Aggregation of a parthenogenetic diploid embryo and a male embryo improves the blastocyst development and parthenogenetic embryonic stem cell derivation

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Abstract: Parthenogenetically derived mammalian embryos, with no paternal genome, are not viable and die, largely from defective placental growth attributed to a lack of paternal effect, resulting in the low blastulation rate and low derivation efficiency of parthenogenetic embryonic stem cells (pESCs). Therefore, the present study, by the optimization of parthenogenetic embryo production and the aggregation of the parthenogenetic diploid embryo and the identified male embryo, aims to investigate a method to improve the development of parthenogenetic embryo and pESC derivation in mice. Using different chemical combinations for the optimization, we found that the heterozygous diploid type had a significantly higher blastulation rate than the haploid type (P < 0.05). The treatment of strontium chloride (SrCl₂) combined with cytochalasin B for 4 h produced the highest heterozygous diploid rate and blastulation rate. Our self-made concave hole system was used for the aggregation of the parthenogenetic heterozygous diploid embryo with the male embryo identified by the duplex PCR method, and we found that the chimeric embryo had an improved rate of blastulation and pESC isolation.

Key words: Parthenogenetic diploid, male embryo, embryo aggregation, blastulation rate, parthenogenetic embryonic stem cell isolation

1. Introduction

Parthenogenetic embryonic stem cells (pESCs) could advance regenerative medicine by avoiding immunorejection (Cibelli et al., 2002; Vrana et al., 2003; Kim et al., 2007). However, previous reports suggested that the potential development of parthenogenetic embryos and pESC derivation efficiency is very low (Liu et al., 1998; Cibelli et al., 2002).

Parthenogenetic embryo production optimization, especially the ploidy (haploid or heterozygous diploid) of parthenogenetic-activated oocytes, is an important factor for the development of parthenogenetic embryos and pESC derivation efficiency in mice (O’Neil et al., 1991; Liu et al., 1998). Studies have suggested that activated heterozygous diploid oocytes (without the extrusion of the second polar body (Pb₂)) compared to the haploid (with the extrusion of Pb₂), have better potential of development to parthenogenetic blastocysts (Ilyin and Pavker, 1992; Prather et al., 1997). Previous studies suggested that pESCs derived from oocytes activated with 6%–7% ethanol, which only triggers a single rise in Ca²⁺, showed limited developmental potential in mice (Allen et al., 1994; Szabo and Mann, 1994; Newman-Smith and Werb, 1995). In contrast, multiple Ca²⁺ elevations induced by strontium (Sr²⁺) treatment fully activate oocytes, similar to fertilization (Swann and Ozil, 1994; Toth et al., 2006), and significantly enhance parthenogenetic embryo development (Bos-Mikich et al., 1995; Moses and Kline, 1995). Besides, it has been suggested that oocytes activated with 6-dimethyl aminopurine (6-DMAP) or cytochalasin B (CB) could effectively prohibit the extrusion of Pb₂ (Czołowska et al., 1986; Szollosi et al., 1993; Sun et al., 1999; Fan and Sun, 2004). Therefore, different chemical combinations (ethanol, SrCl₂, 6-DMAP, and CB) were used in this study to activate MII oocytes, looking at the effect of different chemical combinations on the ploidy of activated oocytes and the development of parthenogenetic blastocysts. By comparing the potential of the embryo development and the pESC derivation efficiency, we aimed to optimize the parthenogenetic embryo production.

Besides the ploidy of activated oocytes, studies suggested that mammalian parthenogenetic embryos are not viable and die (McGrath and Solter, 1984; Surani et al., 1984), attributed to a lack of biparental imprinting.
Parthenogenetic mouse pups have been produced from reconstructed parthenogenetic embryos after genetic modification of the paternal imprinted genes (Kono et al., 2004; Wu et al., 2006, 2016), suggesting the important role of the paternal effect on the development of mammalian parthenogenetic embryos. Therefore, the duplex PCR method for the \textit{SRY} (sex determining region on the Y chromosome) and \textit{ZFX} (zinc finger X) genes was used in this study for male embryo sex identification to investigate the paternal effect on the development of parthenogenetic embryos and pESC derivation.

