Effect of histone deacetylase inhibitor romidepsin on the in vitro growth of foetal fibroblast cells and early development of porcine-cloned embryos

Zhangfan Wang, Chengxue Liu, Renyun Hong, Jie Yang, Hui Li, Yunsheng Li, Hongguo Cao, Yunhai Zhang and Zubing Cao

Anhui Provincial Laboratory of Local Livestock and Poultry, Genetical Resource Conservation and Breeding, College of Animal Science and Technology, Anhui Agricultural University, Hefei, China

ABSTRACT
This study was designed to explore the effects of histone deacetylase inhibitor romidepsin (FK228) treatment on the morphology, proliferation and karyotype of porcine foetal fibroblast cells and early developmental competence of somatic cell nuclear transfer (SCNT) embryos. We found that the treatment of foetal fibroblast cells with 0.1 μM FK228 for 24 h did not alter normal morphology, proliferation rate and chromosome number of donor cells, while other treatments with different doses and durations altered the above characteristics of donor cells seriously. Simultaneously, fusion rate, blastocyst rate and total cell number per SCNT blastocysts from different donor cell treatment groups were similar to the control group. We further found that the treatment of SCNT embryos with low-dose FK228 did not affect their developmental efficiency, but treatment with high dose dramatically caused blastocyst formation failure. In addition, 0.1 μM FK228 treatment for 36 h significantly elevated total cell number per SCNT blastocysts. Finally, combined treatments of both donor cells and embryos significantly improved the cleavage rate of cloned embryos, but did not affect the blastocyst rate and total cell number. Taken together, histone deacetylase inhibitor FK228 with optimal dose and exposure duration can enhance early developmental efficiency of porcine SCNT embryos and blastocyst quality.

ARTICLE HISTORY
Received 25 July 2016
Revised 26 October 2016
Accepted 21 November 2016

KEYWORDS
FK228; foetal fibroblast cells; somatic cell nuclear transfer; embryos

Introduction
Somatic cell nuclear transfer (SCNT) is of profound significance to save endangered animals, create transgenic animals, duplicate elite livestock, and provide tools and materials for basic research in biomedical field (Sameic 2005; Samiec & Skrzyszowska 2011a, 2011b). Transgenic cloned pigs are of great importance in animal husbandry and are also regarded as an ideal model for human diseases due to these characteristics of abundant resources, high fecundity, and physiological and anatomical structure similarities to humans (Samiec & Skrzyszowska 2011a; Samiec et al. 2012; Kurome et al. 2013; Ma et al. 2016). Viable SCNT-derived offspring were successfully obtained in pigs 16 years ago (Betthauser et al. 2000; Onishi et al. 2000; Polejaeva et al. 2000). However, the efficiency of pig cloning remains unsatisfactory. The low efficiency in pig cloning is mainly attributed to incomplete reprogramming of the donor cell-inherited nucleus (Jeanisch et al. 2002; Sameic 2004, 2005; Shi et al. 2015). Previously, some drugs regulating epigenetic status of donor cell nuclear chromatin in cloned embryos, such as non-specific histone deacetylase inhibitors (HDACi) including trichostatin A (TSA) (Li et al. 2008; Meng et al. 2009; Samiec et al. 2015), Scriptaid (Chen et al. 2013), valproic acid (VPA) (Costa-Borges et al. 2010; Xue et al. 2012), sodium butyrate (Das et al. 2010) and non-selective DNA methylation inhibitors such as 5-aza-2’-deoxycytidine (Diao et al. 2013; Huan et al. 2013) and zebularine (Xiong et al. 2013) have been shown to improve cloning efficiency. Although the above-mentioned inhibitors can partially improve the in vitro and/or full-term developmental efficiency of SCNT embryos, these studies are still in their infancy. Moreover, several studies have shown that TSA is a potent HDAC inhibitor with relatively high embryo toxicity that can cause developmental defects during post-implantation stages of embryogenesis and/or fetogenesis (Endo et al. 2008; Zhao et al. 2009).
Therefore, we attempted to investigate whether the novel HDAC inhibitor romidepsin (FK228) can be used as an effective reprogramming modulator in SCNT.

FK228 is a cyclic peptide HDACi that can increase the genomic histone acetylation levels by inhibiting the activity of HDAC1 and HDAC2 (Wang et al. 2010; Bishten et al. 2011; Liu et al. 2012). We speculate that FK228 may potentially be able to improve the efficiency of pig SCNT. Therefore, the present study aimed to investigate the effects of FK228 on the morphology, growth and karyotype of in vitro cultured donor cells, and the in vitro developmental competence of SCNT embryos.

Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO), media were purchased from Gibco (Grand Island, NY) and disposable plasticware were purchased from Nunc (Roskilde, Denmark). The FK228 stock solution and working solution were prepared as follows: storage solution I was prepared by adding 184.9 μL of dimethyl sulfoxide (DMSO, cell culture tested) to 1 mg FK228 (Selleck, 10 mM). Storage solution I was then diluted 1000× to obtain storage solution II (10 μM). All animal experiments were approved by the Animal Care and Use Committee of Anhui Agricultural University.

