denuded oocytes

When pairs of PNs from denuded, in vitro matured oocytes were placed into enucleated in vivo zygotes, the fusion rate was 97%. Of these reconstructed zygotes, 82% cleaved after overnight culture and 61% of the embryos developed to blastocyst stage (group III, Table III). The cleavage and blastocyst formation rates were comparable to those of ‘in vivo control’ zygotes created by exchanging PNs between in vivo matured zygotes (group IV). In contrast, when PNs were exchanged between in vitro matured denuded oocytes (group II) or were transferred from an in vivo zygote into an enucleated in vitro zygote (group I), the cleavage and blastocyst formation rates were significantly lower than those described above (P < 0.001).

Mean cell number per blastocyst was approximately 50% lower whenever a PN, regardless of its origin, was transferred into an enucleated ooplasm from an in vitro matured, denuded oocyte (groups III, IV versus groups I, II; Figure 2). However, when placed into enucleated in vivo zygotes, PNs from in vivo matured oocytes (group IV) developed into blastocysts at a rate comparable to that observed in control zygotes (group I).

### Table II. In vitro maturation and preimplantation development of denuded oocytes from three mouse strains

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>GV (n)</th>
<th>PB (n)</th>
<th>PN (n)</th>
<th>2-Cell (n)</th>
<th>4-Cell (n)</th>
<th>Morula (n)</th>
<th>Blastocyst (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6D2F1</td>
<td>160</td>
<td>147 (92%)b</td>
<td>60 (40%)c</td>
<td>42 (70%)</td>
<td>32 (54%)</td>
<td>25 (42%)</td>
<td>18 (30%)d</td>
</tr>
<tr>
<td>CB6F1</td>
<td>168</td>
<td>141 (84%)b</td>
<td>49 (35%)c</td>
<td>35 (71%)</td>
<td>30 (59%)</td>
<td>26 (54%)</td>
<td>14 (28%)d</td>
</tr>
<tr>
<td>B6SJLF1</td>
<td>178</td>
<td>167 (94%)b</td>
<td>65 (39%)c</td>
<td>47 (73%)</td>
<td>38 (58%)</td>
<td>33 (50%)</td>
<td>18 (28%)d</td>
</tr>
</tbody>
</table>

Denuded oocytes of three mouse strains matured in HTF + FBS. Resultant zygotes cultured in G1, G2 medium. Values in parentheses are percentages.

b: > a (P < 0.03); c, d: in the same column, no significant difference.

GV = germinal vesicle stage oocyte; PB = mature MII oocyte with first polar body; PN = fertilized oocyte with two pronuclei.
blastocysts with larger cell numbers than those following transfer of PNs from denuded oocytes (group III), (88± versus 70±, respectively, P, 0.03; Figure 2). Blastocyst morphology also varied. Whenever a PN, regardless of origin, was placed into ooplasm that had matured in vivo, the developing blastocyst demonstrated a fully expanded blastocoel surrounded by numerous uniformly sized cells and a clearly defined ICM (Figure 3). In contrast, reconstructed embryos that developed from ooplasm subjected to in vitro maturation appeared unhealthy, the resultant blastocysts showed delays in formation, smaller blastocoels, a compromised capacity to hatch. Strikingly, the ICM of blastocysts reconstructed by exchanging of PNs between zygotes of denuded oocytes was apparently small or absent.

Oct-4 expression of oocytes and embryos

Embryos with enucleated blastomeres or fragmented nuclei were eliminated from this analysis. The staining pattern for Oct-4 protein was spatially and temporally regulated during preimplantation development. No Oct-4 staining was observed in any GV-stage or MII-stage oocyte or in 2-cell- or 4-cell-stage embryo. Staining was initially observed in 6–8-cell-stage embryos derived from in vivo matured oocytes (Figure 4). As reported by others, Oct-4 staining was strongest within the nuclei of blastomeres of morulae and early blastocysts (Mitalipov et al., 2003). In blastocysts, Oct-4 signals were weak in the nuclei of cells in the trophectoderm (TE) but strong in cells in the ICM (Figure 4). Comparisons of Oct-4 staining patterns were performed at morula–blastocyst stage because they were clearly defined with a distinct spatial distribution.