Previous studies have shown the effect of embryo aggregation for parthenogenetic or cloned embryo development and embryonic stem cell derivation in pigs and mice. The study by Terashita et al. (2011) found that the aggregation of embryos at the four-cell stage improved the quality of miniature pig somatic cell nuclear transfer blastocysts. Studies by Lee et al. (1994, 2006, 2013) showed that zona-mediated embryo aggregation could improve the efficiency of pig cloned ES-like cells. Similar results were obtained for parthenogenetic embryos and parthenogenetic stem cells in pigs (Saadeldin et al., 2014) and mice (Shan et al., 2012). Therefore, the hypothesis of the present study is that the aggregation of a male embryo and a parthenogenetic diploid embryo can improve the development of parthenogenetic blastocysts and the pESC derivation efficiency in mice, which would contribute to replacement therapies in regeneration medicine.

2. Materials and methods
Unless otherwise indicated, all reagents were purchased from Sigma.

2.1. Animals
We used Kunming mice at about 6–8 weeks of age and of similar live weights (25 ± 2.4 g). All animals were maintained in accordance with the Animal Experiment Hand Book at the Center for Developmental Biology and experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986, and associated guidelines.

2.2. Superovulation
Kunming female mice were superovulated by injection with 10 IU of pregnant mare serum gonadotrophin, followed 48 h later by injection with 10 IU of human chronic gonadotrophin (hCG). The oocytes (18 h after hCG injection) were collected for activation.

2.3. Parthenogenetic activation
The MII oocytes were collected for parthenogenetic activation. Different chemical combinations (5% or 10% ethanol, 10 mM SrCl\textsubscript{2}, 2 mM 6-DMAP, 5 µg/mL CB) were used as different parthenogenetic activation treatments:

\begin{enumerate}
\item 5% or 10% ethanol for 5 min or 10 min;
\item 10% ethanol for 10 min + 2 mM 6-DMAP for 2 h or 4 h or 6 h;
\item 10% ethanol for 10 min + 2 mM 6-DMAP + 5 µg/mL CB for 6 h;
\item 10 mM SrCl\textsubscript{2} for 2 h or 4 h or 6 h;
\item 10 mM SrCl\textsubscript{2} + 5 µg/mL CB for 2 h or 4 h or 6 h.
\end{enumerate}

There are four types of parthenogenetic-activated oocytes (Rougier and Werb, 2001): homogeneous haploid (extrusion of Pb\textsubscript{2} and only one pronucleus), mosaic haploid (sharply divided into two equal cleavages), homogeneous diploid (extrusion of Pb\textsubscript{2} and two pronuclei), and heterozygous diploid (without extrusion of Pb\textsubscript{2} and two pronuclei), which all could be clearly identified under the micromanipulator (Leica DM IRB, Germany) (630×). Karyotype analysis was used for ploidy confirmation (haploid or diploid).

Different types of activated oocytes were cultured in vitro in CZB droplets covered with paroline oil in the incubator (Forma 3131, Thermo Forma Co., USA) at 37 °C with 5% CO\textsubscript{2}. The blastulation rate was calculated.

2.4. Embryo biopsy for sex identification

2.4.1. Duplex PCR method optimization
Four male and 4 female mice (liver tissues) were used to optimize and confirm the correctness of the duplex PCR method for the \textit{SRY} and \textit{ZFX} genes used as the method for sex identification. Mouse liver DNA was isolated using the DNeasy Blood & Tissue Kit (QIAGEN, Germany) according to the instructions provided by the manufacturer. Primers were designed using Primer Premier software (Version 5.0, PREMIER Biosoft, Palo Alto, CA, USA) to amplify a 250-bp product of the \textit{SRY} gene specific for male mice and a 399-bp product of the \textit{ZFX} gene for both male and female mice, based on sequences obtained from GenBank (Table 1). All primers were synthesized by Beijing Biological Technology Co., Ltd., China.

