MG132 Treatment During Oocyte Maturation Improves Embryonic Development After Somatic Cell Nuclear Transfer and Alters Oocyte and Embryo Transcript Abundance in Pigs

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SUMMARY
The objective of this study was to examine the effect of treating pig oocytes during in vitro maturation (IVM) with a proteasome inhibitor, MG132, on oocyte maturation and embryonic development. In one series of experiments, oocytes from medium-sized follicles (3–8 mm in diameter) were untreated (MCO) or treated with MG132 during 0–22 hr (M0–22) or 30–42 hr (M30–42) of IVM. There was no significant effect of MG132 on nuclear maturation or cytoplasmic maturation (as assessed by intracellular amounts of glutathione and p34cdc2 kinase activity). Blastocyst formation after parthenogenetic activation (PA) and somatic cell nuclear transfer (SCNT), however, was increased for M30–42 (65.2% and 27.7% for PA and SCNT, respectively) compared to MCO (42.6% and 13.6%, respectively) and M0–22 (45.3% and 19.5%, respectively; P < 0.05). Expression of PCNA and ERK2 was increased in M30–42 for IVM oocytes while transcript abundance for POUF51, DNMT1, FGFR2, and PCNA was increased in M30–42 for 4-cell SCNT embryos. When oocytes derived from small follicles (<3 mm in diameter) were untreated (SCO) or treated with MG132 during 0–22 hr (S0–22), 30–42 hr (S30–42) of IVM, or 0–22 and 30–42 hr of IVM (S0–22/30–42), expression of POU5F1, DNMT1, FGFR2, and PCNA and blastocyst formation were increased for SCNT embryos derived from S30 to 42 (16.5%) and S0–22/30–42 oocytes (20.8%) as compared to embryos from SCO (8.7%) or S0–22 oocytes (8.8%; P < 0.05). Results demonstrate that treatment of oocytes with MG132 during the later stage of IVM improves embryonic development and alters gene expression in pigs.


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INTRODUCTION
Advances in reproductive technology including in vitro fertilization (IVF), intracytoplasmic sperm injection, and somatic cell nuclear transfer (SCNT), have made the

Abbreviations: IVF, in vitro fertilization; IVM, in vitro maturation; MI/MII, metaphase III; MPF, maturation-promoting factor; PA, parthenogenetic activation; SCNT, somatic cell nuclear transfer; UPP, ubiquitin-proteasome pathway.
production of live offspring from in vitro-produced embryos a routine practice in a wide variety of mammalian species. In vitro embryo production depends on high-quality, matured oocytes for a high success rate. Various modifications have been applied to in vitro maturation (IVM) systems to improve oocyte competence, including use of growth factors and antioxidants (Abeydeera et al., 1998; De Matos and Furnus, 2000; Mito et al., 2009) and synchronization of meiosis using chemicals such as dibutyryl cyclic adenosine monophosphate or cycloheximide (Funahashi et al., 1997; Ye et al., 2002).

Pig oocytes need a relatively long period of culture to mature in vitro compared with bovine and ovine oocytes. The coordinated synthesis and degradation of proteins plays an important role in controlling cell cycle progression in somatic cells and oocytes (Peters, 1999; Moor and Dai, 2001). It has been reported that protein degradation via the ubiquitin-proteasome pathway (UPP) is an essential process in meiotic maturation of pig, rat, and mouse oocytes (Josefsberg et al., 2000; Huo et al., 2004ab; Sun et al., 2004). Moreover, specific proteins such as ubiquitin C-terminal hydrolase-L1 and ubiquitin C-terminal hydrolase-L3 have been implicated in meiotic maturation of pig oocytes (Susor et al., 2007; Yi et al., 2008). Treatment of oocytes with a proteasome inhibitor during oocyte maturation can modulate meiotic maturation by influencing degradation of regulatory protein such as cyclin B1 and mitogen-activated protein kinase phosphorylation (Josefsberg et al., 2000; Huo et al., 2004ab). One specific proteasome inhibitor, MG132, can induce germinal vesicle breakdown and arrest oocytes at the metaphase-I (MI) stage in a dose- and time-dependent manner (Josefsberg et al., 2000; Chmelikova et al., 2004). In other studies on pig SCNT, a beneficial effect of MG132 on development of SCNT embryos has been shown when reconstructed oocytes were treated with MG132 from 0 to 22 hr (42.6% and 45.3%, respectively). Blastocyst cell number was similar for all treatments.

