The proteasomal inhibitor MG132 increases the efficiency of mouse embryo production after cloning by electrofusion

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Abstract

In this study, we cloned mice from ES cells by a post-electrofusion MG132 treatment and improved development of cloned embryos with a sequential cultivation protocol. When 5 μM MG132, a proteasome inhibitor, were used to treat the reconstructed embryos, the capacity of in vitro development, implantation and full-term development were significantly improved. Blastocyst formation rates of the reconstructed embryos from X4 ES cells (F1 strain derived from C57BL/6 × 129sv) and J1 ES cells obtained with or without MG132 treatment were 66.9% and 26.6%, and 66.1% and 34.5% respectively (P < 0.05). A total of 146 two-cell embryos cloned from X4 ES cells with MG132 treatment were transferred to recipients, and five cloned pups (3.4%) were born, of which four survived. When the same numbers of two-cell embryos cloned from X4 ES cells without MG132 treatment were transferred, however, no live-born mice were obtained. When embryos cloned from J1 ES cells without MG132 treatment were cultured in KSOM medium for 54 h followed by culture in CZB medium containing 5.6 mM glucose for 42 h, the blastocyst rate was significantly higher than when they were cultured in KSOM continuously for 96 h (34.5% vs 17.1%). However, sequential cultivation did not improve the development of embryos cloned with MG132 treatment and that of parthenotes. In conclusion, MG132 treatment increased the developmental potential of reconstructed mouse embryos, and sequential cultivation improved development of the embryos cloned by electrofusion without MG132 treatment.

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Introduction

Several protocols have been developed for cloning mice, particularly with respect to the technique for the transfer of the nucleus into the oocyte cytoplasm. Most use a piezo-driven nuclear injection protocol, by which the first cloned mouse was obtained by Wakayama et al. (1998). The conventional electrofusion method, used extensively in livestock cloning, was not successfully applied to cloning of mice until Ogura et al. (2000) produced cloned mice by performing membrane fusion in Ca^{2+}-free fusion solution. Activation of reconstructed oocytes induced by electropulses at the time of fusion reduces the maturation-promoting factor (MPF) level in recipient oocytes, high levels of MPF are very important for nuclear reprogramming. Ca^{2+}-free fusion solution could prevent oocyte activation. However, this is confounded by the low survival rate and fusion after pulsing (Ogura et al. 2000). Therefore, methods to avoid activation of reconstructed oocytes would enhance the application of electrofusion in mouse cloning. MG132, an ubiquitin-mediated proteasome proteolysis inhibitor, inhibited the degradation of cyclin B and hence maintained a high MPF activity of oocytes (Josefsberg et al. 2000, Zhou et al. 2003).

In addition, cultivation conditions, including the composition of medium (Chung et al. 2002), concentration of oxygen (Gao et al. 2003b) and cultivation procedure (Heindryckx et al. 2001), dramatically influenced the in vitro development of cloned embryos. Previous studies demonstrated that the requirement for glucose of cloned mouse embryos is different from normal embryos cultured in vitro, and that sequential cultivation contributes to the development of cloned embryos (Heindryckx et al. 2001, Chung et al. 2002).

In this study, MG132 was used during and after electrofusion to sustain a high MPF level in oocytes, and its effect on the developmental capacity of cloned embryos was evaluated. Cloned embryos produced from different protocols were cultured in KSOM alone or cultured sequentially in KSOM medium and CZB medium, to refine the protocol for culture of cloned embryos.
Materials and Methods

Collection of oocytes

Female B6D2F1 (C57BL/6 × DBA/2 hybrid) mice, 6–8 weeks of age, were superovulated with 7.5 IU pregnant mare serum gonadotropin followed by 5 IU human chorionic gonadotropin (hCG) 48 h later. Mature oocytes were collected from the ampullae of oviducts 14–17 h after hCG injection and placed in M2 medium containing 0.1 mg/ml bovine testicular hyaluronidase. After complete removal of cumulus cells from the oocytes, they were transferred to fresh CZB medium and incubated in a humidified atmosphere of 5% CO₂ in air at 37 °C until enucleation.