\begin{table}
\centering
\caption{Oligonucleotide primers used in PCR amplification for \textit{SRY250} and \textit{ZFX399}.}
\begin{tabular}{|c|c|}
\hline
\textbf{Gene fragment} & \textbf{Gene oligonucleotide sequences (5’- to 3’-)} \\
\hline
\textit{SRY250} & F: CTTTTTTCAGGAGGCGACAGA \\
& R: GACAGGCTGCCAATAAAAGC \\
\hline
\textit{ZFX399} & F: AAGAGAGCGCATCAAGTGTGA \\
& R: GCTACCTTTGTTGCCGAAAT \\
\hline
\end{tabular}
\end{table}

PCR amplification reactions (30 µL) contained 3 µL of 10X PCR buffer (Fisher Biotech), 2 µL of 2.5 mM MgCl\textsubscript{2} (Fisher Biotech), 3 µL of 10 mM dNTPs (Fisher Biotech), 1 µL each of forward and reverse primers (Beijing Biological Technology Co., Ltd., China), 1.5 µL of Taq DNA

(Surani et al., 1984; Sturm et al., 1994; Walsh et al., 1994).
polymerase (5.5 U/µL; Fisher Biotec), 4 µL of DNA, and 14.5 µL of ddH₂O. The fragments of SRY250 and ZFX399 were amplified with an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 35 s, and 72 °C for 40 s with a final extension at 72 °C for 10 min. The PCR products were confirmed by gel electrophoresis.

2.4.2. Embryo biopsy for sex identification This step aimed to investigate how many blastomeres (2 or 4 blastomeres) from 8-cell embryos can be used as the optimal template for embryo sex identification. The 8-cell embryos were collected from mice with pregnancy of 2.5 days. The collected 8-cell embryos were put in droplets of 100 µL covered with paraffin oil and were fixed by self-made fixation needle under a micromanipulator (Leica DM IRB, Germany). Two or 4 blastomeres were sucked out (Figure 1) and covered with paraffin oil in the PCR tube for boiling and ice-bath treatment for 5 min, and then centrifuged at 4000 rpm for 2 min. The supernatant was generated for the PCR amplification. A previously optimized duplex PCR method was used for the sex identification. The PCR products were confirmed by gel electrophoresis.

2.5. Embryo aggregation The remaining blastomeres of the identified male embryo and the parthenogenetic heterozygous diploid 8-cell embryo were aggregated. Thepronase concentration (0.25%, 0.5%) and treatment time (10 min, 20 min) and the aggregation system (ordinary M16 droplet, M16 droplet culture system containing 0.06% phytohemagglutinin (PHA), self-made concave hole culture system (Figure 2)) were optimized.

The rate of bare embryo (evaluation: no obvious spallation after removing the pellucid zone), rate of aggregated embryo (evaluation: no separation within 0.5 h after aggregation), rate of development of aggregated embryo (evaluation: no degeneration and death within 24 h for aggregated embryo), and the blastulation rate (evaluation: blastocyst within 72 h) were observed.

2.6. pESC derivation pESCs from different types of blastocysts were isolated using the method as previously described for ESC isolation (Wang and Liu, 2007; Liu et al., 2015).

A previously optimized duplex PCR method for sex identification was used again to identify pESCs from the ESCs derived from chimeric embryos.

The pESC-like colonies were formed after the inner cell masses were passaged four times. We selected two at random to determine their pluripotency using real-time PCR for pluripotency genes (POUSF1, NANO, CDX-2, REX-1) and the relative expression levels of these genes were compared among the pESCs derived from different types of blastocysts (parthenogenetic haploid (control), heterozygous diploid, and chimeric embryo).