The objective of the present study was to examine the effect of MG132 treatment during IVM on oocyte maturation and embryonic development after parthenogenetic activation (PA) and SCNT in pigs. To this end, we assessed effects of MG132 exposure at the beginning or end of maturation on intracellular amounts of glutathione and maturation-promoting factor (MPF) activity in oocytes, and on embryo cleavage and blastocyst formation of PA and SCNT embryos. In addition, effects of MG132 on transcript abundance for key genes involved in the cell cycle, signal transduction, transcription, and DNA methylation (CDK1, ERK2, PCNA, DNMT1, FGFR2, and POU5F1) were assessed in IVM oocytes and SCNT embryos. Our findings demonstrate that treatment of pig oocytes with MG132 at the later stage of IVM improves oocyte competence for embryonic development, probably by influencing cytoplasmic maturation, and as a result, enhancing transcript abundance in oocytes and SCNT embryos. Moreover, the beneficial effect of MG132 occurs not only for oocytes derived from medium-sized follicles but also for oocytes from small-sized follicles that have lower developmental competence.

**RESULTS**

**Effect of MG132 on Nuclear Maturation, Intracellular Glutathione Content, and MPF Activity of IVM Oocytes Derived From Medium-Sized Follicles (Experiment 1)**

As shown in Table 1, there was no significant effect of MG132 treatment at 0–22 or 30–42 hr on nuclear maturation. The proportion of oocytes that reached metaphase II (MII) were 96.1%, 90.2%, and 94.2% for control oocytes, oocytes treated with MG132 from 0 to 22 hr, and oocytes treated with MG132 from 30 to 42 hr, respectively. There was also no effect of MG132 on intracellular glutathione content or MPF activity.

**Effect of MG132 Treatment During IVM on Embryonic Development After PA (Experiment 2) and SCNT (Experiment 3)**

There was no effect of MG132 on cleavage of PA oocytes, but the proportion that developed to the blastocyst stage was affected by MG132 treatment (P < 0.05; Table 2). Development was higher for oocytes treated with MG132 from 30 to 42 hr (65.2%) than for control oocytes or oocytes treated with MG132 from 0 to 22 hr (42.6% and 45.3%, respectively). Blastocyst cell number was similar for all treatments. MG132 also affected SCNT embryos (Table 3). There was no effect of MG132 on cleavage, but the percent of reconstructed embryos that developed to the blastocyst

<table>
<thead>
<tr>
<th>MG132 treatment</th>
<th>No. of oocytes*</th>
<th>Nuclear status (%)</th>
<th>Relative amount after IVM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GV MI MII</td>
<td>GSH MPF</td>
</tr>
<tr>
<td>None</td>
<td>258</td>
<td>0.0 ± 0.0</td>
<td>3.9 ± 1.4</td>
</tr>
<tr>
<td>0–22 hr</td>
<td>250</td>
<td>0.0 ± 0.0</td>
<td>9.8 ± 2.9</td>
</tr>
<tr>
<td>30–42 hr</td>
<td>256</td>
<td>0.5 ± 0.5</td>
<td>5.3 ± 1.5</td>
</tr>
</tbody>
</table>

GV, germinal vehicle; MI, metaphase I; MII, metaphase II.

*Four replicates.
stage was higher for embryos from oocytes treated with MG132 from 30 to 42 hr (27.7%) than for embryos from control oocytes (13.6%) or oocytes treated with MG132 from 0 to 22 hr (19.5%, \( P < 0.05 \)). There was no effect of MG132 on blastocyst cell number.

Gene Expression of IVM Oocytes and SCNT Embryos After MG132 Treatment (Experiment 4)

Expression level of several genes that are known to be important in oocyte maturation and nuclear reprogramming were analyzed to determine if the effect of MG132 on blastocyst formation was associated with increased amounts of mRNA. Treatment with MG132 from 30 to 42 hr, but not from 0 to 22 hr, increased transcript abundance for PCNA and ERK2 but not for CDK in IVM oocytes (Fig. 1A). In SCNT embryos, expression of all analyzed genes (POU5F1, DNMT1, FGFR2, and PCNA) was increased in embryos derived from oocytes treated with MG132 treatment from 30 to 42 hr of IVM (\( P < 0.05 \)). Expression of POU5F1 and FGFR2 was increased for SCNT embryos derived from oocytes treated with MG132 from 0 to 22 hr (\( P < 0.05 \); Fig. 1B).

Effects of MG132 on Nuclear Maturation, Intracellular Glutathione Content, and MPF activity of IVM Oocytes Derived From Small-Sized Follicles (Experiment 5)

Nuclear maturation and intracellular glutathione content was higher for oocytes from medium-sized follicles than for oocytes derived from small follicles (\( P < 0.05 \); Table 4). There was no effect of MG132 on nuclear maturation or relative intracellular glutathione content after IVM in small follicle-derived oocytes (Table 4). In contrast, MPF activity was increased in oocytes treated with MG132 at 0–22 and 30–42 hr as compared to untreated oocytes (\( P < 0.05 \); Table 4).

