Increased Pre-Implantation Development of Cloned Wuzhishan Miniature Pig Embryos Treated With Scriptaid and Valproic Acid

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ABSTRACT
The aim of the present study was to examine the effects of scriptaid (SCR), a histone deacetylase inhibitor (HDACi), on the in vitro and in vivo development of Wuzhishan miniature pig somatic cell nuclear transfer (SCNT) embryos. Experiment 1 compared the in vitro developmental competence of nuclear transfer embryos treated with various concentrations of SCR for 24 h. Embryos treated with 250 nM SCR showed a significantly increased rate of blastocyst formation compared with controls or embryos treated with 50 nM or 500 nM SCR (20.00% vs 12.16%, 8.89%, and 11.86%, respectively). Experiment 2 examined the in vitro developmental competence of nuclear transfer embryos treated with 250 nM SCR for different time periods following chemical activation. Embryos treated for 24 h showed higher rates of blastocyst formation than controls or embryos treated for 12 h or 48 h (22.22% vs 11.54%, 10.00%, and 18.52%, respectively). Experiment 3 directly compared the effects of SCR and VPA treatment and examined the additive effect of SCR and VPA on nuclear transfer embryos following chemical activation. Embryos treated with 250 nM SCR or 2 mM VPA for 24 h showed a significantly increased rate of blastocyst formation compared with controls (19.85% and 18.32% vs. 9.23%, respectively), but not increased with a combination of 250 nM SCR and 2 mM VPA for 24 h (18.90% vs. 19.85%, 18.32%).

In experiment 4, nuclear transfer embryos treated with 250 nM SCR for 24 h and non-treated controls were transferred to surrogate mothers, resulting in the birth of healthy cloned pups from the controls but no births in the SCR-treated group. These results suggest that SCR treatment increases the rate of blastocyst formation in somatic cell nuclear transfer embryos and affects their subsequent growth. Also, co-treatment with VPA and SCR did not improve the blastocyst formation rate.

Keywords: histone deacetylase inhibitor; somatic cell nuclear transfer; valproic acid; scriptaid; in vivo development; in vitro development

INTRODUCTION
Miniature pigs are an attractive species for use as experimental animal models for biomedical research because their anatomy and physiological characteristics are similar to those of humans, and their small size makes them easier to handle and cheaper to maintain than larger common domestic pigs [1]. The Wuzhishan inbred miniature pig is a Chinese variety with a high inbreeding coefficient and is a candidate experimental model for biomedical research. By combining the technique of homologous recombination in somatic cells with that of somatic cell nuclear transfer (SCNT), it is possible to introduce specific modifications into the pig genome [2-5]. Although several pig breeds have been successfully cloned, the remodeling and reprogramming of differentiated somatic nuclei into a totipotent embryonic state by SCNT is not efficient, and the mechanism underlying the remodeling process is not known. In most mammalian species studied thus far, the survival rate to birth for cloned blastocysts is only about 1–5%, compared with a 30–60% birthrate for in vitro fertilization (IVF) blastocysts [6]. Recently, histone deacetylase inhibitors (HDACi) have been used to treat donor cells or reconstructed embryos to improve the efficiency of somatic cell cloning. The HDACi, 6-(1,3-dioxo-1H, 3H -benzo[de] isoquinolin-2-yl) -hexanoic acid hydroxamide (SCR), has low toxicity and enhances both transcriptional activity and protein expression [7]. In mice, treatment of SCNT oocytes with SCR during the first embryonic cell cycle rescues the development of clones produced from important inbred mouse strains such as C57BL/6 and C3H/He, and significantly increases the number of cloned offspring in the 129/Sv, DBA/2, and B6D2F1 strains. Moreover, treatment of cloned embryos with SCR for 24 h results in the same rate of full-term development as treatment for 10 h [8]. Zhao et al. (2009) reported that the treatment of SCNT embryos with 500 nM SCR significantly enhanced their
development to the blastocyst stage compared with untreated embryos (21% vs. 9%) when NIH inbred fetal fibroblast cells (FFCs) were used as donors [9]. Zhu et al. (2011) also reported that, when using Wuzhishan inbred miniature pig FFCs as donor cells, cloned embryos treated with 100 nM SCR for 24 h supported a higher rate of blastocyst development (30.4%) than controls (17.5%) [10]. However, no reports to date have determined the optimal concentration or treatment times for SCR, and none have examined how the percentage of SCR-treated SCNT embryos positively affects development to full term after transfer to recipient females. One of the most powerful HDACi is valproic acid (VPA), which causes hyperacetylation of histones in cells both in culture and in vivo. VPA inhibits histone deacetylase (HDAC) activity in vitro, most probably by binding to its catalytic center [11]. Recently, VPA was reported to enhance the development of miniature pig SCNT embryos into blastocysts [12]. Also, our own laboratory found that the treatment of cloned embryos with 2 mM VPA for 24 h significantly improved in vitro development and blastocyst formation. However, no direct comparison has been made between SCR and VPA in terms of their ability to increase blastocyst development in vitro.