Cell culture and FK228 treatments

Porcine foetal fibroblasts (WH8/3) of a local breed were established from cells obtained from an ~4-week-old porcine foetus and cryopreserved in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, SH30243.01B) supplemented with 15% foetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO; DS879) in vials. During the experimental study, the cells were thawed and cultured at passages 3–6 to more than 80% confluence in the wells of 6-well or 96-well cell culture plates in the above-mentioned medium (DMEM plus 15% FBS) at 37°C in 5% CO2 and maximum humidity. After the attached cells were cultured for 24 h, the culture medium was replaced with a fresh medium containing different concentrations of FK228 (0, 0.05, 0.1 and 1.0 μM) for 24 h and cell viability was detected for each concentration at 24, 48, 72, 96, 120, 144 and 168 h. The day of FK228 treatment was considered day 0, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide was added on days 0, 1, 2, 3, 4, 5 and 6 to measure the absorbance. Simultaneously, the zero and control wells containing porcine foetal fibroblasts were prepared. These wells contained dissolution medium containing the same concentration of FK228, cell culture medium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide and DMSO, and the A values were measured following the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide assay.

Chromosomal analysis of FK228-treated cells

Chromosomal analysis was carried out as described previously with minor modifications (Makala et al. 2012). Briefly, the adherent cells were fixed with a fixative solution (methanol: acetic acid, 3:1) after treatment with colcemid and KCl; centrifuged to remove 0.5 mL supernatant to resuspend, and the cells were dropped onto the slide to produce metaphase spreads, and the slides were stained with Giemsa. The metaphase chromosomes were viewed under oil emulsion using an Olympus microscope (IX71, Japan).
Samples included cells treated with 0.05, 0.1 and 1.0 μM of FK228 for 24 h and untreated controls.

**Oocyte collection and in vitro maturation (IVM)**

Oocyte maturation was carried out as described previously (Cao et al. 2012). Briefly, porcine ovaries were collected from abattoirs and transported to the laboratory in physiological saline solution previously warmed to 25–35 °C. Follicles were aspirated and the cumulus-oocyte complexes (COCs) were selected, recovered and cultured in a maturation medium. After IVM for 40 ± 2 h, the COCs were vortexed in 1 mg/mL of hyaluronidase to remove cumulus cells. Only oocytes with a visible polar body, intact cell membrane and homogenous cytoplasm were selected and transferred to micromanipulation solution with donor cells. The first polar body (pb1) was adjusted to the 1-o’clock position using an enucleation or microinjection needle with a 15–25 mm inner diameter. Subsequently, from the 3-o’clock position, the enucleation or microinjection needle was inserted to draw the pb1 together with 10–20% of the adjacent cytoplasm, which potentially contained the oocyte nucleus. The donor cells were injected into the incision of enucleated oocytes through the same slit. Then, the reconstructed embryos were incubated in porcine zygote medium-3 (PZM-3).

**Fusion and activation**

Fusion and activation of karyoplast–cytoplast couplets was performed on the basis of protocols as previously reported (Cao et al. 2012). After incubation for 30 min, the reconstructed embryos were quickly washed thrice in electrical activation medium, and groups of 10–15 embryos were placed in the fusion chamber with electrodes set 1 mm apart. The position was adjusted so that the contact surface of donor cells and enucleated oocytes was perpendicular to the direction of the electric field. With a CF-150B cell fusion instrument (BLS, Budapest, Hungary), single pulse direct current of 1.56 kV/cm for 100 ms was administered to induce cell fusion and activation. Before determining the fusion status, the couplets were transferred to PZM-3 to balance for 30 min, and the fused embryos were then activated in PZM-3 containing 10 μg/mL cycloheximide (CHX) and 7.5 μg/mL cytochalasin B (CB) for 4 h before culture in PZM-3 for pre-implantation development.

**FK228-dependent epigenetic modulation of activated SCNT-derived oocytes**

SCNT 1-cell embryos after chemical activation were incubated in PZM-3 medium supplemented with 0.05, 0.1 and 1.0 μM of FK228 for 24 h or 36 h, and then embryos were washed three times in fresh PZM-3 medium and were cultured in fresh PZM-3 for 7 days. Furthermore, SCNT 1-cell-activated embryos were also treated with 5, 50 and 100 μM of FK228 for 24 h, and then FK228 was washed out and embryos were cultured in fresh PZM-3 for 7 days.

**Assessment of embryo developmental efficiency and determination of total cell number per blastocysts**

Embryonic development (cleavage and blastocyst rates) was determined after 48 h and 144–168 h of in vitro culture, respectively. A portion of the blastocysts from both the control and treatment groups was subjected to 4’,6-diamidino-2-phenylindole (DAPI) staining to examine the total cell number per blastocyst. Briefly, the blastocysts were washed in PBS, fixed in 4% paraformaldehyde (PFA) and incubated at 4 °C until use. Prior to staining, the blastocysts were washed in PBS and stained with 1 μg/ml of DAPI for 15 min in the dark, and washed in PBS. The stained blastocysts were mounted in a small drop of glycerol on a microscope slide under a cover slip, and then visualised under ultraviolet light by an inverted microscope and photographed. Cell numbers were counted using the Java-based software Image J (Bethesda, MD).

**Experimental schedule**

All experiments were executed as the following schedule: (1) The morphological evaluation of FK228-treated porcine foetal fibroblasts. (2) The examination of cell viability by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide. (3) Karyotype analysis of FK228-treated porcine fibroblast cells. (4) Effect of FK228 treatment of donor cells on the developmental efficiency and quality of SCNT embryos. (5) Effect of FK228 treatment of embryos on the early developmental competence of SCNT embryos. (6) Effect of combined treatments of both donor cells and embryos on the early development of SCNT embryos.

