CUDC-101, a histone deacetylase inhibitor, improves the in vitro and in vivo developmental competence of somatic cell nuclear transfer pig embryos

Jun-Xue Jin, Suo Li, Yu Hong, Long Jin, Hai-Ying Zhu, Qing Guo, Qing-Shan Gao, Chang-Guo Yan, Jin-Dan Kang*, Xi-Jun Yin*

Department of Animal Science, College of Agriculture, Yanbian University, Yanji, China

**ABSTRACT**

The aim of the present study was to examine the effects of CUDC-101, a novel histone deacetylase inhibitor, on the in vitro development and expression of the epigenetic marker histone H3 at lysine 9 (AcH3K9) in pig SCNT embryos. We found that treatment with 1 μmol/L CUDC-101 for 24 hours significantly improved the development of pig SCNT embryos. Compared with the control group, the blastocyst rate was higher (18.5% vs. 10.3%; P < 0.05). To assess in vivo developmental potency, CUDC-101–treated SCNT embryos were transferred into two surrogate mothers, resulting in one pregnancy with six fetuses. We then investigated the acetylation level of histone H3K9 in SCNT embryos treated with CUDC-101 and compared them only against untreated embryos. The acetylation level of control SCNT embryos was lower than that of CUDC-101–treated embryos at pseudopronuclear stages, and immunofluorescent signal for H3K9ac in CUDC-101–treated embryos in a pattern similar to that of control group. In conclusion, we demonstrated that CUDC-101 can significantly improve in vitro and in vivo developmental competence and enhance the nuclear reprogramming of pig SCNT embryos.

**1. Introduction**

Pig SCNT is a tool useful both for biomedical and basic research. Its usefulness is mainly owing to the special interest in using pigs as a source for human xenotransplantation or as models for certain diseases insofar as SCNT is the practical way to produce targeted genetic modifications in pigs [1,2]. Although the first successful SCNT was achieved more than a decade ago, the efficiency of pig cloning by SCNT remains extremely low, with a success rate of 1% to 5% [3,4]. To obtain developmentally competent SCNT embryos, the differentiated donor cell nucleus should undergo remodeling and reprogramming processes. Analyses of cloned embryos and offspring reveal that abnormal epigenetic modifications such as DNA methylation and histone modifications in SCNT embryos [5,6], rather than genetic abnormalities, might be a key factor affecting cloning efficiency. Dynamic interactions between DNA methylation and acetylation in the amino-terminal domains of core histones are thought to regulate DNA functions and control gene expression [7,8].

Histone acetylation is a type of epigenetic modification [9,10]. Several studies suggest that an elevated level of histone acetylation in cloned embryos improves reprogramming efficiency. Global and local patterns of histone acetylation also contribute considerably to nuclear reprogramming [11]. Several studies demonstrate that different compounds such as trichostatin A [12], scriptaid...
regulate histone acetylation. CUDC-101 is a potent inhibitor of HDAC. CUDC-101 also increases the acetylation of histones H3 and H4, as well as the acetylation of non-histone substrates of HDAC, such as p53 and α-tubulin, in a dose-dependent manner in various cancer cell lines [16]. For this study, we hypothesized that an increase of histone acetylation would improve the efficiency of SCNT pig embryos. Thus, we explored the effects of CUDC-101 on the in vitro and in vivo developmental competence of pig SCNT embryos and examined the histone acetylation level.

The objective of the present study was to examine the global acetylation level of histone H3 at lysine 9 (AcH3K9) of SCNT embryos treated with CUDC-101. Furthermore, we examined the in vitro developmental competence of pig embryos treated with increasing concentrations of CUDC-101 for different durations and then evaluated the in vivo development of fetuses.

2. Materials and methods

This research was carried out in accordance with the Ethics Committee of Yanbian University. All chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise noted. CUDC-101 was purchased from Selleck Chemicals (Houston, TX, USA).