Therefore, the objective of this study was to examine and optimize the use of SCR to reprogram somatic nuclei following SCNT using fibroblast cells derived from the ears of Wuzhishan inbred femal miniature pigs as donor cells, and to test its ability to enhance the in vivo development of porcine SCNT embryos.

MATERIALS AND METHODS
This research was approved by the Ethics Committee of Yanbian University

Chemicals and reagents
All chemicals used in this study were purchased from Sigma Chemical Company (Sigma, St. Louis, MO, USA) unless stated otherwise.

Isolation and culture of porcine somatic cells
Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory at 25–35°C. Antral follicles (2–6 mm in diameter) were aspirated using an 18 gauge needle. Aspirated oocytes showing a uniformly granulated cytoplasm and surrounded by at least three uniform layers of compact cumulus cells were selected and washed three times in Hepes-buffered NCSU37 containing 0.1% polyvinyl alcohol (PVA). Oocytes were cultured in four-well plates (Nunc) each containing 500 µl of NCSU-37 medium supplemented with 10% porcine follicular fluid, 0.6 mmol/l cysteine, 1 mmol/l dibutyryl cyclic adenosine monophosphate (dbcAMP) and 0.1 IU/ml human menopausal gonadotropin (hMG, Teikokuzoki, Tokyo, Japan) for 20 h, followed by culture without dbcAMP and hMG for another 18–24 h as previously described [13].

Nuclear transfer
Nuclear transfer was performed as described by Yin et al. (2002) [13]. Briefly, mature eggs showing the first polar body were cultured in medium supplemented with 0.4 mg/ml demecolcine and 0.05 mol/l sucrose for 1 h. Sucrose was used to enlarge the perivitelline space of the eggs. Treated eggs showing a protruding membrane were moved to medium supplemented with 5 mg/ml cytochalasin B (CB) and 0.4 mg/ml demecolcine, and the protrusion was removed with a beveled pipette. A single donor cell was injected into the perivitelline space of each egg and electrically fused using two direct current pulses of 150 V/mm for 50 ms in 0.28 mol/l mannitol supplemented with 0.1 mM MgSO4 and 0.01% PVA. Fused eggs were cultured in NCSU-37 medium for 1 h before electro activation and then cultured in 5 mg/ml of CB-supplemented medium for 4 h. The reconstructed oocytes were activated by two direct current pulses of 100 V/mm for 20 ms in 0.28 mol/l mannitol supplemented with 0.1 mM MgSO4 and 0.05 mol/l CaCl2. Activated eggs were cultured in the medium for 6 days in an atmosphere of 5% CO2 and 95% air at 39°C.

Experimental design
Experiment 1 examined the development of SCNT embryos treated with different concentrations of SCR. After activation, SCNT embryos were cultured in medium supplemented with 0, 50, 250, or 500 nM SCR for 24 h and then transferred to medium without SCR.