Preparation of donor cells

Two ES cell lines, J1 ES cell line and X4 ES cell line (C57/BL6 × 129/sv), ranging from 25 to 30 and 10 to 15 passages respectively, were used. The X4 ES cell line was established by Dr Xiangyun Li and generated pups when aggregated with tetraploid embryos (Li et al. 2005). The cell lines were cultured in Dulbecco’s modified Eagle’s medium (Gibco BRL) supplemented with 15% heat-inactivated fetal calf serum, 1000 U/ml leukemia inhibitory factor and the following reagents: 2 mM l-glutamine, 1% minimum essential medium nonessential amino-acid solution (Gibco BRL) and 1% β-mercaptoethanol. The ES cells were synchronized at M phase by treatment with 3 ng/ml nocodazole for 3 h prior to manipulation, and only those cells that acquired the characteristic spherical shape were collected by gentle blowing (Fig. 1).

Nuclear transfer

The chromosomes of oocytes were removed mechanically, and the enucleated oocytes were used as recipient cytoplast (Tsunoda & Kato 1997). A single metaphase cell was injected into an oocyte under the zona pellucida, and then membrane fusion was induced by a direct current of 200 V/mm pulsed for 15 μs after a alternative current of 8 V/mm pulsed for 15–20 s in 0.3 M mannitol solution containing 0.1 mM MgCl₂, 0.05 mM CaCl₂, 0.5 mg/ml BSA, 0.5 mM HEPES and 5 μM MG132, or not. Immediately after electrofusion manipulation, the oocytes were cultured in the KSOM medium supplemented with 5 μM MG132 or not for 1 h.

Activation and MG132 treatment of oocytes

The renucleated oocytes were selected and then activated in Ca²⁺-free KSOM medium containing 10 mM Sr²⁺ for 4–6 h (Wakayama et al. 1998). The oocytes with one pronucleus and a second polar body were considered to have diploid chromosomes, and were cultured for 4 days in KSOM or KSOM plus CZBG (CZB medium containing 5.6 mM glucose) in 5% CO₂ in air at 37 °C.

Embryo culture and transfer

All the activated embryos were washed five times with KSOM before being cultured in KSOM in a humidified atmosphere of 5% CO₂ in air at 37 °C. One part of the cloned embryos were continuously cultured in KSOM for 96 h, and the second part were changed into CZBG medium at 54 h after activation and cultured up to 96 h after activation.

Two-cell reconstructed embryos were transferred into oviducts of day-0.5 pseudopregnant mice. The mice were killed on day 19.5 to examine potential development into fetuses. Living young were fostered carefully to other females. Normal, fertilized one-cell embryos were collected and cultured in KSOM for 24 h, and the two-cell embryos obtained were transferred into oviducts of day-0.5 pseudopregnant mice. The birth bodies and placentas were weighed.

Statistical analysis

Results were evaluated by the chi-square test or ANOVA, with a P value of less than 0.05 considered to be statistically significant.

Results

The effect of MG132 treatment on in vitro development of cloned embryos

Reconstructed embryos were pulsed in a fusion medium supplemented with 5 μM MG132 and incubated in KSOM supplemented with 5 μM MG132 for 1 h after pulsing.

Figure 1 Donor cells used for nuclear transfer. ES cells colonies (a) were cultured on feeder cell layer routinely. They were treated with 3 μg/ml nocodazole for 3 h before nuclear transfer, and the spherical cells (b) in M phase were used as donor cells. Magnification: 200 × .
The results showed that MG132 treatment did not affect fusion, pronuclear formation and the first cleavage of cloned embryos, but significantly promoted their development to 4–8-cell, morula and blastocyst stages (Table 1). The J1-strained ES cells were easier to fuse with oocytes than the F1-strained ES cells (89% vs 73.3%, P < 0.05).