We analyzed the Oct-4 staining patterns in morulae from denuded oocytes, in vitro matured CEOs and in vivo matured CEOs (Table IV). In each case ~90% showed strongly stained nuclei evenly distributed across the embryo. There was no apparent difference in staining, either in intensity or number of stained cells, in the morulae of these three groups.

We also analyzed Oct-4 staining patterns blastocysts from denuded oocytes, in vitro matured CEOs and in vivo matured CEOs (Table V). Whereas 80–86% of the blastocysts in the latter two groups showed strong staining in ICM cells and, as they developed, a progressive weakening of staining in TE cells, only 6 of 30 (20%) blastocysts from denuded oocytes showed a similar pattern. Eight other blastocysts from denuded oocytes showed strongly stained cells scattered throughout the TE but no identifiable ICM; the remaining 16

Table III. Preimplantation development of zygotes reconstructed by PN transfer between in vitro matured, denuded oocytes and in vivo matured oocytes from CB6F1 mice

<table>
<thead>
<tr>
<th>Group (PN–ooplasm)</th>
<th>Zygote (n)</th>
<th>Fused zygote (n</th>
<th>2-Cell (n)</th>
<th>Blastocyst (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (in vivo PN–in vitro ooplasm)</td>
<td>66</td>
<td>65 (98%)</td>
<td>45 (69%)</td>
<td>19 (29%)</td>
</tr>
<tr>
<td>II (in vitro PN–in vitro ooplasm)</td>
<td>58</td>
<td>56 (97%)</td>
<td>38 (68%)</td>
<td>15 (27%)</td>
</tr>
<tr>
<td>III (in vitro PN–in vivo ooplasm)</td>
<td>98</td>
<td>95 (97%)</td>
<td>78 (82%)</td>
<td>58 (61%)</td>
</tr>
<tr>
<td>IV (in vivo PN–in vivo ooplasm)</td>
<td>119</td>
<td>114 (96%)</td>
<td>92 (81%)</td>
<td>75 (66%)</td>
</tr>
</tbody>
</table>

In vivo matured oocytes were primed with PMSG for 48 h and hCG 15 h; denuded oocytes were primed with PMSG for 48 h. Embryos in groups I and II utilize ooplasts from denuded oocytes matured in vitro; those in groups III and IV utilize ooplasts from in vivo matured oocytes.

b > a (P < 0.05); d > c (P < 0.001).

Figure 3. Micrographs showing representative blastocysts developed from CB6F1 mice: (A) embryos reconstructed by exchanging of PNs between zygotes of denuded oocytes (group II); (B) embryos reconstructed by placing PNs from zygote of denuded oocytes into enucleated zygotes of in vitro matured oocytes (group III) and (C) embryos reconstructed by exchanging PNs between zygotes of in vivo matured oocytes (group IV). When compared to group III, blastocysts in group II showed delays in formation, a narrower blastocoel, a smaller diameter, poor compaction of cells of various sizes at hatching and a low hatching rate. The diameter of reconstructed mouse blastocysts ranged from 100–130 μm.
blastocysts showed a weak or undetectable staining pattern. There is a significant difference ($P < 0.003$) in these distributions of stained blastocysts suggesting that premature cumulus cell removal adversely affects Oct-4 expression during the morula–blastocyst transition.

Following PN transfer, we further examined the influence of the ooplasm on Oct-4 staining in blastocysts. We analyzed blastocysts from group I (in vivo PN–in vitro ooplasm), group II (in vitro PN–in vitro ooplasm), group III (in vitro PN–in vivo ooplasm) and group IV (in vivo PN–in vivo ooplasm; see Table V and Figure 5). Once again, $\sim 80\%$ of those blastocysts that developed from oocytes with an in vivo-matured ooplasm (groups III and IV) displayed a local concentration of strongly stained cells. In contrast, only 6 and 15 blastocysts from groups I and II (in vitro matured ooplasm) showed a similar pattern ($P < 0.003$); the majority of blastocysts ($\sim 50\%$) in these two groups demonstrated weak staining.

### Discussion

In many species (mouse: Anderiesz and Trouson, 1995; rabbit: Chang, 1955; bovine: Hashimoto et al., 1998; human: Goud et al., 1998), immature oocytes denuded of cumulus cells have been reported to complete meiosis, fertilize...
and initiate early cleavage but then subsequently arrest. However, others have noted that denuded murine oocytes can display the same developmental competence as in vivo matured oocytes (Schroeder and Eppig, 1984; Yamazaki et al., 2001).