2.1. Oocyte collection and maturation

Pig ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory at 35 °C. The contents from follicles (3–6 mm in diameter) were recovered by aspirating with an 18-gauge needle. Cumulus-oocyte complexes (COCs) were pooled and washed three times with HEPES-buffered NCSU-37 (North Carolina State University) medium containing 0.1% polyvinyl alcohol (v:v). Only COCs possessing a compact cumulus mass and evenly granulated ooplasm were selected. The COCs were cultured for 20 to 22 hours at 38.5 °C in an atmosphere of 5% CO2 and 95% air at 38.5 °C. At the end of the culture period, blastocysts were washed three times in PBS, fixed with 4% paraformaldehyde in PBS for 30 minutes, and placed on slides with a drop of mounting medium consisting of 25 µL propidium iodide in glycerol and 0.4 µL/mL 6-dimethylaminopurine. The reconstituted oocytes were activated by two direct pulses of 100 V/mm for 20 μsec in 0.28 mol/L mannitol supplemented with 0.1 mmol/L MgSO4 and 0.01% polyvinyl alcohol (v:v). Activated eggs were cultured in medium for 7 days in an atmosphere of 5% CO2 and 95% air at 38.5 °C. For the immunodetection of AcH3K9, embryos were transferred into the perivitelline space of each egg and electrically fused using two direct pulses of 150 V/mm for 50 μsec in 0.28 mol/L mannitol supplemented with 0.1 mmol/L MgSO4 and 0.01% polyvinyl alcohol (v:v). Fused eggs were cultured for 1 hour in medium containing 0.4 μg/mL cytochalasin B and 0.4 µg/mL demecolcine. Protrusions were then removed by aspirating with a 15-μm inner diameter glass pipette. A single donor cell was inserted into the perivitelline space of each egg and electrically fused using two direct pulses of 150 V/mm for 50 μsec in 0.28 mol/L mannitol supplemented with 0.1 mmol/L MgSO4 and 0.05 mmol/L CaCl2. Activated eggs were cultured in medium for 7 days in an atmosphere of 5% CO2 and 95% air at 38.5 °C. The contents from follicles (3–6 mm in diameter) were recovered by aspirating with an 18-gauge needle. Cumulus-oocyte complexes (COCs) were pooled and washed three times with HEPES-buffered NCSU-37 (North Carolina State University) medium containing 0.1% polyvinyl alcohol (v:v). Only COCs possessing a compact cumulus mass and evenly granulated ooplasm were selected. The COCs were cultured for 20 to 22 hours at 38.5 °C in an atmosphere of 5% CO2 and 95% air in four-well plates (Nunc, Roskilde, Denmark) with each well containing 500 µL maturation medium under mineral oil. The maturation medium was composed of NCSU-37 medium supplemented with 10% pig follicular fluid (v:v), 0.6 mmol/L cysteine, 1 mmol/L dibutyryl cyclic adenosine monophosphate, and 0.1 IU/mL human menopausal gonadotropin (Teikokuzoki, Tokyo, Japan). Thereafter, COCs were cultured without dibutyryl cyclic adenosine monophosphate and human menopausal gonadotropin for another 18 to 24 hours.

2.2. Isolation and culture of pig somatic cells

Fetal fibroblasts were obtained from a hybrid pig on Day 30 of pregnancy. Tissues were cut into small pieces and cultured at 38 °C in an atmosphere of 5% CO2 and 95% air in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum (v:v), 1 mmol/L sodium pyruvate, and 100 U/mL each of penicillin and streptomycin. When fibroblasts were at 90% confluence, they were trypsinized, rinsed, and subcultured into two 25-cm² cell culture flasks (Corning, Kennebunk, MA, USA) for further passaging. Nuclear donor cells for SCNT were derived from passages 4 to 8 and cultured in serum-starved medium (0.5% fetal bovine serum [v:v]) for 3 to 4 days.