2.7. RT-PCR Total RNA was isolated using the Easy-Spin (DNA-free) Total RNA Extraction Kit (QIAGEN) according to the manufacturer's instructions. cDNA was produced from 1 µg of total RNA from pESC samples using the Omniscript RT Kit (QIAGEN). qRT-PCR was performed using a LightCycler 2.0 (Roche, Germany). PCR products for four genes (POUSF1, NANO, CDX-2, REX-1) were detected in three replicates by SYBR Green probes. Each reaction consisted of 2 µL of cDNA, 2 µL of SYBR Green I Master Mix (Roche), and 2 µL each of the forward and reverse primer and was adjusted to a total volume of 20 µL using distilled water. The amplification consisted of initial preincubation at 95 °C for 30 s, followed by 45 amplification cycles at 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 20 s. Primer sequences and the approximate sizes of the amplified fragments of all transcripts (POUSF1, NANO, CDX-2, REX-1) are shown in Table 2. Each transcript sample was quantified in three replicates and their relative expressions were analyzed using the 2^(-ΔΔCt) method (Livak and Schmittgen, 2001).

2.8. Statistic analysis The differences between data (%) were compared using chi-square tests and the reliability of the data was tested using Hardy–Weinberg equilibrium. Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). P < 0.05 was regarded as statistically significant.

3. Results

3.1. Parthenogenetic embryo production optimization The types of parthenogenetic-activated oocytes were identified under the micromanipulator (Leica DM IRB). The number of oocytes with each type was counted directly.
under the micromanipulator (630×). The karyotype analysis showed that the observed haploid oocytes had a normal 20,X karyotype, while the observed diploid oocytes had a normal 40,XX karyotype (Figure 3), which confirmed the ploidy (haploid or diploid) of the oocytes identified under the micromanipulator.

There was an effect of different chemical combinations on the types of activated oocytes. Oocytes treated separately with ethanol or SrCl₂ produced significantly higher percentages of the haploid type (homozygous or mosaic haploid) than the diploid type (homozygous diploid; no heterozygous diploid produced) (P < 0.05; Table 3). Treatment with 10% ethanol for 10 min produced the highest percentage of the mosaic haploid type (41.7%; Table 3), while ethanol combined with 6-DMAP or CB and SrCl₂ combined with CB produced significantly more of the heterozygous diploid type than the homozygous or mosaic haploid type (P < 0.05; Table 3).

There was an effect of different chemical combinations on the development potential of parthenogenetic embryo. Oocytes treated with ethanol separately had a significantly lower rate of blastulation than other treatments (Table 3). Compared to separate ethanol treatment, oocytes treated with SrCl₂ separately had improved rates of blastulation.
Table 3. The effect of different chemical combinations on the type of activated oocytes and the development potential of embryos (no. and mean ± standard deviation, percentage).