Effect of MG132 on Development of PA and SCNT Embryos Derived From Oocytes From Small-Sized Follicles (Experiments 6 and 7)

The percent of activated oocytes that became blastocysts was higher for embryos derived from oocytes from medium-sized follicles than for embryos derived from oocytes from small-sized follicles (\( P < 0.05 \)), but there was no difference in cleavage or in blastocyst cell number due to origin of oocytes (Table 5). The beneficial effect of MG132 was not found for embryonic development of PA oocytes that were derived from small follicles (Table 5). In general, MG132 did not affect the developmental competence of oocytes from small follicles, although there was a tendency (\( P = 0.07 \)) for blastocyst formation to be higher for embryos from oocytes treated with MG132 from 30 to 42 hr and 0–22 and 30–42 hr (24.0%) than for embryos from control oocytes (15.5%).

Results for SCNT embryos are shown in Table 6. The percent of constructed embryos that became blastocysts was higher for embryos derived from oocytes from medium-sized follicles than for embryos derived from oocytes from small-sized follicles (\( P < 0.05 \)), but there was no difference between medium- and small-sized follicles in other measurements. In contrast to the effect for PA embryonic development, development of SCNT embryos to the blastocyst stage and blastocyst cell number were significantly affected by MG132 treatment during IVM of recipient oocytes. In particular, blastocyst formation was higher for embryos from oocytes treated with MG132 from 30 to 42 hr (16.5%) and 0–22 and 30–42 hr (20.8%) than for embryos

### TABLE 2. Effect of MG132 Treatment During In Vitro Maturation of Oocytes on Embryonic Development After Parthenogenetic Activation

<table>
<thead>
<tr>
<th>MG132 treatment</th>
<th>No. of embryos*</th>
<th>% of Embryos developed to</th>
<th>Blastocyst cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>224</td>
<td>89.8 ± 1.7</td>
<td>37.0 ± 1.7</td>
</tr>
<tr>
<td>0–22 hr</td>
<td>250</td>
<td>93.8 ± 1.8</td>
<td>38.5 ± 1.3</td>
</tr>
<tr>
<td>30–42 hr</td>
<td>218</td>
<td>94.7 ± 2.3</td>
<td>40.7 ± 1.4</td>
</tr>
</tbody>
</table>

Different superscript letters (a,b) within a column differ (\( P < 0.05 \)).

*Four replicates.

### TABLE 3. Effect of MG132 Treatment During In Vitro Maturation of Oocytes on Embryonic Development After Somatic Cell Nuclear Transfer

<table>
<thead>
<tr>
<th>MG132 treatment</th>
<th>No. of embryos*</th>
<th>% of Embryos developed to</th>
<th>Blastocyst cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>156</td>
<td>85.2 ± 3.5</td>
<td>39.4 ± 4.0</td>
</tr>
<tr>
<td>0–22 hr</td>
<td>154</td>
<td>88.0 ± 1.4</td>
<td>33.0 ± 2.8</td>
</tr>
<tr>
<td>30–42 hr</td>
<td>147</td>
<td>89.3 ± 1.4</td>
<td>37.4 ± 2.7</td>
</tr>
</tbody>
</table>

Different superscript letters (a,b) within a column differ (\( P < 0.05 \)).

*Four replicates.
from control oocytes (8.7%) or oocytes treated with MG132 from 0 to 22 hr (8.8%, P < 0.05). In addition, blastocyst cell number was greater for embryos from oocytes treated with MG132 from 30 to 42 hr (55.2 cells) and 0–22 and 30–42 hr (52.0 cells) than for embryos from control oocytes (44.5 cells) or oocytes treated with MG132 from 0 to 22 hr (23.8 cells; P < 0.05).

Effect of MG132 Treatment on Gene Expression of IVM Oocytes and SCNT Embryos Derived From Small Follicles (Experiment 8)

As compared to oocytes from medium-sized follicles, expression of all genes analyzed was reduced for IVM oocytes from small-sized follicles (Fig. 2A). Expression of PCNA and ERK2 in IVM oocytes from small-sized follicles were increased by MG132 treatment from 30 to 42 hr and 0–22 and 30–42 hr of IVM compared to untreated oocytes (P < 0.05).

Transcript abundance was greater in all cases for embryos derived from oocytes from medium-sized follicles than for embryos from control oocytes from small-sized follicles (P < 0.05; Fig. 2B). Treatment with MG132 from 0 to 22, 30–42, or 0–22 and 30–42 hr increased transcript abundance for all genes analyzed (POU5F1, DNMT1, FGFR2, and PCNA) compared to embryos derived from untreated oocytes from small follicles (P < 0.05).