2.3. Nuclear transfer

Nuclear transfer was performed as described by Yin, et al. (2002) [17]. In vitro-matured eggs with the first polar body were cultured in medium supplemented with 0.05 mol/L sucrose and 0.4 µg/mL demecolcine for 1 hour. Sucrose was used to enlarge the perivitelline space of eggs. Treated eggs with a protruding membrane were transferred to medium containing 5 µg/mL cytochalasin B and 0.4 µg/mL demecolcine. Protrusions were then removed by aspirating with a 15-μm inner diameter glass pipette. A single donor cell was inserted into the perivitelline space of each egg and electrically fused using two direct pulses of 150 V/mm for 50 μsec in 0.28 mol/L mannitol supplemented with 0.1 mmol/L MgSO4 and 0.01% polyvinyl alcohol (v:v). Fused eggs were cultured for 1 hour in medium containing 0.4 µg/mL demecolcine before electro-activation and then cultured for 4 hours in medium supplemented with 2 mmol/L 6-dimethylaminopurine. The reconstructed oocytes were activated by two direct pulses of 100 V/mm for 20 μsec in 0.28 mol/L mannitol supplemented with 0.1 mmol/L MgSO4 and 0.05 mmol/L CaCl2. Activated eggs were cultured in medium for 7 days in an atmosphere of 5% CO2 and 95% air at 38.5 °C. The embryos were then transferred to PBS containing 4% paraformaldehyde in PBS for 30 minutes, and placed on slides with a drop of mounting medium consisting of 25 µL/mL propidium iodide in glycerol and PBS (9:1). A cover slip was placed on top of the blastocysts, and the edges were sealed with nail polish. The number of nuclei was counted under ultraviolet light.

2.4. Transfer of embryos and pregnancy determination

Cloned embryos at the one-cell stage after activation or at the two- to four-cell stage after 1 day of culture were transferred into the oviducts of naturally cycling gilts on the first day of standing estrus. The pregnancy status of recipients was determined by ultrasonography on Days 25, and fetuses were recovered on Day 25 or 26 post transfer.

2.5. Analysis of the histone acetylation level

For the immunodetection of Ach3K9, embryos were washed three times in PBS and fixed with 4% paraformaldehyde (w:v) in PBS for 30 minutes. Embryos were then transferred to PBS containing 1% Triton X-100 (v:v) at 37 °C for 30 minutes. After blocking nonspecific sites with 2% BSA (w:v) in PBS overnight at 4 °C, embryos were incubated with primary antibodies (rabbit polyclonal antibody against histone H3K9ac [Upstate Biotechnology, Lake Placid, NY, USA], diluted to 1:200) at 37 °C for 3 hours. Goat anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (1:200; Jackson ImmunoResearch
Laboratories Inc., West Grove, PA, USA) was then applied for 3 hours at room temperature. After washing three times in PBS, the DNA was counterstained with 25 μg/mL propidium iodide for 20 minutes. Stained embryos were then mounted under a coverslip with antifade mounting medium to retard photobleaching. Each experiment was repeated at least three times, and at least five randomly selected, reconstructed embryos were examined each time. Slides were scanned by using an epifluorescent microscope (IX71 Olympus, Tokyo, Japan) with 488-nm fluorescein isothiocyanate.