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Time</th>
<th>Oocyte (n)</th>
<th>Activated oocytes (%)</th>
<th>Homozygous haploid (%)</th>
<th>Mosaic haploid (%)</th>
<th>Homozygous diploid (%)</th>
<th>Heterozygous diploid (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% ethanol</td>
<td>5 min</td>
<td>48</td>
<td>41.7 ± 4.2a</td>
<td>20.8 ± 2.5a</td>
<td>18.8 ± 1.2a</td>
<td>2.1 ± 0.2ab</td>
<td>0ab</td>
<td>6.3 ± 0.6c</td>
</tr>
<tr>
<td></td>
<td>10 min</td>
<td>80</td>
<td>46.2 ± 3.9a</td>
<td>25.0 ± 3.3a</td>
<td>18.8 ± 1.8a</td>
<td>2.5 ± 0.2ab</td>
<td>0ab</td>
<td>7.5 ± 0.5s</td>
</tr>
<tr>
<td>10% ethanol</td>
<td>5 min</td>
<td>40</td>
<td>52.5 ± 5.5ab</td>
<td>27.8 ± 2.2ab</td>
<td>27.8 ± 2.1ab</td>
<td>2.7 ± 0.3ab</td>
<td>0ab</td>
<td>7.5 ± 0.6s</td>
</tr>
<tr>
<td></td>
<td>10 min</td>
<td>60</td>
<td>81.7 ± 8.6d</td>
<td>38.3 ± 3.3ab</td>
<td>41.7 ± 3.9ab</td>
<td>1.7 ± 0.2ac</td>
<td>0ac</td>
<td>13.3 ± 0.9b</td>
</tr>
<tr>
<td>10% ethanol + 6-DMAP</td>
<td>2 h</td>
<td>66</td>
<td>60.6 ± 7.0b</td>
<td>4.5 ± 0.5A</td>
<td>4.5 ± 0.5A</td>
<td>6.1 ± 0.6A</td>
<td>45.5 ± 4.8b</td>
<td>12.1 ± 1.6s</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>72</td>
<td>66.7 ± 5.8c</td>
<td>4.2 ± 0.5A</td>
<td>4.2 ± 0.4A</td>
<td>4.2 ± 0.4A</td>
<td>54.2 ± 4.4b</td>
<td>13.9 ± 1.8b</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>36</td>
<td>66.7 ± 6.0c</td>
<td>2.8 ± 0.2A</td>
<td>2.8 ± 0.1A</td>
<td>2.8 ± 0.3A</td>
<td>58.3 ± 6.1b</td>
<td>22.2 ± 3.1c</td>
</tr>
<tr>
<td>10% ethanol + 6-DMAP + CB</td>
<td>6 h</td>
<td>40</td>
<td>75.0 ± 7.8d</td>
<td>5.0 ± 0.4A</td>
<td>5.0 ± 0.5A</td>
<td>7.5 ± 0.5A</td>
<td>57.5 ± 5.3b</td>
<td>22.5 ± 2.0d</td>
</tr>
<tr>
<td>SrCl2</td>
<td>2 h</td>
<td>60</td>
<td>55.0 ± 6.0b</td>
<td>20.0 ± 1.6A</td>
<td>30.0 ± 2.6A</td>
<td>5.0 ± 0.4Ab</td>
<td>0ab</td>
<td>10.0 ± 1.2b</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>38</td>
<td>63.1 ± 5.2c</td>
<td>26.3 ± 3.1Ab</td>
<td>31.6 ± 3.6Ab</td>
<td>5.3 ± 0.3Ab</td>
<td>0ab</td>
<td>13.2 ± 1.5b</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>40</td>
<td>62.5 ± 5.9c</td>
<td>32.5 ± 2.3Ab</td>
<td>25.0 ± 2.9Ab</td>
<td>5.0 ± 0.4Ab</td>
<td>0ab</td>
<td>12.5 ± 0.9b</td>
</tr>
<tr>
<td>SrCl2 + CB</td>
<td>2 h</td>
<td>48</td>
<td>75.0 ± 7.1d</td>
<td>4.2 ± 0.3A</td>
<td>6.2 ± 0.6A</td>
<td>6.2 ± 0.6A</td>
<td>58.3 ± 5.0b</td>
<td>22.9 ± 2.0b</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>54</td>
<td>85.2 ± 7.2b</td>
<td>3.7 ± 0.4A</td>
<td>5.6 ± 0.5A</td>
<td>5.6 ± 0.5A</td>
<td>70.4 ± 7.1b</td>
<td>29.6 ± 3.0c</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>50</td>
<td>76.0 ± 5.9d</td>
<td>6.0 ± 0.7A</td>
<td>6.0 ± 0.5A</td>
<td>8.0 ± 0.7A</td>
<td>56.0 ± 5.9b</td>
<td>22.0 ± 1.8c</td>
</tr>
</tbody>
</table>

Different uppercase superscripts in the same line indicate difference at P < 0.05.
Different lowercase superscripts in the same column indicate difference at P < 0.05.

(P < 0.05; Table 3), while the rate was still significantly lower than that of 10% ethanol combined with 6-DMAP treated for 6 h (22.2%; Table 3), 10% ethanol combined with 6-DMAP and CB treated for 6 h (22.5%; Table 3), and SrCl2 combined with CB treated for 2 h (22.9%) or 4 h (29.6%) or 6 h (22.0%) (P < 0.05; Table 3), which all produced significantly more of the heterozygous diploid type. Oocytes treated with 10% ethanol combined with 6-DMAP for 6 h had significantly higher blastulation rates than those treated for 2 h and 4 h (P < 0.05; Table 3). Oocytes treated with SrCl2, combined with CB for 2 h, 4 h, and 6 h had no significant difference in blastulation rates (P > 0.05; Table 3). Oocytes activated by SrCl2, combined with CB for 4 h produced the highest amount of the diploid type (70.4%; Table 3) and the highest blastulation rate (29.6%; Table 3).