When intact cumulus–oocyte connections persist through the first meiotic division, gonadotropins exert advantageous effects on an oocyte’s competence to mature, fertilize and support normal preimplantation development (Eppig et al., 1992; Anderiesz et al., 2000). Gonadotropins produce these actions by interacting with specific receptors expressed in granulosa cells but not in oocytes (Ulloa-Aguirre et al., 1995). Therefore, it is not surprising that we observed that priming with either PMSG or PMSG plus hCG, had no beneficial effect on the maturation, fertilization and the subsequent embryonic development. This phenomenon was noted in three different strains of mice (CB6F1, B6D2F1, B6SJLF1) even when these strains matured in media in which denuded oocytes were reported to display the same developmental competence as in vivo matured oocytes, e.g., B6D2F1 in Waymouth (Yamazaki et al., 2001) and B6SJLF1 in MEM (Schroeder and Eppig, 1984). Significantly, >70% of these embryos arrested at an early cleavage stage, an outcome compatible with that reported by Takeuchi et al., (2004). Interestingly, each of these mouse strains is hybrids with a B6 gene (i.e., Ped fast; Warner et al., 1998). Whether oocytes from Ped slow strains respond differently to premature cumulus cell denuding remains to be determined.

After in vitro maturation, the denuded mouse oocytes displayed low fertilization rates, that are comparable to those reported for Swiss, B6SJLF1 and B6D2F1 mice (Cross and Brinster, 1970; Schroeder and Eppig, 1984; Yamazaki et al., 2001). These low rates may be attributed to difficulties encountered by the sperm when penetrating zona and ooplasmic membrane, difficulties that would require ICSI (Takeuchi et al., 2004). Inadequacy of male PN formation may also occur and may be related to glutathione concentrations in the oocyte. Glutathione is vital for PN formation and is present in higher levels in cumulus-intact oocytes than in denuded oocytes (Bing et al., 2002). As a result, CEO that mature in vitro would be expected to be more able to decondense the sperm nucleus normally into well-developed PN than oocytes denuded prior to maturation in vitro (mouse: Cecconi et al., 1996; rat: Vanderhyden and Armstrong, 1989; bovine: Zhang et al., 1995; Hashimoto et al., 1998).

When PNs of zygotes from either in vitro or in vivo matured oocytes were transferred into ooplasm from zygotes from denuded oocytes, only 27–29% developed to blastocyst stage. In contrast, when PNs of zygotes from denuded oocytes were placed into ooplasm from zygotes from in vivo matured oocytes, 61–66% developed to blastocyst. Taken together, these results clearly indicate that the ooplasm of in vitro matured, denuded oocytes is less competent to support early embryonic development. This conclusion is supported further by the observations that the blastocysts originating from ooplasm from in vivo matured oocytes demonstrate twice the blastomere number than those that originate from ooplasm of in vitro matured, denuded oocytes. It would appear that, although maturational competency is achieved when the diameter of an immature oocyte reaches 80 μm, the ooplasm is still ‘immature’ and requires further developmental changes to be able to support normal embryogenesis. Recently, the ooplasm of in vitro matured oocytes from mouse preantral follicles has been shown to be similarly incompetent to support embryonic development (Liu et al., 2003b). Identification of the physiologic and biochemical events underlying this developmental event(s) in the ooplasm is crucial to design culture strategies to improve the embryonic potential of immature oocytes.

Although Oct-4 expression was similar in the morulae of all reconstructed embryos, significant differences were observed when the embryos developed to blastocyst stage. Significantly more blastocysts that arose from ooplasm from in vivo matured zygotes (groups III, IV) displayed a normal pattern of Oct-4 expression than those supported by ooplasm from in vitro matured zygotes (groups I, II). Therefore, the oocyte’s ooplasm and not its nucleus appear to determine the change in the distribution pattern of Oct-4 expression during the morula–blastocyst transition. Aberrant Oct-4 expression would be expected to change the fate of a blastomere’s lineage thereby negatively impacting on an embryo’s developmental competence. Oct-4 deficient embryos develop into a blastocyst-like structure but without a pluripotent ICM; they subsequently die from an inability to differentiate into a fetal pole (Nichols et al., 1998). In addition, Boiani et al., (2002) reported a significant association between aberrant Oct-4 expression and poor developmental potential in mouse clones. The blastocysts that developed from ooplasm from denuded oocytes demonstrated patterns of abnormalities including a generalized lack of Oct-4 expressing blastomeres, random expression in blasto-