**DISCUSSION**

Significant changes in protein synthesis and degradation occur in mammalian oocytes during the growing phase and meiotic maturation. Biosynthesis of many proteins decreases during meiotic maturation while some proteins become increased in amount (Sutor et al., 2007). Previous studies that examined the effect of proteasome inhibitors on oocyte maturation suggest that meiotic maturation is controlled by timely degradation of particular proteins mediated by the UPP (Josefsberg et al., 2000; Huo et al., 2004a; Sun et al., 2004; Yi et al., 2008). We found in this study that treatment of immature oocytes with the proteasomal inhibitor MG132 in the later stages of maturation improved gene expression in IVM oocytes and SCNT embryos. Thus, regulation of proteosomal degradation during later stages of maturation may be a useful method for improving the yield of pig embryos produced by PA and SCNT. Here, we used two endpoints to evaluate the ability of MG132 to improve oocyte competence for supporting embryonic development: The first was to measure development to the blastocyst stage, while the second measured transcript abundance for several genes because it has been reported that developmental competence of SCNT embryos is correlated with expression level of genes such as PCNA, OCT4, and DNMT1 (Lee et al., 2006; You et al., 2010a).

Meiotic maturation was not altered in this study by any MG132 treatment. This result was not surprising for oocytes treated at 30 hr of maturation. More than 70% of oocytes reach the MII stage by 32 hr of IVM (Ikeda and Takahashi, 2008).

**TABLE 4. Effect of MG132 Treatment During In Vitro Maturation on Meiotic Maturation, Intracellular Content of Glutathione (GSH), and p34cdc2 Kinase (Maturation-Promoting Factor; MPF) Activity in Pig Oocytes Derived From Small Follicles**

<table>
<thead>
<tr>
<th>Follicle size (diameter)</th>
<th>MG132 treatment</th>
<th>No. of oocytes cultured*</th>
<th>% of Oocytes that reached MII</th>
<th>Relative amount after IVM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium (3–8 mm)</td>
<td>None</td>
<td>259</td>
<td>89.1 ± 1.9a</td>
<td>1.0 ± 0.02b</td>
</tr>
<tr>
<td>Small (&lt;3 mm)</td>
<td>None</td>
<td>217</td>
<td>69.9 ± 2.5b</td>
<td>0.9 ± 0.02b</td>
</tr>
<tr>
<td></td>
<td>0–22 hr</td>
<td>202</td>
<td>67.9 ± 3.4b</td>
<td>0.9 ± 0.02b</td>
</tr>
<tr>
<td></td>
<td>30–42 hr</td>
<td>221</td>
<td>71.4 ± 3.2b</td>
<td>0.9 ± 0.02b</td>
</tr>
<tr>
<td></td>
<td>0–22 + 30–42 hr</td>
<td>217</td>
<td>71.7 ± 2.5b</td>
<td>0.9 ± 0.02b</td>
</tr>
</tbody>
</table>

Different superscript letters (a,b,c) within a column differ (P < 0.05).

*Four replicates.
The failure to observe an inhibitory effect of MG132 when added early in maturation, however, is in contrast to other results that MG132 inhibited germinal vesicle breakdown of pig oocytes (Sun et al., 2004) and that addition of MG132 to the IVM medium, even just before polar body extrusion, inhibited the first metaphase-to-anaphase transition and prevented polar body emission in rat oocytes (Josefsberg et al., 2000). Different concentration of MG132 used in this study would be a probable reason for this discrepancy as the 1 mM of MG132 used herein was lower than that used in other studies (10–100 mM). We chose 1 mM MG132 because this concentration was sufficient to maintain MPF activity of SCNT oocytes in the post-fusion period, and also significantly increased embryonic development in our previous study (You et al., 2010a).

Also, there was a report that prolonged (4–6 hr) treatment of reconstructed oocytes with 5 mM MG132 inhibited embryonic development in mice (Gao et al., 2005).

Cytoplasmic maturation in terms of intracellular glutathione content and MPF activity in IVM oocytes was not altered by MG132. It was not established in this study how MG132 influenced embryonic development. It is likely that MG132 inhibited UPP-mediated degradation of maternal proteins important for embryonic development. Further proteomic analysis could lead to the identification of specific proteins whose abundance is altered by MG132. It is also likely that one consequence of altered protein degradation is concomitant changes in transcript abundance in the oocyte and early embryo. Reactivation of several genes such as POU5F1, DNMT1, and FGFR2 is an essential step to allow...