**Statistical analysis**

All experiments were replicated at least three times. Unless otherwise stated, the experimental data were expressed as means ± standard error of mean (means ± SEM). The percentage data were converted by arcsin transform before statistical analysis. The differences between the means for the converted percentage data of fibroblast growth, euploidy or aneuploidy,
fusion, cleavage, and blastocyst formation rates and total cell number per blastocyst were analysed by one-way analysis of variance (ANOVA) using SPSS software (version 17.0 for Windows, Chicago, IL). Post-hoc multiple comparison among groups was then carried out using the least significant differences (LSD) test. Student’s t test was applied to compare the converted percentage data of cleavage, blastocyst rate and total cell number per blastocyst between the control group and combined treatment group, as shown in Table 7. Differences were considered significant at $p < .05$.

Results

Effect of FK228 on the morphology of porcine foetal fibroblast cells

Control cells that were not treated with FK228 showed steady proliferation. These cells had uniform size, and exhibited short spindle morphology and even distribution. With increasing concentration of FK228 and duration of exposure, a greater proportion of treated cells showed abnormally morphological changes such as elongated fusiform, irregular shape and the gradual emergence of death (Figure 1). There were fewer adherent cells in the group of 1.0 $\mu$M of FK228 for 24 h than that in groups of 0.05 $\mu$M and 0.1 $\mu$M of FK228 for 24 h. When the cells were subjected to FK228 treatment for more than 48 h, the cells became thinner, longer and the proportion of cell death was higher (Figure 1). However, there was no negative effect on cell morphology in groups of 0.05 $\mu$M and 0.1 $\mu$M of FK228 for 24 h. Therefore, 0.05 $\mu$M or 0.1 $\mu$M FK228 treatment for 24 h could be optimal treatment strategy in the subsequent experiments.

Effect of FK228 treatment on the growth and survival of porcine foetal fibroblast cells

The growth curve (Figure 2(A)) showed a clear pattern with a relatively obvious log phase growth, and

![Figure 1. Effect of FK228 treatment on the morphology of porcine foetal fibroblast cells. (A, B, and C) denote the morphology of cells before treatment; (A', A'', and A''') are the morphologies of cells treated with 0.05 $\mu$M of FK228 for 24 h, 48 h and 72 h, respectively; (B', B'', and B''') are the morphologies of cells treated with 0.1 $\mu$M of FK228 for 24 h, 48 h and 72 h, respectively; (C', C'', and C''') are the morphologies of cells treated with 1 $\mu$M of FK228 for 24 h, 48 h and 72 h, respectively.](image-url)
plateau phase were relatively obvious when a cell density of 6000 cells/well was used. When the cell density was 4000 cells/well, an erratic growth curve was found due to the low cell density. However, the density of 8000 cells/well was too high, which caused the arrest of cells growth. Therefore, a cell density of 6000 cells/well was used in the subsequent experiment. Specifically, cell survival rate significantly increased and peaked in groups of 0.1 and 1 μM of FK228 for 24 h compared with the group of 0.05 μM FK228 treatment for 24 h and the control group (Figure 2(B)). Furthermore, the cell survival rate significantly decreased with increase in the exposure duration of FK228 compared to the control group, namely that cell survival rate in FK228 treatment for 48 and 72 h were significantly lower than that in the control group. When FK228 was removed after treatment for 24 h, cell survival rate in all treatment groups gradually decreased. These results indicated 0.1 μM FK228 treatment for 24 h could maintain the normal proliferation activity of porcine foetal fibroblast cells.

Effect of FK228 treatment for 24 h on the karyotype of porcine foetal fibroblast cells

The percentage of cells with normal karyotypes in the control (75%) and in the group of 0.05 μM (62%), 0.1 μM (63%) and 1 μM (52%), respectively, are shown in Table 1. Of these, the incidence of chromosomal abnormalities in groups of 0.05 and 1 μM of FK228 for 24 h were significantly higher than the control group (p < .05), but there was no significant difference in the incidence between group of 0.1 μM of FK228 for 24 h and the control group. Chromosome number in the

Table 1. Effect of FK228 treatment for 24 h on the karyotype of porcine foetal fibroblast cells.

| Groups          | Total cell numbers | Euploidy, % | Aneuploidy, % |
|-----------------|--------------------|-------------|---------------|
| Control         | 100                | 75          | 25            |
| 0.05 μM         | 100                | 62          | 38            |
| 0.1 μM          | 100                | 63          | 37            |
| 1 μM            | 100                | 52          | 48            |

Different superscripts in the same column mean significant difference exists (p < .05).

Euploidy or Aneuploidy rate = number of cells with normal karyotype/number of cells analysed.

Figure 2. Effect of FK228 treatment on the growth and survival of porcine foetal fibroblast cells. Growth curve of porcine foetal fibroblast cells at different densities (A) and with 6000 cells per well following treatment with different concentrations of FK228 for 24, 48, and 72 h (B). The A value is the absorbance value (also called optical density, OD), which was used to indirectly determine the concentration of a substance by enzyme-linked immunosorbent detector (BioTek-ELx800). (A) The ‘A’ value reflects the proliferation of cells and the starting zero well only contained cell culture medium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide, and DMSO. (B) B1, B2, and B3 are the survival of cells treated with 0.05, 0.1 and 1 μM of FK228 for 24, 48, and 72 h, respectively. The ‘A’ value reflects the proliferation of cells with an initial cell density of 6000 cells/well and the different standard shoulder indicate significant difference in the same day (p < .05).
control group, groups of 0.05 μM and 0.1 μM of FK228 for 24 h were in line with the normal chromosome number of pigs (38); however, group of 1 μM of FK228 for 24 h had a chromosome number of less than 38 (Figure 3). These results demonstrated that 0.1 μM FK228 treatment for 24 h could maintain the normal karyotypes of porcine foetal fibroblast cells.