In experiment 2, SCNT embryos were cultured in medium supplemented with 250 nM SCR for 0, 12, 24, or 48 h and then transferred to medium without SCR.

Experiment 3 examined the combined effects of VPA and SCR on the development of cloned embryos. Cloned embryos were treated with SCR, VPA, or both. Treatment with 2 mM VPA significantly improves embryo development and blastocyst formation (manuscript submitted); therefore, a combination of 250 nM SCR and 2 mM VPA was selected for the study.

In experiment 4, SCNT embryos cloned from Wuzhishan miniature pig ear-derived fibroblasts treated without (control) or with 250 nM SCR for 24 h after activation were transferred to the oviducts of each two surrogates on the day of, or 1 day after, the onset of estrus. Pregnancy was diagnosed on Day 25 (Day 0 was the day of SCNT) and checked regularly at 1 week intervals by ultrasound examination. The cloned piglets were delivered naturally or induced by an intramuscular injection of Prostaglandin F2 Alpha (PGF2α) on Day 117 of gestation (if labor did not start spontaneously).

Statistical analysis
Each experiment was repeated at least three times. Data were expressed as percentages and analyzed using the chi-squared test. Cell numbers were analyzed by analysis of variance (ANOVA) using SAS 6.12 (SAS Institute, Cary, NC).

RESULTS
Experiment 1
SCNT embryos derived from Wuzhishan miniature inbred donor cells were treated with 0, 50, 250 or 500 nM SCR for 24 h after activation. The percentage of SCNT embryos developing to the blastocyst stage significantly increased after treatment with 250 nM SCR compared with controls or embryos treated with 50 nM or 500 nM SCR (21.51% vs. 10.51%, 12.63% and 17.16%, respectively; p < 0.05); however, SCR treatment had no effect on the cleavage percentage (78.38–84.75%) at 48 h or blastocyst quality as determined by the mean number of cells (30.33 ± 2.65–33.67 ± 5.13) (Table 1).
### Table 1

**In vitro** development of miniature pig Somatic Cell Nuclear Transfer embryos treated with different concentrations of SCR for 24 h.

<table>
<thead>
<tr>
<th>Concentration of SCR (nM)</th>
<th>No. of embryos cultured</th>
<th>No. (%) of 2-4-cell embryos</th>
<th>No. (%) of blastocysts</th>
<th>Mean no ± SEM of cells in blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>148</td>
<td>116 (78.38)</td>
<td>18 (12.16)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.00 ± 2.65</td>
</tr>
<tr>
<td>50</td>
<td>90</td>
<td>74 (82.22)</td>
<td>8 (8.89)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.67 ± 3.21</td>
</tr>
<tr>
<td>250</td>
<td>370</td>
<td>300 (81.08)</td>
<td>74 (20.00)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.33 ± 8.96</td>
</tr>
<tr>
<td>500</td>
<td>118</td>
<td>100 (84.75)</td>
<td>14 (11.86)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.67 ± 5.13</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Values with different superscripts in the same column were significantly different (p < 0.05).

### Table 2

**In vitro** development of miniature pig Somatic Cell Nuclear Transfer embryos treated with 250 nM SCR for different times.

<table>
<thead>
<tr>
<th>Duration of SCR treatment (h)</th>
<th>No. of embryos cultured</th>
<th>No. (%) of 2-4-cell embryos</th>
<th>No. (%) of blastocysts</th>
<th>Mean no ± SEM of cells in blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>156</td>
<td>132 (84.62)</td>
<td>18 (11.54)&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>28.33 ± 6.51</td>
</tr>
<tr>
<td>12</td>
<td>180</td>
<td>154 (85.56)</td>
<td>18 (10.00)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29.00 ± 8.54</td>
</tr>
<tr>
<td>24</td>
<td>180</td>
<td>158 (87.78)</td>
<td>40 (22.22)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28.60 ± 7.47</td>
</tr>
<tr>
<td>48</td>
<td>162</td>
<td>144 (88.89)</td>
<td>30 (18.52)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.60 ± 8.65</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup>Values with different superscripts in the same column were significantly different (p < 0.05).