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**TABLE 5. Effect of MG132 Treatment During In Vitro Maturation on Embryonic Development of Pig Oocytes After Parthenogenetic Activation**

<table>
<thead>
<tr>
<th>Follicle size (diameter)</th>
<th>MG132 treatment</th>
<th>No. of oocytes*</th>
<th>≥2-Cell</th>
<th>Blastocyst</th>
<th>Blastocyst cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium (3–8 mm)</td>
<td>None</td>
<td>212</td>
<td>87.2 ± 1.4</td>
<td>32.9 ± 2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.3 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Small (&lt;3 mm)</td>
<td>None</td>
<td>156</td>
<td>89.4 ± 1.4</td>
<td>15.5 ± 2.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.3 ± 4.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0–22 hr</td>
<td>156</td>
<td>81.2 ± 6.0</td>
<td>15.7 ± 4.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.5 ± 3.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>30–42 hr</td>
<td>143</td>
<td>90.8 ± 2.6</td>
<td>22.4 ± 3.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.5 ± 4.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>0–22 + 30–42 hr</td>
<td>146</td>
<td>87.0 ± 3.4</td>
<td>24.0 ± 2.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>36.5 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Different superscript letters (a,b) within a column differ (P < 0.05).

*Six replicates.

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**TABLE 6. Effect of MG132 Treatment During In Vitro Maturation on Embryonic Development of Pig Oocytes After Somatic Cell Nuclear Transfer**

<table>
<thead>
<tr>
<th>Follicle size (diameter)</th>
<th>MG132 treatment</th>
<th>No. of embryos*</th>
<th>≥2-Cell</th>
<th>Blastocyst</th>
<th>Blastocyst cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium (3–8 mm)</td>
<td>None</td>
<td>112</td>
<td>85.3 ± 2.0</td>
<td>14.5 ± 1.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>37.3 ± 5.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Small (&lt;3 mm)</td>
<td>None</td>
<td>74</td>
<td>80.1 ± 3.8</td>
<td>8.7 ± 1.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>44.5 ± 4.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0–22 hr</td>
<td>69</td>
<td>83.9 ± 3.7</td>
<td>8.8 ± 0.7&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>23.8 ± 3.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>30–42 hr</td>
<td>77</td>
<td>87.5 ± 1.7</td>
<td>16.5 ± 2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.2 ± 9.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>0–22 + 30–42 h</td>
<td>74</td>
<td>90.0 ± 0.8</td>
<td>20.8 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.0 ± 5.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Different superscript letters (a,b,c) within a column differ (P < 0.05).

*Three replicates.

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**Figure 2.** Relative mRNA abundance for in vitro-matured oocytes (A) and 4-cell SCNT embryos (B) that were treated with 1 mM MG132 during various stages of maturation. Immature oocytes were collected from small-sized follicles (<3 mm in diameter) and matured in vitro. Oocytes from medium-sized follicles (3–8 mm) served as a positive control (None-medium). Bars with different letters (a–d) are different (P < 0.05). 2001). The failure to observe an inhibitory effect of MG132 when added early in maturation, however, is in contrast to other results that MG132 inhibited germinal vesicle breakdown of pig oocytes (Sun et al., 2004) and that addition of MG132 to the IVM medium, even just before polar body extrusion, inhibited the first metaphase-to-anaphase transition and prevented polar body emission in rat oocytes (Josefsberg et al., 2000). Different concentration of MG132 used in this study would be a probable reason for this discrepancy as the 1 mM of MG132 used herein was lower than that used in other studies (10–100 mM). We chose 1 mM MG132 because this concentration was sufficient to maintain MPF activity of SCNT oocytes in the post-fusion period, and also significantly increased embryonic development in our previous study (You et al., 2010a). Also, there was a report that prolonged (4–6 hr) treatment of reconstructed oocytes with 5 mM MG132 inhibited embryonic development in mice (Gao et al., 2005).
normal development in mouse (Bortvin et al., 2003; Kawasumi et al., 2009), bovine (Golding et al., 2011), and human embryos (Cauffman et al., 2005). These genes have been used as markers for evaluating nuclear reprogramming of cloned embryos in pigs and mice (Boiani et al., 2002; Lee et al., 2006; You et al., 2010b). In this study, treatment of oocytes with MG132 at 30–42 hr of IVM enhanced transcript abundance for PCNA and ERK2 in IVM oocytes, and for all analyzed genes in SCNT 4-cell embryos. In pigs, an in vitro developmental block is observed at the 4-cell stage. Thus, we analyzed gene expression at the 4-cell stage to correlate transcript abundance with later development of SCNT embryos. It was not clear if the increased transcript abundance of genes in cloned embryos contributed directly to increased embryonic development, although this result was consistent with our previous study (You et al., 2010b) that blastocyst formation was higher in 4-cell SCNT pig embryos with higher expression of candidate genes (DNMT1, PCNA, FGFR2, and POU5F1). Mouse-specific Y-box protein 2 (MSY2), a germ cell-specific RNA-binding protein, is implicated in mRNA stability and MSY2-associated mRNAs are protected from degradation in oocytes (Yu et al., 2003; Medvedev et al., 2008). It is not clear in pigs if RNA-stabilizing proteins such as MSY2 in mice are present and play a role in stabilizing RNA in oocytes. Perhaps MG132 treatment inhibited degradation of proteins like MSY2 that stabilize maternal mRNA, and thereby increase steady stage amounts of key mRNA and promote proper nuclear reprogramming and embryonic development.