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**Table V. Distribution of Oct-4 staining patterns in blastocysts from CB6F1 mice**

<table>
<thead>
<tr>
<th>Blastocyst type</th>
<th>n</th>
<th>Strong staining with normal distribution (%)</th>
<th>Strong staining M but aberrant distribution (%)</th>
<th>Weak staining (%)</th>
<th>No detectable staining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (in vivo PN–in vitro ooplasm)</td>
<td>20</td>
<td>3 (15)</td>
<td>6 (30)</td>
<td>11 (55)</td>
<td>0</td>
</tr>
<tr>
<td>Group II (in vitro PN–in vitro ooplasm)</td>
<td>24</td>
<td>3 (13)</td>
<td>8 (33)</td>
<td>12 (50)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Group III (in vitro PN–in vivo ooplasm)</td>
<td>24</td>
<td>18 (75)</td>
<td>4 (17)</td>
<td>1 (4)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Group IV (in vivo PN–in vivo ooplasm)</td>
<td>17</td>
<td>15 (88)</td>
<td>1 (6)</td>
<td>1 (6)</td>
<td>0</td>
</tr>
<tr>
<td>Denuded oocytes, <em>in vitro</em> maturation</td>
<td>30</td>
<td>6 (20)</td>
<td>8 (27)</td>
<td>14 (46)</td>
<td>2 (7)</td>
</tr>
<tr>
<td>Cumulus enclosed oocytes, <em>in vitro</em> maturation</td>
<td>25</td>
<td>20 (80)</td>
<td>2 (10)</td>
<td>2 (10)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Cumulus enclosed oocytes, <em>in vivo</em> maturation</td>
<td>28</td>
<td>24 (86)</td>
<td>3 (11)</td>
<td>1 (3)</td>
<td>0</td>
</tr>
</tbody>
</table>

b > a (P < 0.003); c > d (P < 0.002).
meres in the ICM and TE and diminished expression in the ICM. Oct-4 has become a useful marker of pluripotency in mouse and human embryos; if Oct-4 expression falls 30% below the normal level, embryonic stem cells lose their pluripotency and undifferentiate into TE lineage (van Eijk et al., 1999; Niwa et al., 2000). Our Oct-4 observations may explain our previous findings that only one live pup was recovered when 79 2-cell embryos generated from denuded oocytes were transferred into pseudopregnant mothers (Liu et al., 2001). The finding that these changes were first noticeable at the morula–blastocyst transition suggests that important developmental events take place at this time. Such a conclusion is underscored by clinical observations in our IVF laboratory that ~40% of the human embryos that progress to morula stage do not develop further into blastocysts with a definable ICM (unpublished observations).

Changes in protein and mRNA patterns have been observed during progression from GV to MII (Schultz et al., 1965).

**Figure 5.** DNA and Oct-4 staining patterns in morula–blastocysts derived from reconstructed zygotes in group I (in vivo PN, in vitro ooplasm) and group III (in vitro PN, in vivo ooplasm). Oct-4 staining patterns in the morulae appeared comparable in both groups. In contrast, Oct-4 staining in Bls generated from in vitro ooplasts was appreciably weaker and displayed aberrant spatial patterns when compared to that seen in the Bls generated from in vivo ooplasts. The diameter of reconstructed mouse cleavage-stage embryos and Bls were 80–90 and 100–130 μm, respectively.
1988). Several ooplasmic proteins are present at higher levels in oocytes that matured in vivo than in in vitro matured CEOs (mouse: Schultz et al., 1978; sheep: Warnes et al., 1977; porcine: Meinecke and Schoter, 1996; rabbit: Van Blerkom and McGaughey, 1978). Such differences in the protein profile may be exaggerated in denuded oocytes; ooplasmic proteins abundant in in vitro matured human MII oocytes are completely absent in MII oocytes denuded prior to the onset of meiosis I (Trounson et al., 2001). Identification of these proteins will provide more clues about their role(s) in fertilization and early embryogenesis.

