REPRODUCTION
RESEARCH

Inhibition of RNA synthesis during Scriptaid exposure enhances gene reprogramming in SCNT embryos

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Abstract

Insufficient epigenetic reprogramming is incompatible with normal development of embryos produced by somatic cell nuclear transfer (SCNT), but treatment with histone deacetylases inhibitors (HDACi) enhances development of SCNT embryos. However, the mechanisms underpinning HDACi benefits in SCNT embryos remain largely uncharacterized. We hypothesized that, in addition to enhancing reprogramming, HDACi treatment may promote expression of genes not required for early development of SCNT embryos. To test this hypothesis, RNA synthesis was inhibited by treating bovine SCNT embryos with 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DBR), which were concomitantly treated or not with Scriptaid (Scrip; an HDACi). Development to the blastocyst stage was significantly increased by treatment with Scrip alone (26.6%) or associated with DRB (28.6%) compared to Control (17.9%). The total number of nuclei was significantly improved only in embryos that were treated with both Scrip + DRB. Nuclear decondensation after SCNT was significantly increased by DRB treatment either alone or associated with Scrip. The relative mRNA expression, evaluated during the embryo genome activation (EGA) transition, revealed that some KDMs (*KDM1A*, *KDM3A*, *KDM4C* and *KDM6A*) and *DNMT1* where prematurely expressed in Scrip-treated embryos. However, treatment with Scrip + DRB inhibited early mRNA expression of those genes, as well as several other KDMs (*KDM4A*, *KDM4B*, *KDM5A*, *KDM5B*, *KDM5C* and *KDM7A*) compared to embryos treated with Scrip alone. These findings revealed that HDACi improved development in SCNT embryos compared to Control, but altered the expression of genes involved in epigenetic regulation and did not improve embryo quality. Inhibition of RNA synthesis during HDACi treatment enhanced nuclear chromatin decondensation, modulated gene expression and improved SCNT embryo quality.

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Introduction

Somatic cell nuclear transfer (SCNT) has been used to investigate cell reprogramming mechanisms during embryo development (Meissner & Jaenisch 2006). In addition, SCNT has been applied to clone animals of several species and for different purposes, including animal production, conservation and creation of disease models for biomedical research (Gutierrez *et al.* 2015, Whitelaw *et al.* 2016). However, the efficiency of SCNT-based reprogramming remains low, since only less than 5% of the embryos created by SCNT generally develop to term (Keefer 2015).

The low efficiency of SCNT cloning has been attributed to incomplete epigenetic reprogramming in the transplanted nuclei (Niemann 2016). Indeed, normal gene expression during embryo development is coordinated by epigenetic factors including DNA methylation and post-translational modification of histones (Sepulveda-Rincon *et al.* 2016). Insufficient epigenetic reprogramming may result in the expression of somatic genes in the SCNT embryo, which can perturb cell function and differentiation (Ng & Gurdon 2005, 2008). Thus, for successful development of SCNT embryos, the epigenetic memory in the transplanted nuclei must undergo sufficient remodeling to allow embryo cells to attain a totipotent state and recapitulate gene expression patterns of normally developing embryos (Pichugin *et al.* 2010). So far, research efforts to enhance epigenetic reprogramming in SCNT embryos have produced inconsistent results.

Inhibitors of histone deacetylase enzymes (HDACi) were the first epigenetic modulators shown to improve cell reprogramming in SCNT embryos (Kishigami *et al.* 2006, 2007). Treatment with HDACi increases histone acetylation in SCNT, which promotes an open chromatin state by reducing histone–DNA interaction, thus facilitating chromatin access to reprogramming factors present in the host oocyte cytoplasm (Rybouchkin *et al.* 2006, Bui *et al.* 2011). It has been shown that HDACi

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treatment facilitates DNA synthesis (Bui *et al.* 2010), mRNA expression (Van Thuan *et al.* 2009) and DNA damage repair (Bohrer *et al.* 2014) in SCNT embryos. HDACi treatment also modulates expression of genes important for reprogramming (Miyamoto *et al.* 2017) and decreases DNMT1 expression and DNA methylation in SCNT embryos (Liang *et al.* 2015, Sun *et al.* 2015). Nonetheless, besides increasing embryo development (Kang *et al.* 2013), the exact mechanism by which HDACi treatment enhances nuclear reprogramming in SCNT embryos remains to be elucidated.

Recent studies proposed that histone 3 lysine 9 trimethylation (H3K9me3)-rich domains present in the genome of somatic cells are critical epigenetic barriers for cell reprogramming and SCNT cloning success. Indeed, removal of H3K9me3 through expression of specific lysine demethylases (e.g., *KDM4D*, *KDM4A*, *KDM4E*) improved cell reprogramming, SCNT embryo development, derivation of embryonic stem cell and increased animal cloning efficiency (Matoba *et al.* 2014, Dahl *et al.* 2016, Ruan *et al.* 2018, Liu *et al.* 2018*a*). Trimethylation of histone 3 lysine 4 (H3K4me3) and histone 3 lysine 27 (H3K27me3) are other potential critical components of epigenetic reprogramming in SCNT embryos. Demethylation of broad H3K4me3 domains by KDM5 family demethylases is required for embryo genome activation (EGA) and development of mouse embryos produced by fertilization (Dahl *et al.* 2016, Zhang *et al.* 2016*a*). Moreover, SCNT embryos with higher developmental potential showed a peak of KDM5B expression during the EGA period (Liu *et al.* 2016*a*), implying that H3K4me3 removal is also critical for SCNT reprogramming. Similarly, H3K27me3 was identified as a barrier for cell reprogramming and generation of porcine-induced pluripotent stem (iPS) cells (Xie *et al.* 2016). In addition, inhibition of the KDM6B, which demethylates H3K27me3, compromised bovine embryo development and blastocyst formation (Canovas *et al.* 2012, Chung *et al.* 2017).