**Effect of FK228 treatment of donor cells on the developmental efficiency and quality of SCNT embryos**

Before performing SCNT, porcine foetal fibroblast cells were treated with 0, 0.05, 0.1 and 1.0 of FK228 for 24 h. The fusion rate of reconstructed embryos in different treatment groups did not significantly differ from the control group (Table 2). The cleavage rate also did not significantly differ between the treatment groups and the control group. Interestingly, the blastocyst rate was highest in 0.1 μM FK228 treatment group. However, total cell numbers of blastocysts increased with increasing concentration and reached the highest when FK228 concentration was 1.0 μM (Table 3). So, these results revealed that 0.05 and 0.1 μM FK228 treatment of donor cells for 24 h did not improve developmental efficiency and quality of SCNT embryos.

**Effect of FK228 treatment of embryos on the early developmental competence of SCNT embryos**

There were no significant difference between treatment groups (0.05, 0.1 and 1.0 μM of FK228 for 24 h) and control group for cleavage rate, blastocyst rate and total cell number of blastocysts (Table 4). Unexpectedly, higher dose treatment (5, 50 and 100 μM of FK228 for 24 h) of embryos lead to the developmental arrest of SCNT embryos (Table 5). Furthermore, although significant difference was not observed between treatment groups (0.05, 0.1 and 1.0 μM of FK228 for 36 h) and control group for cleavage rate and blastocyst formation rate, 0.1 μM FK228 treatment for 36 h significantly improved the total cell number in blastocysts compared to the control group (Table 6). These results indicated 0.1 μM FK228 treatment for 36 h could improve developmental quality of SCNT embryos.

**Effect of combined treatments of both donor cells and embryos on the early development of SCNT embryos**

Donor cells were treated with 0.1 μM of FK228 for 24 h, after which SCNT embryos derived from these cells were further treated with 0.1 μM of FK228 for 36 h. This combined treatment strategy significantly improved the cleavage rate of SCNT embryos compared to the control group, but did not affect the blastocyst formation rate and total cell number per blastocysts (Table 7).

**Discussion**

The fundamental role of FK228 in fibroblast cells is improving the acetylation level of histones in the genome during cell reprogramming (Zhang et al. 2010). Previous studies have confirmed that FK228 is a novel

---

**Table 2.** Effect of treatment of donor cells using FK228 with different concentrations on fusion rate of porcine cloned embryos.

| Groups   | No. of cultured oocytes | Replicates | No. of fused oocytes, % (mean ± SEM)* |
|----------|-------------------------|------------|-------------------------------------|
| Control  | 149                     | 5          | 123 (82.74 ± 3.06)†                  |
| 0.05 μM  | 145                     | 5          | 123 (84.85 ± 1.31)†                  |
| 0.1 μM   | 147                     | 5          | 133 (89.67 ± 1.51)†                  |
| 1.0 μM   | 115                     | 4          | 103 (89.49 ± 2.22)†                  |

Different superscripts in the same column mean significant difference exist (p < .05). SEM: standard error of mean.

*Fusion rate = No. oocytes fused/No. oocytes cultured.

---

![Figure 3](image-url). Mitotic metaphase chromosomes distribution of porcine foetal fibroblast cells treated with FK228. (A) Control group; (B) 0.05 μM – 24 h group; (C) 0.1 μM – 24 h group; (D) 1.0 μM – 24 h group. This figure illustrates the metaphase chromosomes of porcine foetal fibroblasts (38). (A) is the control group without FK228, while (B, C, and D) are the groups treated with 0.05, 0.1 and 1.0 μM of FK228 for 24 h, respectively.
HDACi that could effectively inhibit the proliferation of tumour cells (Coiffier et al. 2014; Mummaneni & Shord 2014; Wei et al. 2014) and suffer more cytotoxicity in cancer cells than normal cells (Kasman et al. 2007; Emanuele et al. 2008). This study showed that FK228 treatment has a concentration- and time-dependent effect on the morphology and growth of porcine foetal fibroblast cells; and these findings are consistent

### Table 3. Effect of treatment of donor cells using FK228 with different concentrations on developmental competence and quality of porcine cloned embryos.

| Groups | No. of cultured embryos | Replicates | No. of cleaved embryos, % (mean ± SEM) | No. of blastocysts, % (mean ± SEM) | Total cell numbers of blastocysts (mean ± SEM) |
|--------|--------------------------|------------|---------------------------------------|-----------------------------------|-----------------------------------------------|
| Control | 95                       | 4          | 66 (76.6 ± 5.7)*                      | 24 (25.7 ± 4.7)*                  | 35.5 ± 5.0*                                  |
| 0.05 µM | 99                       | 4          | 72 (77.5 ± 3.2)*                      | 16 (16.7 ± 1.8)*                  | 34.9 ± 4.6*                                  |
| 0.1 µM  | 103                      | 4          | 77 (79.3 ± 3.1)*                      | 29 (29.0 ± 2.1)*                  | 39.3 ± 2.8*                                  |
| 1.0 µM  | 101                      | 4          | 82 (80.3 ± 3.8)*                      | 24 (25.6 ± 3.4)*                  | 48.3 ± 4.5*                                  |

Different superscripts in the same column mean significant difference exist (p < .05); SEM: standard error of mean.