2.6. Experimental design

Experiments were designed to study the effects of CUDC-101 on the in vitro and in vivo development and the histone acetylation level in pig SCNT embryos. In experiment 1, SCNT embryos were treated with various concentrations of CUDC-101 (0–100 μmol/L) for 24 hours after activation. Cleavage (48 hours) and blastocyst (Day 7) rates were recorded to assess the in vitro developmental capacity of embryos. In experiment 2, SCNT embryos were treated with 1 μmol/L CUDC-101 for different durations (0, 12, 24, or 36 hours) after activation. Cleavage (48 hours) and blastocyst (Day 7) rates were recorded to assess the in vitro developmental capacity of embryos. In experiment 3, CUDC-101 treated and untreated SCNT embryos were transferred into oviducts of surrogates on day of, or 1 day after, the onset of estrus. In experiment 4, SCNT embryos were treated with CUDC-101 for 24 hours. The SCNT embryos were treated with increasing concentrations (0–100 μmol/L) of CUDC-101 for 24 hours. There were no differences in cleavage or blastocyst rates, or in blastocyst cell number. As shown in Table 1, the rate of blastocyst formation was significantly higher (P < 0.05) in the 1 μmol/L CUDC-101 group than in the control group (18.5% vs. 10.3%; Fig. 1A). However, CUDC-101 did not affect the number of cells (41.8/C6 vs. 48.0/C6) at 48 hours or blastocyst quality, as determined by the mean number of cells (41.8 ± 9.7 vs. 41.8 ± 11.0; Fig. 1B). By contrast, the cleavage rate was significantly lower (P < 0.05) than the control when SCNT embryos were treated with high concentrations of CUDC-101 (>10 μmol/L). High concentrations of CUDC-101 (>10 μmol/L) also inhibited the development of SCNT embryos to blastocysts (Table 2).

2.7. Statistical analysis

Each experiment was repeated at least three times. Data expressed as proportions (percentages) were analyzed using chi-square test, and nuclei numbers were analyzed by ANOVA using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). The total fluorescence intensity emitted by each individual nucleus was measured on antibody-stained images, after background subtraction, by Image-Pro Plus 6.0. P < 0.05 was regarded as significant.

### Table 1

<table>
<thead>
<tr>
<th>Concentration of CUDC-101 (μmol/L)</th>
<th>No. of embryos cultured</th>
<th>Two-, four-cell, n (%)</th>
<th>No. of blastocysts (%)</th>
<th>Mean ± SEM of cells in blastocysts (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>146</td>
<td>122 (83.6)</td>
<td>15 (10.3)</td>
<td>41.8 ± 11.0 (4)</td>
</tr>
<tr>
<td>0.005</td>
<td>95</td>
<td>85 (89.5)</td>
<td>9 (9.5)</td>
<td>46.8 ± 10.4 (4)</td>
</tr>
<tr>
<td>0.05</td>
<td>68</td>
<td>59 (88.8)</td>
<td>6 (8.8)</td>
<td>43.5 ± 10.5 (4)</td>
</tr>
<tr>
<td>0.5</td>
<td>68</td>
<td>60 (88.2)</td>
<td>6 (8.8)</td>
<td>48.0 ± 10.8 (4)</td>
</tr>
<tr>
<td>1</td>
<td>157</td>
<td>136 (86.6)</td>
<td>29 (18.5)</td>
<td>41.8 ± 9.7 (4)</td>
</tr>
<tr>
<td>2.5</td>
<td>141</td>
<td>122 (86.5)</td>
<td>26 (18.4)</td>
<td>40.0 ± 15.1 (4)</td>
</tr>
<tr>
<td>5</td>
<td>172</td>
<td>151 (87.8)</td>
<td>31 (18.0)</td>
<td>40.3 ± 13.0 (4)</td>
</tr>
</tbody>
</table>

Values with different superscripts in the same column were significantly different (P < 0.05).

### 3. Results

#### 3.1. Experiment 1

We examined the effects of CUDC-101 treatment on the in vitro development of SCNT embryos. To determine the optimal concentration of CUDC-101, SCNT embryos were treated with increasing concentrations (0–100 μmol/L) of CUDC-10 for 24 hours. The SCNT embryos were treated with increasing concentrations (0–500 nmol/L) of CUDC-101 for 24 hours. There were no differences in cleavage or blastocyst rates, or in blastocyst cell number. As shown in Table 1, the rate of blastocyst formation was significantly higher (P < 0.05) in the 1 μmol/L CUDC-101 group than in the control group (18.5% vs. 10.3%; Fig. 1A). However, CUDC-101 (1 μmol/L) did not affect cleavage (86.6% vs. 83.6%) at 48 hours or blastocyst quality, as determined by the mean number of cells (41.8 ± 9.7 vs. 41.8 ± 11.0; Fig. 1B). By contrast, the cleavage rate was significantly lower (P < 0.05) than the control when SCNT embryos were treated with high concentrations of CUDC-101 (>10 μmol/L). High concentrations of CUDC-101 (>10 μmol/L) also inhibited the development of SCNT embryos to blastocysts (Table 2).