### Table 3

**In vitro** development of miniature pig Somatic Cell Nuclear Transfer embryos treated with 2 mm of VPA and 250 nM of SCR or co-treated with SCR and VPA for 24 h.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of embryos cultured</th>
<th>No. (%) of 2-4-cell embryos</th>
<th>No. (%) of blastocysts</th>
<th>Mean no ± SEM of cells in blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>260</td>
<td>226 (86.92)</td>
<td>24 (9.23)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.50 ± 9.47</td>
</tr>
<tr>
<td>SCR</td>
<td>262</td>
<td>228 (87.02)</td>
<td>52 (19.85)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.25 ± 4.03</td>
</tr>
<tr>
<td>VPA</td>
<td>262</td>
<td>220 (83.97)</td>
<td>48 (18.32)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.00 ± 4.97</td>
</tr>
<tr>
<td>SCR+VPA</td>
<td>254</td>
<td>196 (77.17)</td>
<td>48 (18.90)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.00 ± 5.50</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Values with different superscripts in the same column were significantly different (p < 0.05).

### Table 4

Full-term development of nuclear transfer-derived embryos using Wuzhishan miniature ear fibroblast cells as donors following SCR treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cloning date</th>
<th>No. of transferred embryos</th>
<th>Pregnancy status</th>
<th>No. of piglets born</th>
<th>Av. offspring body weight (g ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2011-8-17</td>
<td>271</td>
<td>+</td>
<td>8 live and 1 dead</td>
<td>945.75 ± 202.42&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2011-8-23</td>
<td>210</td>
<td>+</td>
<td>5 live</td>
<td>713.17 ± 136.50&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SCR</td>
<td>2011-8-31</td>
<td>208</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2011-9-8</td>
<td>270</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Body weight of naturally bred Wuzhishan miniature piglets

486.50 ± 61.83<sup>c</sup>

<sup>a,b,c</sup>Values with different superscripts in the same column were significantly different (p < 0.05).

### Experiment 2

SCNT embryos were treated with 250 nM SCR for 0, 12, 24 and 48 h after activation. Development to the blastocyst stage (Fig. 1) increased significantly (p < 0.05) when SCNT embryos were treated with 250 nM SCR for 24 h and 48 h compared with that in controls or embryos treated for 12 h (22.22% and 18.52% vs. 11.54% and 10.00%, respectively). However, SCR treatment had no effect on percentage cleavage (84.62–88.89%) at 48 h or blastocyst quality as determined by the mean number of cells (28.60 ± 7.47–31.60 ± 8.64) (Table 2).

### Experiment 3

Embryos treated with 250 nM SCR or 2 mM VPA for 24 h...
showed a significantly increased rate of blastocyst formation compared with controls (19.85% and 18.32%, respectively, vs. 9.23%), but combinations of SCR and VPA showed no additive effects in terms of improving the development of cloned embryos compared with SCR and VPA groups (18.90% vs. 19.85% and 18.32%) (Table 3).