*Cleavage rate = No. embryos cleaved/No. embryos cultured.

**Blastocyst rate = No. blastocysts/No. embryos cultured.

### Table 4. Effect of FK228 treatment of embryos with low dose for 24 h on developmental efficiency and quality of porcine cloned embryos.

| Group   | No. of cultured embryos | Replicates | No. of cleaved embryos, % (mean ± SEM) | No. of blastocysts, % (mean ± SEM) | Total cell numbers of blastocysts (mean ± SEM) |
|---------|--------------------------|------------|---------------------------------------|-----------------------------------|-----------------------------------------------|
| Control | 153                      | 6          | 117 (79.4 ± 4.5)                      | 25 (25.8 ± 4.1)                  | 44.3 ± 7.6                                   |
| 0.05 µM | 159                      | 6          | 121 (79.6 ± 5.1)                      | 33 (26.3 ± 3.2)                  | 45.8 ± 5.9                                   |
| 0.1 µM  | 156                      | 6          | 118 (80.3 ± 4.2)                      | 34 (27.8 ± 2.4)                  | 53.0 ± 6.6                                   |
| 1.0 µM  | 152                      | 6          | 116 (80.6 ± 4.0)                      | 35 (29.4 ± 3.5)                  | 39.7 ± 5.6                                   |

*Cleavage rate = No. embryos cleaved/No. embryos cultured.

**Blastocyst rate = No. blastocysts/No. embryos cultured.

### Table 5. Effect of FK228 treatment of embryos with high dose for 24 h on developmental efficiency and quality of porcine cloned embryos.

| Group   | No. of cultured embryos | Replicates | No. of cleaved embryos, % (mean ± SEM) | No. of blastocysts, % (mean ± SEM) | Total cell numbers of blastocysts (mean ± SEM) |
|---------|--------------------------|------------|---------------------------------------|-----------------------------------|-----------------------------------------------|
| Control | 155                      | 4          | 102 (79.3 ± 6.9)                      | 36 (29.6 ± 3.7)*                  | 39.8 ± 4.0*                                  |
| 5 µM    | 155                      | 4          | 107 (82.8 ± 5.4)                      | 0*                                | 0*                                            |
| 50 µM   | 157                      | 4          | 101 (80.4 ± 7.8)                      | 0*                                | 0*                                            |
| 100 µM  | 160                      | 4          | 89 (69.1 ± 2.1)                       | 0*                                | 0*                                            |

Different superscripts in the same column mean significant difference exist (p < .05); SEM: standard error of mean.

*Cleavage rate = No. embryos cleaved/No. embryos cultured.

**Blastocyst rate = No. blastocysts/No. embryos cultured.

### Table 6. Effect of FK228 treatment of embryos for 36 h on developmental efficiency and quality of porcine cloned embryos.

| Group   | No. of cultured embryos | Replicates | No. of cleaved embryos, % (mean ± SEM) | No. of blastocysts, % (mean ± SEM) | Total cell numbers of blastocysts (mean ± SEM) |
|---------|--------------------------|------------|---------------------------------------|-----------------------------------|-----------------------------------------------|
| Control | 102                      | 4          | 80 (83.1 ± 3.5)                       | 37 (39.2 ± 3.8)*                  | 38.6 ± 3.9*                                  |
| 0.05 µM | 101                      | 4          | 83 (84.8 ± 3.5)                       | 37 (37.7 ± 3.1)*                  | 48.3 ± 4.5*                                  |
| 0.1 µM  | 103                      | 4          | 84 (85.7 ± 1.2)                       | 40 (41.6 ± 5.5)*                  | 50.6 ± 3.6*                                  |
| 1.0 µM  | 102                      | 4          | 76 (77.3 ± 2.1)                       | 4 (4.0 ± 2.7)*                    | 52.5 ± 9.0*                                  |

Different superscripts in the same column mean significant difference exist (p < .05); SEM: standard error of mean.

*Cleavage rate = No. embryos cleaved/No. embryos cultured.

**Blastocyst rate = No. blastocysts/No. embryos cultured.

### Table 7. Effect of FK228 treatments of both donor cells and embryos on developmental efficiency and quality of porcine cloned embryos.

| Group            | No. of cultured embryos | Replicates | No. of cleaved embryos, % (mean ± SEM) | No. of blastocysts, % (mean ± SEM) | Total cell numbers of blastocysts (mean ± SEM) |
|------------------|--------------------------|------------|---------------------------------------|-----------------------------------|-----------------------------------------------|
| Control          | 158                      | 5          | 105 (71.3 ± 6.3)*                     | 33 (25.0 ± 2.5)                  | 44.3 ± 4.8                                    |
| Combined treatment | 168                     | 5          | 131 (81.0 ± 1.7)*                     | 44 (29.6 ± 4.8)                  | 37.4 ± 4.9                                    |

Different superscripts in the same column mean significant difference exist (p < .05); SEM: standard error of mean.

*Cleavage rate = No. embryos cleaved/No. embryos cultured.