It is common in pigs to culture immature oocytes for 40–44 hr. Oocyte maturation and developmental competence are influenced by sequential treatments during the first and the second half of IVM (Algrany et al., 2004; Kawashima et al., 2008; Grupen and Armstrong, 2010). In this study, we treated immature oocytes with MG132 at two different stages (0–22 and 30–42 hr) of IVM to test for any stage-specific effect of MG132 treatment. Compared to the beneficial effect of MG132 treatment at the later stage of IVM, neither a stimulatory nor inhibitory effect was shown in oocyte maturation and embryonic development when oocytes were treated during the first 22 hr of IVM. This result indicates differential proteosomal activity that corresponds with distinct stages of IVM, consistent with the observation in mice that different levels of proteosomal activity are required to initiate and to sustain translational activation of mRNA during early and later stages of oocyte maturation (Yang et al., 2010).

As described earlier (Yoon et al., 2000; Marchal et al., 2002; Wu et al., 2006; Kim et al., 2010) and shown here, oocytes derived from small-sized follicles have lower developmental competence than those from larger follicles. Development of a system capable of increasing developmental competence of small follicle-derived oocytes would be helpful for securing a sufficient number of oocytes for large-scale production of in vitro-produced embryos. In this study, treatment of oocytes derived from medium-sized follicles with MG132 at the later stage of IVM improved embryonic development after PA and SCNT. Based on this result, another series of experiments was performed to examine if the low developmental competence of oocytes that were derived from small follicles could be improved by MG132 treatment during IVM. Indeed, embryonic development to the blastocyst stage after SCNT embryos from oocytes derived from small follicles was increased by MG132 treatment at the later stage of IVM (30–042 or 0–20 and 30–42 hr). Using MG132, blastocyst development for SCNT embryos made using oocytes from small follicles was comparable to that for untreated SCNT embryos made using oocytes from medium-sized follicles. In contrast to the increased development in SCNT embryos, development of PA oocytes derived from small follicles was not increased by MG132 treatment. The reason for this difference is unclear. Gene expression in IVM oocytes that were derived from small follicles was stimulated by MG132 treatment, although overall transcript abundance remained lower than for untreated oocytes that were from medium-sized follicles. It could be that low transcript abundance has a greater impact on developmental competence of PA oocytes than for embryos formed as a result of SCNT.

In summary, our results clearly demonstrate that treatment of oocytes with a proteasomal inhibitor, MG132, at the later stage of IVM improves embryonic development after PA and SCNT, probably by inhibiting UPP-mediated degradation of proteins and then creating a favorable cytoplasmic microenvironment for nuclear reprogramming and embryonic development. Production of normal live offspring resulting from transfer of in vitro-produced embryos is the most important parameter predictive of embryo normality and viability. Unfortunately, in vivo developmental competence of embryos derived from MG132-treated oocytes could not be examined in this study, but the beneficial effect of MG132 should be assessed by embryo transfer in the future.

MATERIALS AND METHODS

Culture Media

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise noted. The IVM medium was tissue culture medium 199 (Invitrogen, Grand Island, NY) that was supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 0.91 mM pyruvate, 10 ng/ml mouse epidermal growth factor, 75 μg/ml kanamycin, and 1 μg/ml insulin (Song et al., 2009). The in vitro culture (IVC) medium for embryo development was Porcine Zygote Medium-3 containing 0.3% (w/v) fatty acid-free bovine serum albumin (BSA; Yoshioka et al., 2002), which was supplemented with 2.77 mM myo-inositol, 0.34 mM tri-sodium citrate, and 10 μM β-mercaptoethanol. The proteosomal inhibitor, MG132, was purchased from Sigma–Aldrich.

In Vivo Maturation of Oocytes

Pig ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in saline. After washing in fresh saline, the follicles present in the ovarian cortex were examined and categorized into two...
groups according to their size: small (<3 mm in diameter) and medium (3–8 mm). Follicles larger than 8 mm in diameter were not included in these studies. Cumulus–oocyte complexes (COCs) were aspirated from superficial follicles using an 18-gauge needle and a 10-ml syringe. After removing apparently degenerated oocytes, COCs with a thick layer of cumulus cells were washed twice in HEPES-buffered Tyrode’s medium (TLH) containing 0.05% (w/v) polyvinyl alcohol (PVA; TLH-PVA; Bavister et al., 1983) and once in IVC medium. Groups of 40–70 COCs from each category were placed into individual wells of a 4-well multi-dish (Nunc, Roskilde, Denmark) that contained 500 µl IVC medium supplemented with 10 IU/ml equine chorionic gonadotropin (Intervet International BV, Boxmeer, Holland) and 10 IU/ml human chorionic gonadotropin (Intervet), and cultured at 39 °C in a humidified atmosphere of 5% CO2. After 22 hr of maturation, the COCs were washed three times in fresh, hormone-free IVC medium, and then cultured for an additional 20 hr. COCs were untreated or treated with 1 mM MG132 during IVM according to the experimental design.