**DISCUSSION**

The limited success of SNCT is attributed to incomplete reprogramming of the transferred nuclei. The objective of the present study was to determine whether SCR improved the development of SNCT embryos. The results showed that, relative to untreated controls, the treatment of cloned embryos with SCR resulted in a significantly increased rate of blastocyst development. Although the mechanism underlying the SCR-mediated improvement in cloning efficiency remains unknown, it is thought that HDACi induce the hyperacetylation of core histones, resulting in structural changes in chromatin that permit transcription, and enhanced DNA demethylation of the somatic cell-derived genome after nuclear transfer [14]; both are a necessary part of genetic reprogramming [15, 16]. Increased acetylation of histones leads to relaxed binding of the nucleosome to DNA and/or linker histones, relaxation of chromatin structures, and formation of a transcriptionally permissive state [17-20]. Histone deacetylation, frequently followed by histone methylation, establishes a base for highly repressive chromatin structures such as heterochromatin [21]. SCR-induced hyperacetylation decreases DNA methylation and, thus, the activation of genes that are key for development [22]. Zhao et al. (2009) reported that reconstructed embryos treated with 500 nM SCR for 14-16 h showed significantly increased development to the blastocyst stage when compared with untreated embryos (21% vs. 9%; p < 0.05). SCR treatment resulted in eight pregnancies from 10 embryo transfers and 14 healthy NIH miniature pigs from eight litters. However, no viable piglets were obtained from nine embryo transfers in the untreated group [9]. Zhu et al. (2011) reported that, when using Wuzhishan inbred miniature pig FFCs as donor cells, cloned embryos treated with 100 nmol/L SCR for 24 h showed a higher rate of blastocyst development than controls (30.4% vs. 17.5%; p < 0.05). Embryos treated with 100 nmol/L SCR for 24 h, or control embryos, were transferred into four recipient gilts. The average litter size and cloning efficiency (piglets born/transferred embryos) for the treatment group were significantly higher (5% and 2.4%, respectively) than those for the control group (1.5% and 0.7%, respectively). Treatment with 100 nmol/L SCR showed the best effect on embryo development. In the present study, the highest level of *in vitro* development was seen in embryos treated with 250 nM SCR for 24 h; treatment with 500 nM SCR for 24 h actually showed adverse effects. We did not test SCR at 100 nmol/L for 24 h, but we did test it at 50 nmol/L for 24 h, which did not improve SCNT embryo development. This apparent inconsistency between our results and those of Zhu et al might be due to species differences.

We also examined whether combined treatment with SCR and VPA after nuclear transfer would further improve the pre-implantation development of cloned embryos. Based on previous experiments, doses of 250 nM SCR and 2 mM VPA were selected for the treatment of nuclear transfer embryos. The development of cloned embryos after combined treatment did not improve over those treated with 250 nM SCR or 2 mM VPA alone. This may be because SCR and VPA affect histone acetylation levels via the same mechanism. In cows, the treatment of donor cells and early cloned embryos with a combination of two epigenetic modification drugs (5-aza-2/-Deoxycytidine and Trichostatin A) significantly increased both the *in vitro* and *in vivo* development of cloned embryos [23, 24].

Several HDACi, such as Trichostatin A, SCR and VAP, have been used to improve cloning efficiency during porcine SCNT. Trichostatin A enhances the developmental competence of SCNT embryos in pigs [25-30]. However, cloned piglets derived from Trichostatin A -treated embryos have significant problems [26, 30].

![Blastocysts derived from the SCR-treated group.](Image)

![Wuzhishan miniature cloned piglets born using female ear fibroblast cells as donors (Day 2 after birth).](Image)
When the SCR-treated embryos were transferred to surrogates, no cloned piglets were obtained, whereas the SCR untreated control embryos were transferred to surrogates produced healthy piglets. Because the sample numbers were small, we cannot attribute this to SCR treatment; however, the data show that SCR treatment did not improve the cloning efficiency. Similar results have been published by other groups. Li et al. (2008) found that cloned piglets derived from TSA-treated embryos did not survive [26]; the same result was obtained with rabbits. Another study reported that TSA-treated and untreated SCNT embryos developed to term; however, all offspring from TSA-treated embryos died within 1 h to 19 d [31].

In conclusion, the present study focused on the effects of a novel HDACi on the in vitro and in vivo development of pig SCNT embryos. SCR treatment improved the in vitro development of cloned pig embryos but showed no positive effects when the cloned embryos were transferred to surrogate mothers. Combined treatment with SCR and VPA showed no additive effects in terms of improved embryo development in vitro.

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