**The effect of MG132 treatment on implantation and full-term development**

To compare implantation and full-term development of embryos cloned with or without MG132 treatment, two-cell-stage, cloned embryos were transferred into the oviducts of day-0.5 pseudopregnant mice. A total of 146 embryos cloned from X4 ES cells with MG132 treatment were transferred, from which eight dead fetuses and five live-born cloned pups (3.4%) were obtained (Table 2, Fig. 2c). Four of them survived to adulthood normally (Fig. 2d) and produced normal offspring. Large offspring were not found in these five fetuses, the birth body weight of them was in normal range and there was no significant difference from controls (1.8 ± 0.4 g vs 1.5 ± 0.1 g, P > 0.05). However, the weight of placentas was much higher than controls (0.5 ± 0.2 g vs 0.09 ± 0.01, P < 0.05; Table 3); moreover, the placentas presented a dramatic variety of size (Fig. 2a). No live births or dead fetuses were obtained from the 146 transferred embryos without MG132 treatment. Although many implantation sites were obtained, there were no fetuses in them (Fig. 2b). Neither live pups nor dead fetuses were obtained from transferred embryos cloned from J1 ES cells with or without MG132 treatment, although the blastocyst formation was similar to that in embryos cloned from X4 ES cells.

**Development of cloned embryos cultured with different protocols**

In this experiment, embryos reconstructed from J1 ES cells were either cultured continuously in KSOM for 96 h or cultured sequentially in KSOM for the first 54 h and then cultured in CZBG (CZB medium supplemented with 5.6 mM glucose) for the remaining 42 h. The results showed that sequential cultivation significantly increased blastocyst formation of embryos cloned without MG132 treatment compared to KSOM continuous culture (34.5% vs 17.1%, P < 0.05). However, sequential culture did not improve the development of embryos cloned with MG132 treatment (Table 4).

**Development of parthenotes cultured with different protocols**

When the Sr⁺-activated oocytes that had been treated with or without MG132 were cultured in KSOM alone or sequentially cultured in KSOM plus CZBG, no significant difference was found at any stage of development of parthenotes (Table 5).
Discussion

Routine nuclear transfer using electrofusion is inherently defective when performed in the mouse, because the electrical stimulus activates the recipient oocytes. Consequently, the exposure time of the donor nucleus to the inactivated ooplasm is reduced (Kono 1997). However, prolonging the duration of interaction between nucleus and MII-phase oocytes would potentially contribute to reprogramming (Wakayama et al. 1998, Koo et al. 2001, Shin et al. 2001). Therefore, a mechanism to avoid activation of recipient oocytes at the time of electrofusion is important for increasing the efficiency of mouse cloning by an electrofusion technique. Ca\(^{2+}\) influx is a mediator for MPF inactivation induced by electropulses. Ogura et al. (2000) avoided oocyte activation by using Ca\(^{2+}\)-free electrofusion solution and successfully obtained cloned mice. However, we found that many oocytes and donor cells died when electrofusion was performed in Ca\(^{2+}\)-free solution. It is therefore necessary to find an alternative method to allow the application of electrofusion in mouse cloning.

Proteasome serves as the main cellular protein degradation pathway (Goldberg 1995, Coux et al. 1996). MG132, a proteasome inhibitor, inhibits the degradation of cyclin B and sustains a high level of MPF. Another action of MG132 treatment on the cloned embryos that requires consideration is its contribution to nuclear reprogramming (Sutovsky & Prather 2004). Previous research demonstrated that MG132 treatment induced accumulation of proteins (Meriin et al. 1998, Grossin et al. 2004), changes in gene expression (Jiang et al. 2004, Wu et al. 2004) and chromosomal remodeling (Gao et al. 2005). By using MG132 to inhibit the autoactivation of rat oocytes, Zhou et al. (2003) successfully generated cloned rat. Recently, Gao et al. (2005) reported that injection of MG132 significantly increased blastocyst formation, but did not improve implantation of cloned mouse embryos. In this study, treatment of reconstructed embryos with MG132 during and for 1 h after electrofusion increased not only blastocyst formation but also implantation and full-term development. One reasonable explanation is that the length of treatment with MG132 is crucial to the full-term development of cloned embryos. In fact, in the study, Gao et al. (2005) also proved that prolonged treatment with MG132 (4–6 h) decreases the development capacity