RNA synthesis is virtually undetectable when meiosis resumes and remains so until the time when the embryonic genome is activated (2-cell stage in the mouse and 4–6-cell stages in humans; Heikinheimo and Gibbons, 1998). However, translation of the existing ooplasmic mRNA pools continues throughout the final stages of meiosis (Wassarman, 1996). Because the activation of transcriptional events occurs gradually, maternal transcripts are needed almost until blastocyst stage (Bachvarova and Moy, 1985). Many early embryonic events, including activation of embryonic transcription depend on maternal proteins. There are intracellular mechanisms that regulate the expression of different mRNA transcripts. RNAs masked by deadenylation for storage have to be unmasked by polyadenylation to resume translation; other mRNAs are already unmasked before GV breakdown and must undergo amplified translation (Stutz et al., 1998). Regulatory factors responsible for unmasking the mRNAs and subsequent translation for various mRNAs are therefore integral not only for meiotic progression but also for subsequent embryonic development (Sirard et al., 1989). The number of specific mRNA transcripts has been reported to be lower in ‘defective’ bovine oocytes and embryos (Wrenzycki et al., 1999; Lonergan et al., 2000). El Moutaissim et al. (1999) demonstrated correlations between the developmental progression of mouse and human preimplantation embryos in culture and the expression of anti-oxidation enzymes. Finally, Brevini-Gandolfi et al. (1999) reported that the two groups of in vitro matured bovine oocytes that display different developmental capacities also display different poly(A) tail lengths of several maternal transcripts, including those for β-actin (cytoskeletal proteins), Oct-4 (involved in early differentiation and totipotency) and Cx-43 (important for blastocyst formation). Recently, this relationship between developmental competence of cattle oocyte and mRNA polyadenylation was confirmed from GV stage to 2-cell stage (Brevini et al., 2002). The foregoing studies suggest that inadequate ooplasmic maturation is linked to anomalies in the regulation of mRNA polyadenylation which in turn compromises developmental competence; similar events may be taking place during the final maturation stages of denuded oocytes. Future studies should focus on whether either or both of these mechanisms play a role in establishing intracellular Oct-4 levels during embryogenesis.

Although we have focused on mRNA translation as a mechanism to explain the adverse effects of premature cumulus cell denuding, cellular metabolism may also be compromised. Cumulus cells dramatically influence tricarboxylic acid cycle activity, oxygen consumption and pyruvate utilization in the final stages of oocyte maturation (Downs et al., 2002). In addition, fully-grown CEOs take up significantly more uridine, guanosine, choline, and deoxyglucose from culture medium than do denuded oocytes (Heller and Schultz, 1980).

Although there was no difference in the blastocyst development rates in groups III and IV in the PN transfer study, there was a significant difference in cell number in the resultant blastocysts (Figure 2). Such observations suggest that there might also be subtle defects in nuclear function during the MII–interphase transition when denuded oocytes mature and are fertilized; we have noted similar observations when oocytes are chemically activated in vitro (Liu et al., 2001). This may be the reason why investigators have reported live birth using the nucleus from denuded mouse oocytes only when nuclear transfer is performed at the MII spindle stage, but not at the PN stage (Kono et al., 1996; Liu et al., 2003b).

In summary, we have evaluated the maturation, fertilization and preimplantation development of oocytes denuded at the GV stage, matured in vitro and then normally inseminated by exposure to sperm. Embryonic development was significantly poorer in these oocytes when compared to CEOs that matured in vitro or in vivo. Neither PMSG nor PMSG + hCG improved the rate of blastocyst formation or the quality of the developed blastocysts. Using PN transfer procedures, we established that defects in the ooplasm are primarily responsible for these developmental anomalies. In addition we report that Oct-4 expression is aberrant when embryos originating from the ooplasm of prematurely denuded oocytes reach the blastocyst stage. Such findings are important if we are to understand the intracellular mechanisms responsible for ooplasmic maturation and address them in clinical in vitro maturation protocols.

Acknowledgements
The authors wish to thank Mengchen Hsieh and Dan Sheng for their assistance with the statistical analyses.

References


Submitted on August 17, 2004; resubmitted on February 17, 2005; accepted on March 3, 2005