#### 3.2. Experiment 2

In SCNT embryos treated with CUDC-101 for different durations, development to the blastocyst stage increased significantly when SCNT embryos were treated with 1 μmol/L CUDC-101 for 24 hours compared with the control (19.0% vs. 9.5%; Table 3). However, CUDC-101 did not affect

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Fig. 1. (A) Pig blastocysts derived from the CUDC-101–treated group. (B) An image of a Day 7 embryo after SCNT stained with propidium iodide.
cleavage (84.5% vs. 85.3%) at 48 hours or blastocyst quality, as determined by the mean number of cells (45.5 ± 12.8 vs. 37.3 ± 10.9).

### 3.3. Experiment 3

CUDC-101–treated SCNT embryos were transferred to surrogate mothers, resulting in one pregnancy out of two surrogate mothers. Six fetuses were counted in the pregnant surrogate (Fig. 2). Untreated SCNT embryos were transferred to three surrogate mothers, resulting in one pregnancy with four fetuses (Table 4).

### 3.4. Experiment 4

To determine how CUDC-101 improves the development potential of SCNT embryos, the acetylation level of AcH3K9, an epigenetic marker, was examined in pseudo-pronuclear, two-, four-, and eight-cell, morula, and blastocyst stage embryos. The acetylation level of AcH3K9 in untreated (−) SCNT embryos at the pseudo-pronuclear stage was lower than that of CUDC-101–treated (+) embryos (Fig. 3). In addition, histone acetylation of AcH3K9 was apparent in two- and eight-cell, morula, and blastocyst stage embryos treated with 1 μmol/L CUDC-101 for 24 hours, but not in four-cell stage embryos (Fig. 4).

### 4. Discussion

The SCNT pig has been used in biomedical research for decades as a model for human diseases, because its anatomy and physiology are similar to those of the human, as a genetically defined model for surgery and xenotransplantation, and as a source of human disease therapeutics. However, the efficiency of producing normal offspring remains low, owing in part to the high number of good quality embryos that are needed to produce cloned offspring [18]. Therefore, if the quantity and quality of SCNT embryos developing to blastocysts were higher in vitro, it may be possible to produce more cloned piglets by embryo transfer.

Previous studies suggest that incomplete reprogramming is the main cause of low SCNT efficiency. Abnormal epigenetic modifications such as histone modifications and DNA methylation occur in SCNT embryos [19]. Histone acetylation, a type of epigenetic modification, plays a significant role in reprogramming and affects the development of SCNT embryos [20]. DNA methylation, another key epigenetic modification, changes and regulates the chromatin structure and also plays a crucial role in somatic nuclear reprogramming [21].

Recently, several epigenetic remodeling drugs such as the histone deacetylase inhibitors trichostatin A, valproic acid, scriptaid, sodium butyrate, suberoylanilide hydroxamic acid, m-carboxycinamic acid bishydroxamide, examflatin, and LBH589 have been used with the aim of improving the developmental competence of SCNT embryos. CUDC-101 is a novel small molecule that simultaneously inhibits histone deacetylase and the receptor kinases EGF receptor and human EGF receptor 2 in cancer cells [16]. The use of CUDC-101 with the objective of improving nuclear reprogramming and developmental competence of pig SCNT embryos in vitro and in vivo has not yet been reported.