<table>
<thead>
<tr>
<th>Donor cells</th>
<th>MG132</th>
<th>No. of embryos transferred</th>
<th>No. (%) of dead fetuses</th>
<th>No. (%) of births</th>
<th>No. of surviving pups</th>
</tr>
</thead>
<tbody>
<tr>
<td>X4 ES</td>
<td>+</td>
<td>146</td>
<td>8 (5.4)</td>
<td>5 (3.4)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>146</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>J1 ES</td>
<td>+</td>
<td>150</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>153</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2 Full-term development of two-cell embryos cloned from different ES cells with or without MG132 treatment.

Figure 2 Mice cloned from X4 ES cells and their placenta. (a) The placentas of cloned mice present a variety of weight (the left 0.84 g, the right 0.32 g). (b) Implantation sites derived from reconstructed embryos generated with MG132 treatment were observed in a recipient mouse in which there were no fetuses. (c) All three mice cloned from X4 ES cells on the day of delivery with body weight in normal range (1.8 ± 0.4 g) were normal and fertile (d).
of cloned mouse embryos; therefore, the 1-h duration of MG132 treatment in our study might be more appropriate. Secondly, such an effect might be attributed to the stronger action of MG132 on MPF level sustaining in electrofusion procedure than directly injection, because electropulse could induce the decrease of MPF level which would potentially contribute to nuclear reprogramming (Wakayama et al. 1998, Koo et al. 2001, Shin et al. 2001). However, our data also showed that although the full-term development of cloned embryos produced by electrofusion with treatment with MG132 was improved and the weight of birth body was in the normal range, the placenta was still much bigger than in controls, one of the factors contributing to the failure of animal cloning.

In the present study, two-step cultivation significantly improved blastocyst formation of the embryos cloned without MG132 treatment. The effect is possibly due to changes in the concentration of glucose in the media and in the energy metabolism pathway of preimplantation embryos. Glucose is widely used as a major energy substrate in embryo culture media. Although exposure to high concentrations of glucose during early embryonic stages causes developmental retardation in many species (Thompson et al. 1993, Kim et al. 1993), and a two-cell block in mouse and hamster embryos (Schini & Bavister 1988, Lawitts & Biggers 1991), glucose is known to be an important energy substrate for blastocyst formation in the post-compaction period of bovine embryos (Rieger et al. 1992). This dramatic diversity of the effect of glucose is related to the switch of the energy metabolism pathway in embryos. At the time of activation of the embryonic genome, energy metabolism switches from the use of lactate and pyruvate via the Krebs cycle and oxidative phosphorylation to the primary use of glucose via the Embden–Meyerhof pathway (Carney & Bavister 1987, Rieger et al. 1992). At earlier stages (before embryo genomic activation), high concentrations of glucose lead to an accumulation of Krebs cycle metabolites, which may inhibit embryo development (Kwun et al. 2003).