**Blastocyst rate = No. blastocysts/No. embryos cultured.
with the results reported previously (Savickiene et al. 2006; Hoshino et al. 2008). From the results of the growth curve, 0.1 μM FK228 for 24 h promoted the survival and proliferation of porcine foetal fibroblast cells, but it is interesting that 0.05 μM FK228 treatment for 24 h inhibited the proliferation of foetal fibroblast cells and this inhibition is reversible, and the property gradually weakened with increased concentration and time, which was contrary to the results of study (Kosugi et al. 2001). This may have a dose- and time-dependent manner of FK228 treatment, and further exploration is required to determine the type and generations of cells influenced by FK228 treatment. Various concentrations of FK228 and durations of exposure to FK228 could cause an abnormal number of chromosomes. The potential mechanisms might be as follows: (1) FK228 suppresses the mitotic checkpoint kinase Chk1, thereby increasing the sensitivity of normal cells to HDACi. At this time, a mitosis would be blocked in a large proportion of cells, along with sister chromatid adhesion proteins and chromosome destruction, and finally cell death (Lee et al. 2011). (2) FK228 might affect multiple epigenetically modified features on centromeres, such as enhanced H3K9 acetylation, and lower trimethylation level, which cause the mislocalization of chromosomal passenger proteins on the centromere, so that centromere assembly and function are severely impaired and result in abnormal mitosis (Zhang et al. 2010), which may be another important factor for the abnormal number of chromosomes.

The SCNT technique has been used in basic research as well as interspecies nuclear transfer to rescue endangered animals (Galli et al. 2003). But, the low cloning efficiency and reconstructed embryo dysplasia resulting from incomplete reprogramming of the donor nucleus are responsible for the slow pace of development in the field of porcine SCNT (Hill et al. 2000; Yang et al. 2007). Different studies have shown that the most important and effective way to promote epigenetic reprogramming of donor cell nuclei and restore their totipotency is to change the histone acetylation or DNA methylation status of somatic cell genome before and/or after its transplantation into a cytoplasm of enucleated recipient oocyte (Samiec et al. 2012; Staszkiewicz et al. 2013; Zhou et al. 2014; Samiec et al. 2015). Alone treatment of donor cells with different concentrations of FK228 for 24 h did not obviously improve the cleavage rate of cloned embryos. Furthermore, the blastocyst rate was the lowest in the group of 0.05 μM, consistent with the results of the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide test, while the fusion rate of reconstructed embryos had improved slightly in treatment groups of 0.1 and 1 μM HF228. There were significant improvements in the quality of the embryos treated with increasing concentrations of FK228, which matched the evidence from the cell growth test. This is the first study to report the influence of FK228 on the development efficiency of cloned embryos derived from FK228-treated donor cells. When the results were compared with those seen with other histone deacetylase inhibitors such as TSA and Scriptaid, FK228 treatment only slightly improved the blastocyst rate of cloned embryos derived from FK228-treated donor cells.

This was the first study in which porcine-cloned embryos were treated with FK228. Treatment of SCNT embryos with low or high dose alone for 24 h did not affect the cleavage rate, blastocyst formation rate and total cell number in cloned blastocysts. However, when we extended from 24 to 36 h of exposure to 0.1 μM FK228 for cloned embryos, such strategy of prolonged epigenetic modulation gave rise to improvement of the blastocyst quality, which might be attributed to provide more extending time window for somatic genome to promote more effective epigenetic reprogramming of genome in cloned embryos exposed to FK228 HDACi for 36 h. Therefore, in the next step of investigations, we decided to examine whether simultaneous treatment of both nuclear donor cells and activated SCNT-derived oocytes with the use of FK228 could improve the developmental competences and quality of cloned embryos. Although combined treatment strategy did not facilitate the blastocyst formation rate and total cell number in blastocysts, it dramatically improved the cleavage rate of SCNT embryos, which might be due to better epigenetic reprogramming of cloned embryos exposed to FK228. However, further studies should be performed to determine the specific mechanisms of FK228 in the development of porcine SCNT embryos.

**Conclusions**

In conclusion, the efficient strategies of romidepsin-dependent epigenetic modulation of nuclear donor cells and/or activated SCNT-derived oocytes that encompass optimal FK228 concentration and exposure time not only can maintain the normal cellular characteristics of porcine foetal fibroblast cells, but can also improve early developmental potential and quality of porcine-cloned embryos.

**Acknowledgements**

The authors are thankful to Ms. Ronghua Wu, Jia Tao, and Xing Liu for excellent technical assistance. This study was
supported by the National Natural Science Foundation of China (31272442), National Transgenic Organisms Breeding Major Project (2014ZX08006-01B), and National Training Program of Innovation and Entrepreneurship for Undergraduates (201410364005). Useful comments on the manuscript from Dr. Yong Pu, Dr. Meiling Zhang and Mr. Fei Zhang are appreciated. The authors also express their sincere appreciation to the excellent research teams of Anhui Provincial Laboratory for Local Livestock and Poultry, Genetic Resource Conservation and Bio-Breeding, Anhui Agricultural University for their valuable contribution.

Disclosure statement

The authors declare no conflicts of interest in the research.

Funding

This study was supported by the National Natural Science Foundation of China (31272442), National Transgenic Organisms Breeding Major Project (2014ZX08006-01B), and National Training Program of Innovation and Entrepreneurship for Undergraduates (201410364005).

References

Bethhauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, Forsythe T, Golueke P, Jurgella G, Koppang R, et al. 2000. Production of cloned pigs from in vitro systems. Nature Biotechnology. 18:1055–1059.

Bishton MJ, Harrison SJ, Martin BP, McLaughlin N, James C, Josefsson EC, Henley KJ, Kile BT, Prince HM, Johnstone RW. 2011. Deciphering the molecular and biologic processes that mediate histone deacetylase inhibitor-induced thrombocytopenia. Blood. 117:3658–3668.