In this study, we determined whether CUDC-101 treatment can improve the somatic nuclear reprogramming and developmental competence of pig SCNT embryos in vitro.
and in vivo. We also investigated whether CUDC-101 can enhance the nuclear reprogramming and developmental potential of SCNT embryos by altering epigenetic status and expression, and increasing blastocyst quality. We found that treatment with 1 μmol/L CUDC-101 for 24 hours significantly improved the in vitro development of pig SCNT embryos. Thus, the blastocyst rates of CUDC-101–treated group and control group are 18.5% and 10.3%, respectively (Table 1).

**Table 4**
Recovery of CUDC-101(+) and CUDC-101(−) SCNT fetuses.

<table>
<thead>
<tr>
<th>CUDC-101 treatments</th>
<th>Recipient no.</th>
<th>No. of embryos transferred</th>
<th>Pregnancy status</th>
<th>No. of fetuses recovered (fetus collection day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CUDC-101(+)</td>
<td>1</td>
<td>177</td>
<td>+</td>
<td>6 (D 25)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>211</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>CUDC-101(−)</td>
<td>3</td>
<td>241</td>
<td>−</td>
<td>4 (D 26)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>241</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>224</td>
<td>−</td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 3](J.-X. Jin et al. / Theriogenology 81 (2014) 572–578)

**Fig. 3.** (A) CUDC-101(−) and (+) SCNT embryos at the pseudo-pronuclear stage were labeled for acetyl histone H3K9 (green) and DNA (red). The merged images of histone H3K9 and DNA are yellow. Original magnification, ×200. (B) Fluorescent intensity for H3K9ac in CUDC-101–treated SCNT embryos, and untreated SCNT embryos at pseudo-pronuclear stage. The pseudo-pronuclear stage shows significant differences (P < 0.05).
AcH3K9 was stained in pseudo-pronuclear, two-, four- and eight-cell, morula, and blastocyst stage embryos. Histone acetylation is a type of epigenetic modification that affects the development of donor cells [22] and SCNT embryos [23]. The AcH3K9 signal was greater in the CUDC-101–treated group than in the control group (Fig. 3). In addition, our results show that histone acetylation of AcH3K9 was present in two- and eight-cell, morula, and blastocysts treated with 1 μmol/L CUDC-101 for 24 hours, but not in four-cell stage embryos (Fig. 4). A similar histone acetylation pattern was seen in bovine SCNT embryos [24,25]. Although it is unclear how CUDC-101 improved cloning, we hypothesize that CUDC-101 induced histone hyperacetylation, which is important for nuclear programming, and changed the chromatin structure after nuclear transfer. In addition, SCNT embryos treated with trichostatin A [26], scriptaid [27], or oxamflatin [20] show an improved DNA methylation level. Further studies are necessary to determine whether CUDC-101 improve histone acetylation and DNA methylation.

Lee et al. [28] reported that EGF improved the cleavage rate and total cell number in pig SCNT embryos. However, Lai et al. [16] showed that CUDC-101 inhibited key regulators of the EGF receptor signaling pathway in cancer cell lines, suggesting that CUDC-101 downregulates EGF receptor protein. CUDC-101 might block key regulators of the EGF receptor signaling pathway, resulting in a disruption of the ligand–receptor interaction. Another possibility is that a high concentration of, or a long incubation with, CUDC-101 affects the cleavage rate and developmental competence of SCNT embryos, and might cause developmental defects after SCNT (Table 2). At low doses and short incubations, CUDC-101 is nontoxic to pig SCNT embryos.

In summary, our results suggested that 1 μmol/L CUDC-101 treatment for 24 hours improves pig SCNT preimplantation development. This improvement may be owing to enhanced epigenetic modification of SCNT embryos caused by CUDC-101–induced hyperacetylation. We also determined the pregnancy status of recipients by ultrasonography on Day 25, and assessed fetal development. Future studies should focus on the relationship between modifications in DNA methylation and developmental competence.

Acknowledgments

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References