However, the two-step culture had no marked effects on the development of parthenotes. In fact, despite a low concentration of glucose, KSOM was able to support the development of normal preimplantation embryos. This suggests that the development of cloned mouse embryos is different from normal embryos and needs higher concentrations of glucose at the late preimplantation stage. This result is consistent with the report that cloned mouse embryos maintain some characteristics of somatic cells and require a higher concentration of glucose when cultured in vitro (Gao et al. 2003a). In embryos cloned with MG132 treatment, however, the requirement for a higher glucose level disappears. This may result from an improvement of reprogramming with MG132 treatment, possibly because of the prolonged exposure of the donor nuclei to a high MPF level (Wakayama et al. 1998, Koo et al. 2001, Shin et al. 2001), or accumulation of crucial proteins (Meriin et al. 1998, Grossin et al. 2004) and changes in gene expression (Jiang et al. 2004, Wu et al. 2004).

Table 3 The weight of birth body and placenta.

<table>
<thead>
<tr>
<th>Source of fetus</th>
<th>Body weight (g)</th>
<th>Mass of placenta (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>1.5 ± 0.11a</td>
<td>0.09 ± 0.01b</td>
</tr>
<tr>
<td>Cloned*</td>
<td>1.8 ± 0.4a</td>
<td>0.5 ± 0.2b</td>
</tr>
</tbody>
</table>

* Eight two-cell embryos were transferred into the oviduct of pseudopregnant mice.

Table 4 Development of embryos cloned from JI ES cells with or without MG132 treatment when cultured in different protocols.

<table>
<thead>
<tr>
<th>MG132</th>
<th>Culture protocols</th>
<th>No. of pronuclear embryos</th>
<th>No. (%) of cleaved embryos</th>
<th>No. (%) of 4–8 cell embryos</th>
<th>No. (%) of morulae</th>
<th>No. (%) of blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>K*</td>
<td>61</td>
<td>56 (91.8)a</td>
<td>53 (86.9)a</td>
<td>51 (83.6)a</td>
<td>42 (68.9)a</td>
</tr>
<tr>
<td></td>
<td>K + G**</td>
<td>121</td>
<td>112 (92.6)a</td>
<td>102 (84.3)a</td>
<td>94 (77.7)a</td>
<td>80 (66.1)a</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>152</td>
<td>133 (87.5)a</td>
<td>66 (43.4)b</td>
<td>51 (36.2)b</td>
<td>26 (17.1)b</td>
</tr>
<tr>
<td></td>
<td>K + G</td>
<td>168</td>
<td>148 (88)a</td>
<td>108 (64.3)a</td>
<td>89 (53)b</td>
<td>58 (34.5)c</td>
</tr>
</tbody>
</table>

* Cultured in KSOM.

** Sequentially cultured in KSOM and CZBG (see text).

Table 5 Development of parthenotes cultured with different protocols.

<table>
<thead>
<tr>
<th>MG132</th>
<th>Culture protocols</th>
<th>No. of pronuclear embryos</th>
<th>No. (%) of cleaved embryos</th>
<th>No. (%) of 4–8 cell embryos</th>
<th>No. (%) of morulae</th>
<th>No. (%) of blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>K</td>
<td>106</td>
<td>104 (98.1)a</td>
<td>100 (94.3)a</td>
<td>98 (92.5)a</td>
<td>90 (84.9)a</td>
</tr>
<tr>
<td></td>
<td>K + G</td>
<td>91</td>
<td>91 (100)a</td>
<td>91 (100)a</td>
<td>87 (96)a</td>
<td>81 (88)a</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>106</td>
<td>106 (100)a</td>
<td>104 (98.1)a</td>
<td>100 (94.3)a</td>
<td>92 (86.8)a</td>
</tr>
<tr>
<td></td>
<td>K + G</td>
<td>92</td>
<td>92 (100)a</td>
<td>92 (100)a</td>
<td>89 (97)a</td>
<td>83 (89)a</td>
</tr>
</tbody>
</table>

a–b Values in the same columns with different superscripts were significantly different (P < 0.05).
In summary, treatment with MG132 improved the developmental potential of mouse embryos cloned by electrofusion and produced cloned mice. A sequential cultivation with KSOM followed by CZB supplemented with glucose enhanced the development of cloned embryos.

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