The in vitro development of parthenogenetic embryos is seen in Figure 4.

3.2. Sex identification for male embryos

3.2.1. Optimization of duplex PCR method for sex identification

The duplex PCR amplification results (Figure 5) showed that there were two bands in Lanes 1, 2, 3, and 7 (4 male mice): one band of 250 bp in length and another band between 250 bp and 500 bp, which were consistent with the expected lengths of 250 bp and 399 bp. There was only one band in Lanes 4, 5, 6, and 8 (4 female mice) between 250 bp and 500 bp, which was consistent with the expected 399 bp. It suggested that optimized duplex PCR for the SRY and ZFX genes could be used as the method for sex identification.

3.2.2. Optimal number of blastomeres for embryo sex identification

There was a significant difference for the sex identification rate between 2 and 4 blastomeres from 8-cell embryos. Using 2 blastomeres as the template, the sex of 22 out of 32 embryos could be identified (10 male embryos and 12 female embryos) by the previously optimized duplex PCR method, while using 4 blastomeres as the template, the sex of 35 out of 38 embryos could be identified (17 male embryos and 18 female embryos). The sex identification rate using 4 blastomeres was significantly higher than that using 2 blastomeres (P < 0.05; Table 4). Therefore, 4 blastomeres is the optimal template for sex identification of embryos.

3.3. Embryo aggregation

There was no effect of pronase concentration or treatment time on the rate of bare embryos (P > 0.05; Table 5), while there was an effect of different aggregation systems on the aggregation and development potential of the chimeric embryo. For each type of pronase treatment, the embryos aggregated in our self-made concave hole had significantly higher development rates and blastulation rates (P < 0.05; Table 5). Aggregation of the embryos treated by 0.25%...
pronase for 10 min in our self-made concave hole had the highest aggregation rate, development potential, and blastulation rate (90%, 70%, and 40%; Table 5).

3.4. pESC derivation

pESCs have an obvious boundary with the surrounding MEF cells and exhibit morphology typical of undifferentiated embryonic stem cells (Figure 6). We compared the isolation rate of pESCs derived from the parthenogenetic haploid blastocyst, heterozygous diploid blastocyst, and the chimeric embryo.

In this study, we generated 3 pESC colonies from 29 parthenogenetic haploid embryos (10.3 ± 0.9%; Table 6), 7 pESC colonies from 35 parthenogenetic heterozygous diploid embryos (20 ± 1.8%; Table 6), and 9 ESC colonies from 14 chimeric embryos. Out of the 9 ESCs, 5 ESCs were identified to be pESCs using the previously optimized duplex PCR method for sex identification. The two pESC colonies that we randomly selected had similar results. There was an effect of different types of embryos on the blastulation rate and pESC isolation rate (P < 0.05; Table 6). For the parthenogenetic embryos, the heterozygous diploid type had a significantly higher blastocyst rate and pESC isolation rate than the haploid type (P < 0.05; Table 6), which were both significantly lower than those of the chimeric embryos (P < 0.05; Table 6). Therefore, the chimeric embryo produced by the male embryo and parthenogenetic embryo improved the development potential and the pESC derivation. qRT-PCR for pluripotency genes (POUSF1, NANOG, CDX-2, REX-1) also showed that the relative expression levels of these genes were different among pESCs from these three types.
of blastocysts: except for REX-1, the relative expression levels of POU5F1, NANOG, and CDX-2 in pESCs from the chimeric embryo were all higher than those from the heterozygous diploid blastocyst, which were both significantly higher than that from the parthenogenetic haploid blastocyst (control) (Figure 7).

4. Discussion
Mice parthenogenetic embryonic stem cell lines have been well established (Lee et al., 2008; Ju et al., 2008); however, the potential development of parthenogenetic embryos and the pESC derivation efficiency is still very low (Liu et al., 1998; Cibelli et al., 2002). Therefore, the present study, by the optimization of parthenogenetic embryo production and the aggregation of the parthenogenetic diploid embryo (produced by the optimized method) and the identified male embryo, aimed to investigate a method to improve the development of parthenogenetic embryos and pESC derivation in mice.