Experimental Design
In Experiments 1–4, oocytes from medium-sized follicles were untreated or treated with 1 µM MG132 during 0–22 or 30–42 hr of IVM. In Experiment 1, effects on nuclear maturation, intracellular glutathione content, and MPF (p34cdc2 kinase) activity of IVM oocytes were examined. Subsequently, effects of MG132 on embryonic development after PA (Experiment 2) and SCNT (Experiment 3) were determined. Finally, effects of MG132 on transcript abundance in Experiment 4.

Experiments 5–8 were conducted to determine if beneficial effects of MG132 would be seen in oocytes that were derived from small follicles. For each experiment, oocytes derived from small follicles were untreated or treated with 1 µM MG132 during 0–22, 30–42, or 0–22 hr, and, then 30–42 hr of IVM. In addition, a control group of oocytes from medium-sized follicles was included. The same measurements as for Experiments 1–4 were made. Each experiment was conducted with 4–6 replicates.

Measurement of Intracellular Glutathione Content
Intracellular glutathione content of oocytes was measured using CellTracker Blue CMF-H2C (4-chloromethyl-6,8-difluoro-7-hydroxycoumarin; Invitrogen), as described (Sakatani et al., 2007). A group of 20 denuded oocytes at the MII stage from each group was collected 44 hr after IVM and incubated for 30 min in the dark in TLH-PVA that was supplemented with 10 µM CellTracker. After incubation, oocytes were washed with Dulbecco’s phosphate-buffered saline (Invitrogen) containing 0.1% (w/v) PVA, placed into 10-µl droplets, and observed for fluorescence under an epifluorescence microscope (TE-300; Nikon, Tokyo, Japan) with a UV filter (370 nm). Fluorescent images were recorded and saved as graphic files in TIFF format. The fluorescence intensity of oocytes was analyzed by ImageJ software and normalized to that of untreated control oocytes.

Measurement of p34cdc2 Kinase (MPF) Activity
MPF activity was measured with a MESACUP cdc2 kinase assay kit (Medical & Biological Laboratories; MBL, Nagoya, Japan) as described previously (Anas et al., 2000). Briefly, a group of 20 oocytes at the MII stage was washed twice with the cdc2 kinase sample buffer consisting of 50 mM Tris–HCl, 0.5 M NaCl, 5 mM EDTA, 2 mM EGTA, 0.01% (v/v) polyoxyethylene lauryl ether (Brij 35), 1 mM phenylmethylsulfonyl fluoride, 0.05 mg/ml leupeptin, 50 mM Na-mercaptoethanol, 25 mM β-glycerophosphate, and 1 mM Na-orthovanadate. Oocytes were then transferred to micro-tubes containing 5 µl cdc2 kinase buffer and lysed by repeated freezing in liquid nitrogen and thawing in warm water. The oocyte lysates were stored at −80 °C until used for the assay. Lysates (5 µl) were mixed with 45 µl kinase assay buffer containing 25 mM Hepes buffer 10 mM MgCl2, 10% (v/v) biotinylated MV peptide (SLTSSSPGGATC), and 0.1 mM ATP, and then the mixture was incubated for 30 min at 30 °C. The phosphorylation reaction was terminated by addition of 200 µl stop reagent (phosphate-buffered saline containing 50 mM EGTA) and centrifuged for 15 sec at 14,000 rpm. The phosphorylation of biotinylated MV peptide was detected at 492 nm using a plate reader. Data were expressed relative to p34cdc2 kinase activity in untreated control oocytes.

Gene Expression Analysis by Real-Time PCR (RT-PCR)
Expression of genes in IVM oocytes (CDK1, PCNA, and ERK2) and 4-cell SCNT embryos (POU5F1, DMNT1, FGFR2, and PCNA) were analyzed by real-time polymerase chain reaction (RT-PCR) as described previously (Zhu and Yu, 2004). Total RNA was isolated from IVM oocytes and SCNT embryos on day 2 of IVC using AccuZol™ (Bioneer, Alameda, CA) according to the manufacturer’s instructions. cDNAs were synthesized from total RNA using the Reverse Transcription system (Promega, Madison, WI). Expression levels of specific genes in oocytes were quantified by RT-PCR (Rotor-Gene 3000 system; Corbett, Mortlake, Australia) using the PrimeScript RT-PCR kit (Takara, Shiga, Japan). Primer3 software (Whitehead Institute, MIT Center for Genome Research, Cambridge, MA) was used to design primers used in the study (Table 7). The PCR specificity was checked by melting curve data analysis. All results were normalized to 18S mRNA. Relative mRNA level was presented as 2−ΔΔCt, where Ct = threshold cycle for target amplification, ΔCt = Ct_target gene − Ct_internal reference (18S), and ΔΔCt = ΔCt_sample − ΔCt_calibrator (Matusuoka et al., 1999; Pfaffl, 2001). The experiment was repeated four times.
Examination of Nuclear Status of Oocytes After IVM