Cao Z, Sui L, Li Y, Ji S, Zhang X, Zhang Y. 2012. Effects of chemically defined medium on early development of porcine embryos derived from parthenogenetic activation and cloning. Zygote. 20:229–236.

Chen CH, Du F, Xu J, Chang WF, Liu CC, Su HY, Lin TA, Ju JC, Cheng WT, Wu SC, et al. 2013. Synergistic effect of trichostatin A and scriptaid on the development of cloned rabbit embryos. Theriogenology. 79:1284–1293.

Coiffier B, Pro B, Prince HM, Foss F, Sokol L, Greenwood M, Caballero D, Morschhauser F, Wilhelm M, Pinter-Brown L, et al. 2014. Romidepsin for the treatment of relapsed/refractory peripheral T-cell lymphoma: pivotal study update demonstrates durable responses. J Hematol Oncol. 7:11.

Costa-Borges N, Santalo J, Ibanez E. 2010. Comparison between the effects of valproic acid and trichostatin A on the in vitro development, blastocyst quality, and full-term development of mouse somatic cell nuclear transfer embryos. Cell Reprogram. 12:437–446.

Das ZC, Gupta MK, Uhm SJ, Lee HT. 2010. Increasing histone acetylation of cloned embryos, but not donor cells, by sodium butyrate improves their in vitro development in pigs. Cellular Reprogramming. 12:95–104.

Diao YF, Naruse KJ, Han RX, Li XX, Oqani RK, Lin T, Jin DI. 2013. Treatment of fetal fibroblasts with DNA methylation inhibitors and/or histone deacetylase inhibitors improves the development of porcine nuclear transfer-derived embryos. Animal Reprod Sci. 141:164–171.

Emanuele S, Lauricella M, Tesoriere G. 2008. Histone deacetylase inhibitors: apoptotic effects and clinical implications (Review). Int J Oncol. 33:637–646.

Endo T, Kano K, Naito K. 2008. Nuclear histone deacetylases are not required for global histone deacetylation during meiotic maturation in porcine oocytes. Biol Reprod. 78:1073–1080.

Galli C, Lagutina I, Lazzari G. 2003. Introduction to cloning by nuclear transplantation. Cloning Stem Cells. 5:223–232.

Hill JR, Burchardt RC, Jones K, Long CR, Looney CR, Shin T, Spencer TE, Thompson JA, Winger QA, Westhusin ME. 2000. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. Biol Reprod. 63:1787–1794.

Hoshino I, Matsubara H, Akutsu Y, Nishimori T, Yoneyama Y, Murakami K, Sakata H, Matsushita K, Komatsu A, Brooks R, et al. 2008. Role of histone deacetylase inhibitor in adenovirus-mediated p53 gene therapy in esophageal cancer. Anticancer Res. 28:665–671.

Huan YJ, Zhu J, Xie BT, Wang JY, Liu SC, Zhou Y, Kong QR, He HB, Liu ZH. 2013. Treating cloned embryos, but not donor cells, with 5-aza-2′-deoxycytidine enhances the developmental competence of porcine cloned embryos. J Reprod Dev. 59:442–449.

Jeanisch R, Eggan K, Humphrys D, Rideout W, Hochedlinger K. 2002. Nuclear cloning, stem cells, and genomic reprogramming. Cloning Stem Cells. 4:389–396.

Kasman L, Lu P, Voelkel-Johnson C. 2007. The histone deacetylase inhibitors depsipeptide and MS-275, enhance TRAIL gene therapy of LNCaP prostate cancer cells without adverse effects in normal prostate epithelial cells. Cancer Gene Therapy. 14:327–334.

Kosugi H, Ito M, Yamamoto Y, Towatari M, Ueda R, Saito H, Naoe T. 2001. In vivo effects of a histone deacetylase inhibitor, FK228, on human acute promyelocytic leukemia in NOD/Shi-scid/scid mice. Jpn J Cancer Res Gann. 92:529–536.

Kurome M, Geistlinger L, Kessler B, Zakhartchenko V, Klymiuk N, Wuenesch A, Richter A, Baehr A, Kraehe K, Burkhardt K, et al. 2013. Factors influencing the efficiency of generating genetically engineered pigs by nuclear transfer: multi-factorial analysis of a large data set. BMC Biotechnology. 13:43.

Lee JH, Choy ML, Ngo L, Venta-Perez G, Marks PA. 2011. Role of checkpoint kinase 1 (Chk1) in the mechanisms of resistance to histone deacetylase inhibitors. Proc Natl Acad Sci USA. 108:19629–19634.

Li X, Kato Y, Tsuji Y, Tsunoda Y. 2008. The effects of trichostatin A and scriptaid on mRNA expression of chromatin structure-, DNA methylation-, and development-related genes in cloned mouse blastocysts. Cloning Stem Cells. 10:133–142.

Liu XY, Wang C, Cheng YQ. 2012. FK228 from Burkholderia thailandensis MSMB43. Acta Crystallogr Sect E. 68:o2757–o2758.

Ma J, Li Q, Li Y, Wen X, Li Z, Zhang Z, Zhang J, Yu Z, Li N. 2016. Expression of recombinant human α-lactalbumin in milk of transgenic cloned pigs is sufficient to enhance intestinal growth and weight gain of suckling piglets. Gene. 584:7–16.
Makala L, Di Maro S, Lou TF, Sivanand S, Ahn JM, Pace BS. 2012. FK228 analogues induce fetal hemoglobin in human erythroid progenitors. Anemia. 2012:428137.