Regarding the parthenogenetic embryo production optimization, under normal physiological conditions, oocytes are activated by signals and factors released by sperm, which elevates the level of endogenous Ca²⁺ and activates protein kinase C to decrease the level of maturation-promoting factor (MPF), resulting in the initiation of meiosis. It is suggested that oocytes activated by artificial activation methods cause changes similar to those caused by fertilization. All factors that could cause Ca²⁺ oscillation are able to activate oocytes (Han and Gao, 2013). A single Ca²⁺ transient elevation is only able to reduce MPF activity and multiple transient Ca²⁺ stimulations are required to maintain low levels of this

Table 4. Sex identification rate between 2 and 4 blastomeres from 8-cell embryos.

<table>
<thead>
<tr>
<th>Number of 8-cell embryos (n)</th>
<th>Number of templates (blastomeres) (n)</th>
<th>Sex identification rate (%)</th>
<th>Misdetection rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>2</td>
<td>68.75% (22/32) a</td>
<td>31.25% (10/32) a</td>
</tr>
<tr>
<td>38</td>
<td>4</td>
<td>92.10% (35/38) b</td>
<td>7.90% (3/38) b</td>
</tr>
</tbody>
</table>

Different lowercase superscripts in the same column indicate difference at P < 0.05.

Table 5. The effect of different pronase treatments and different aggregation systems on the aggregation and development potential of chimeric embryos (no. and mean ± standard deviation, percentage).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>8-Cell embryos (n)</th>
<th>Rate of bare embryos (%)</th>
<th>Pairs of embryos for aggregation (n)</th>
<th>Aggregation systems</th>
<th>Rate of aggregation (%)</th>
<th>Rate of development (%)</th>
<th>Rate of blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25% pronase, 10 min</td>
<td>185</td>
<td>32.9±3.0 a</td>
<td>30</td>
<td>Ordinary M16 droplet</td>
<td>40.0±3.8 a</td>
<td>20.0±1.7 a</td>
<td>0 a</td>
</tr>
<tr>
<td>0.25% pronase, 20 min</td>
<td>176</td>
<td>34.1±2.9 a</td>
<td>30</td>
<td>Ordinary M16 droplet</td>
<td>40.0±2.8 a</td>
<td>20.0±2.5 a</td>
<td>0 a</td>
</tr>
<tr>
<td>0.5% pronase, 10 min</td>
<td>142</td>
<td>38.7±2.9 a</td>
<td>27</td>
<td>Ordinary M16 droplet</td>
<td>33.3±3.3 a</td>
<td>11.1±2.0 a</td>
<td>0 a</td>
</tr>
<tr>
<td>0.5% pronase, 20 min</td>
<td>167</td>
<td>40.1±4.3 a</td>
<td>33</td>
<td>Ordinary M16 droplet</td>
<td>36.4±4.1 a</td>
<td>18.2±2.1 a</td>
<td>0 a</td>
</tr>
</tbody>
</table>

Different lowercase superscripts in the same column indicate difference at P < 0.05.
of parthenogenetic embryos and for the developmental potential of pESCs in mice (O’Neil et al., 1991; Liu et al., 1998). Therefore, this study first investigated the effect of different chemical combinations on the ploidy (haploid or diploid) of activated oocytes and the development potential of parthenogenetic embryos, making an optimization for parthenogenetic embryo production.