At 44 hr of IVM, oocytes were mounted on glass slides, fixed for 24 hr in 25% (v/v) acetic acid in ethanol at 4°C, and stained with 1% (w/v) orcein in 45% (v/v) acetic acid. Oocytes were analyzed under a phase-contrast microscope and classified according to the nuclear maturation stage as germinal vehicle breakdown, pro-MI-to-MI, and MII.

Preparation of Donor Cells

Skin fibroblasts were cultured in 4-well dishes and grown in Dulbecco’s modified Eagle’s medium with F-12 (Invitrogen), supplemented with 15% (v/v) fetal bovine serum, until a complete monolayer of cells had formed. The cell cycle of the donor cells was synchronized at the G0/G1 stage by contact inhibition for 48 hr, respectively (Song et al., 2009). The SCNT and PA embryos were treated with 0.4 μg/ml demecolcine or 5 μg/ml cytochalasin B in IVC medium for 4 hr, respectively (Song et al., 2009). The SCNT and PA embryos were washed three times in fresh IVC medium, transferred into 30-μl IVC droplets under mineral oil, and then cultured at 39°C in a humidified atmosphere of 5% CO2, 5% O2, and 90% N2 for 7 days. Cleavage and blastocyst formation were evaluated on days 2 and 7, respectively, with the day of SCNT or PA designated as Day 0. The total blastocyst cell count was performed using Hoechst 33342 staining under an epifluorescence microscope.

Nuclear Transfer

The base medium for oocyte manipulation was calcium-free TLH-BSA containing 5 μg/ml cytochalasin B. After 42 hr of IVM, denuded oocytes were incubated for 15 min in a manipulation medium that contained 5 μg/ml Hoechst 33342, washed twice in fresh medium, and then placed into a manipulation medium droplet that was overlaid with mineral oil. MII oocytes were enucleated by aspirating the first polar body and MII chromosomes using a 17-μm beveled glass pipette (Humagen, Charlottesville, VA). Enucleation was confirmed under an epifluorescent microscope. After enucleation, a single donor cell was inserted into the perivitelline space of each oocyte. Cell–oocyte coupling were placed on a 1-mm manipulation cell was overlaid with 1 ml of 280 mM mannitol that contained 0.001 mM CaCl2 and 0.05 mM MgCl2. Membrane fusion was induced by applying an alternating current field of 2 V, 1 MHz for 2 sec, followed by two direct current pulses of 170 V for 25 μsec using a cell fusion generator (LF101; NepaGene, Chiba, Japan). The oocytes were incubated for 1 hr in TLH-BSA and examined for fusion under a stereomicroscope prior to activation.

Oocyte Activation and Embryo Culture

Reconstructed oocytes were activated by two pulses of a 120 V/mm direct current for 60 μsec in 280 mM mannitol that contained 0.01 mM CaCl2 and 0.05 mM MgCl2. For PA, the oocytes with polar bodies at 42 hr of IVM were activated using a pulse sequence identical to that used to activate SCNT oocytes. Following electrical activation, the SCNT and PA embryos were treated with 0.4 μg/ml demecolcine or 5 μg/ml cytochalasin B in IVC medium for 4 hr, respectively (Song et al., 2009). The SCNT and PA embryos were washed three times in fresh IVC medium, transferred into 30-μl IVC droplets under mineral oil, and then cultured at 39°C in a humidified atmosphere of 5% CO2, 5% O2, and 90% N2 for 7 days. Cleavage and blastocyst formation were evaluated on days 2 and 7, respectively, with the day of SCNT or PA designated as Day 0. The total blastocyst cell count was performed using Hoechst 33342 staining under an epifluorescence microscope.

Statistical Analysis

All statistical analyses were performed using the Statistical Analysis System (version 9.1; SAS Institute, Cary, NC). Data were analyzed using the General Linear Models procedure followed by the least significant difference mean separation procedure when treatments differed at P < 0.05. Percentage data were subjected to arcsine transformation before analysis to maintain homogeneity of variance. The results are expressed as mean ± standard error of the mean (SEM).

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REFERENCES