Meng Q, Polgar Z, Liu J, Dinnyes A. 2009. Live birth of somatic cell-cloned rabbits following trichostatin A treatment and cotransfer of parthenogenetic embryos. Cloning Stem Cells. 11:203–208.

Mummaneni P, Shord SS. 2014. Epigenetics and oncology. Pharmacotherapy. 34:495–505.

Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H, Perry AC. 2000. Pig cloning by microinjection of fetal fibroblast nuclei. Science. 289:1188–1190.

Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, et al. 2000. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature. 407:86–90.

Samec M. 2004. Development of pig cloning studies: past, present and future. J Animal Feed Sci. 13:211–238.

Samec M. 2005. The effect of mitochondrial genome on nuclear-reprogramming of donor cell nuclei in mammalian nuclear transfer-derived embryos. J Animal Feed Sci. 14:393–422.

Samiec M, Opiela J, Lipinski D, Romanek J. 2015. Trichostatin A-mediated epigenetic transformation of adult bone marrow-derived mesenchymal stem cells biases the in vitro developmental capability, quality, and pluripotency extent of porcine cloned embryos. BioMed Res Int. 2015:814686.

Samiec M, Skrzyszowska M. 2011a. The possibilities of practical application of transgenic mammalian species generated by somatic cell cloning in pharmacology, veterinary medicine and xenotransplantology. Pol J Vet Sci. 14:329–340.

Samiec M, Skrzyszowska M. 2011b. Transgenic mammalian species, generated by somatic cell cloning, in biomedicine, biopharmaceutical industry and human nutrition/diets–recent achievements. Pol J Vet Sci. 14:317–328.

Samiec M, Skrzyszowska M, Lipinski D. 2012. Pseudophysiological transcomplementary activation of reconstructed oocytes as a highly efficient method used for producing nuclear-transferred pig embryos originating from transgenic foetal fibroblast cells. Pol J Vet Sci. 15:509–516.

Samiec M, Skrzyszowska M. 2005. Molecular conditions of the cell nucleus remodelling/reprogramming process and nuclear-transferred embryo development in the intracellular karyoplast injection technique: a review. Czech J Animal Sci. 50:185–195.

Savickiene J, Treigyte G, Borutinskaite V, Navakauskiene R, Magnusson KE. 2006. The histone deacetylase inhibitor FK228 distinctly sensitizes the human leukemia cells to retinoic acid-induced differentiation. Ann NY Acad Sci. 1091:368–384.

Shi J, Zhou R, Luo L, Mai R, Zeng H, He X, Liu D, Zeng F, Cai G, Ji H, et al. 2015. Influence of embryo handling and transfer method on pig cloning efficiency. Animal Reprod Sci. 154:121–127.

Staszkiewicz J, Power RA, Harkins LL, Barnes CW, Strickler KL, Rim JS, Bondioli KR, Eilersten KJ. 2013. Silencing histone deacetylase-specific isoforms enhances expression of pluripotency genes in bovine fibroblasts. Cell Reprogram. 15:397–404.

Wang N, Le F, Zhan QT, Li L, Dong MY, Ding GL, Xu CM, Jiang SW, Huang HF, Jin F. 2010. Effects of in vitro maturation on histone acetylation in metaphase II oocytes and early cleavage embryos. Obstet Gynecol Int. 2010:989278.

Wei DG, Chiang V, Fyne EL, Balakrishnan M, Barnes T, Graupe M, Hesselgesser J, Irrinki A, Murry JP, Stepan G, et al. 2014. Histone deacetylase inhibitor romidepsin induces HIV expression in CD4 T cells from patients on suppressive antiretroviral therapy at concentrations achieved by clinical dosing. PLoS Pathog. 10:e1004071.

Xiong X, Lan D, Li J, Zhong J, Zi X, Ma L, Wang Y. 2013. Zebularine and scriptaid significantly improve epigenetic reprogramming of yak fibroblasts and cloning efficiency. Cell Reprogram. 15:293–300.

Xu W, Wang Y, Li Y, Wang L, Xiong X, Su J, Zhang Y. 2012. Valproic acid improves the in vitro development competence of bovine somatic cell nuclear transfer embryos. Cell Reprogram. 14:138–145.

Yang X, Smith SL, Tian XC, Lewin HA, Renard JP, Wakayama T. 2007. Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. Nature Genet. 39:295–302.

Zhang X, Zhang Z, Chen G, Zhao M, Wang D, Du Z, Xu Y, Yu X. 2010. FK228 induces mitotic catastrophe in A549 cells by mistargeting chromosomal passenger complex localization through changing centromeric H3K9 hypoacetylation. Acta Biochim Biophys Sin. 42:677–687.

Zhao J, Ross JW, Hao Y, Spate LD, Walters EM, Samuel MS, Rieke A, Murphy CN, Prather RS. 2009. Significant improvement in cloning efficiency of an inbred miniature pig by histone deacetylase inhibitor treatment after somatic cell nuclear transfer. Biol Reprod. 81:525–530.

Zhou N, Cao Z, Wu R, Liu X, Tao J, Chen Z, Song D, Han F, Li Y, Fang F, et al. 2014. Dynamic changes of histone H3 lysine 27 acetylation in pre-implantational pig embryos derived from somatic cell nuclear transfer. Animal Reprod Sci. 148:153–163.