We concluded that the combination of SrCl₂ and CB for 4 h was the best activation method, which was consistent with the study by Ma et al. (2005). Oocytes treated with SrCl₂ separately for 2 h, 4 h, or 6 h had significantly higher blastulation rates than those treated with 5% ethanol separately for 5 min or 10 min and those treated with 10% ethanol for 5 min (Table 3), which was consistent with previous studies (Allen et al., 1994; Swann and Ozil, 1994; Szabo and Mann, 1994; Newman-Smith and Werb, 1995; Toth et al., 2006). Oocytes activated by 10% ethanol for 10 min had a 13.3% blastulation rate in this study, which was similar to the study by Deng et al. (1996) (12.3%). In this study, oocytes treated by ethanol or SrCl₂ separately produced significantly more of the parthenogenetic haploid type, with significantly lower blastulation rates. While the combinations of chemicals (ethanol + 6-DMAP + CB; SrCl₂ + CB) produced significantly more of the heterozygous diploid type and higher blastulation rates (Table 3), which was consistent with previous studies (Czołowska et al., 1986; Szollosi et al., 1993; Han and Gao, 2013), the reason for this is that 6-DMAP or CB could contribute to the formation of the heterozygous diploid type by the effective prohibition of Pb²⁺ (Sun et al., 1999; Latham et al., 2002; Fan and Sun, 2004). Studies concluded that the heterozygous diploid type generated significantly higher blastulation rates than the haploid type (O’Neil et al., 1991; Liu et al., 1998; Latham et al., 2002), which was validated by the present study.

For the sex identification of male embryos, the duplex PCR method with two primer sets for the SRY (sex determining region Y) and ZFX (zinc finger X) genes used

Table 6. The effect of different types of embryos on the isolation rate of pESCs (no. and mean ± standard deviation, percentage).

<table>
<thead>
<tr>
<th>Number of oocytes/embryos (n)</th>
<th>Treatment</th>
<th>Type and number (n)</th>
<th>Blastulation rate (%)</th>
<th>pESC isolation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>302 oocytes</td>
<td>10% ethanol, 10 min</td>
<td>Parthenogenetic haploid (242)</td>
<td>12.0 ± 1.1ᵃ (n: 29)</td>
<td>10.3 ± 0.9ᵇ (n: 3)</td>
</tr>
<tr>
<td>175 oocytes</td>
<td>SrCl₂ + CB, 4 h</td>
<td>Heterozygous diploid (123)</td>
<td>28.4 ± 2.3ᵇ (n: 35)</td>
<td>20 ± 1.8ᵇ (n: 7)</td>
</tr>
<tr>
<td>35 pairs (35 male + 35 heterozygous diploid)</td>
<td>0.25% pronase, 10 min</td>
<td>Chimeric embryo (30)</td>
<td>46.7 ± 5.0ᶜ (n: 14)</td>
<td>35.7 ± 3.7ᶜ (n: 5)</td>
</tr>
</tbody>
</table>

Different lowercase superscripts in the same column indicate difference at P < 0.05.
in the present study was suggested to be effective. Sex differentiation of mammals is primarily dependent on the SRY gene, which plays a key role in male development; the ZFX gene is present in both males and females (Han et al., 2007). This duplex PCR method has been used in previous studies for sex identification in deer (Han et al., 2007; Lindsay and Belant, 2008).

In the present study, we concluded that the aggregation of the male and parthenogenetic embryos improved the developmental potential of embryos and the pESC derivation in mice (Table 6), which was consistent with previous studies for sex identification in deer (Han et al., 2007; Lindsay and Belant, 2008).

Studies by Lee et al. (1994, 2006, 2013) showed that zona-mediated embryo aggregation could improve the efficiency of pig cloned ES-like cells. Similar results were obtained for parthenogenetic embryos and parthenogenetic stem cells in pigs and mice (Shan et al., 2012; Saadeldin et al., 2014).

In conclusion, aggregation of the parthenogenetic diploid embryo (produced by 10 mM SrCl₂ + 5 µg/mL CB for 4 h) and the male embryo (identified by duplex PCR method) in our self-made concave hole system could improve the blastocyst development and pESC derivation in mice.

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**Figure 7.** Expression profiles of the pluripotency-related genes POU5F1 (A), NANOG (B), CDX-2 (C), and REX-1 (D) among pESCs derived from three types of blastocysts (parthenogenetic haploid (control), heterozygous diploid, and chimeric embryo). Each bar represents the relative fold-change among groups. Data shown in the figure are from three replicates and values are presented as mean ± SEM. Values with different superscripts (a, b) within groups are significantly different (P < 0.05).
References