Altered expression of the X-inactivate-specific transcript (*XIST*) gene, which regulates X chromosome inactivation, was detected as another important component for nuclear reprograming in SCNT embryos because it downregulates the expression of many X-linked genes. Suppression or attenuation of *XIST* gene dramatically increased SCNT efficiency for cloning mice (Inoue *et al.* 2010, Matoba *et al.* 2011). Increased SCNT cloning efficiency was also reported in pigs when *XIST* was attenuated along with H3K9me3 demethylation (Ruan *et al.* 2018). In bovine SCNT embryos, aberrant patterns of H3K27 methylation were found to be associated with X chromosome inactivation (Breton *et al.* 2010). Together, findings from those studies indicate that methylation of H3K4, 9 and 27 control crucial mechanisms necessary for successful cell reprogramming in SCNT embryos.

In a recent study, we have found that the expression of several histone demethylases of the lysine 4, 9 and 27 of the histone H3 during the EGA transition was altered in bovine and porcine SCNT embryos compared with IVF embryos (Glanzner *et al.* 2018). Because histone acetylation allows an open chromatin state, thus promoting transcriptional activity (Bannister & Kouzarides 2011), it is possible that the reported benefits of HDACi treatment on development of SCNT embryos are linked to a better modulation in the expression of demethylases of H3K4, 9 and 27. Notably, HDACi treatment is generally applied to SCNT embryos for 10–15 h starting immediately after nuclear transfer (Kishigami *et al.* 2014). At this developmental stage, embryos have low transcriptional activity since EGA occurs at later cleavage stages. In cattle, major EGA occurs 3–4 days after fertilization during the transition from 8- to 16-cell stages (Frei *et al.* 1989, Graf *et al.* 2014*b*). Based on the premise that the epigenetic memory of the donor nucleus must be erased to ensure proper nuclear reprogramming in SCNT embryos (Firas *et al.* 2014), it is possible that HDACi treatment may promote transcription of somatic genes having adverse consequences on development of SCNT embryos. Indeed, there is evidence suggesting low transcriptional activity during cell reprogramming in early-stage embryos derived by SCNT (Smith *et al.* 1996, Liu *et al.* 2018*a*). Based on this, we hypothesized that the positive effect of HDACi treatment on cell reprograming after SCNT would be further improved by inhibiting mRNA transcription. Therefore, this study was conceived to evaluate the consequences of inhibiting mRNA transcription during HDACi treatment on SCNT embryo development and quality, nuclear chromatin decondensation and expression of important KDMs of H3K4, H3K9 and H3K27 at different developmental stages spanning the EGA transition.

Materials and methods

Chemicals

Unless otherwise indicated, chemicals and reagents were purchased from Sigma Chemical Company (Sigma-Aldrich).

Oocyte collection and in vitro *maturation*

Cow ovaries were obtained from a local abattoir and transported to the laboratory in saline solution (0.9% NaCl; 30°C) containing 100 IU/mL penicillin and 50 mg/mL streptomycin sulfate. Cumulus-oocyte complexes (COCs) from 3 to 8 mm diameter follicles were aspirated with a vacuum pump (vacuum rate of 20 mL of water/minute). The COCs were recovered and selected under a stereomicroscope. Grade 1 and 2 COCs were randomly distributed into 400 µL of maturation medium in four-well plates (Nunc, Roskilde, Denmark) and cultured in an incubator at 39°C in a saturated humidity atmosphere containing 5% CO_2 and 95% air, for 22–24 h. The maturation medium consisted of TCM-199 containing

Earle's salts and L-glutamine (Gibco Labs), supplemented with 25 mM HEPES, 0.2 mM pyruvic acid, 2.2 mg/mL sodium bicarbonate, 5.0 µg/mL LH (Lutropin-V, Vetoquinol, Belleville, ON, Canada), 0.5 µg/mL FSH (Folltropin-V, Vetoquinol), 10% fetal bovine serum (FBS; Gibco), 100 IU/mL penicillin and 50 µg/mL streptomycin sulfate.

Somatic cell nuclear transfer and embryo culture

Fibroblast cells derived from a male fetus were cultured *in vitro* in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM-F12), supplemented with 10% FBS and 1% antibiotics (10,000 IU/mL penicillin and 10,000 µg/mL streptomycin) at 37°C in 5% CO_2 and 95% air. For nuclear transfer, cells were maintained in culture for at least 48 h after reaching confluence. Cumulus-free oocytes with an extruded polar body (metaphase II) were cultured in TCM-199 supplemented with 0.4 µg/mL demecolcine and 0.05 M sucrose for 60 min. This treatment resulted in a small protrusion in the ooplasmic membrane that contained the metaphase chromosomes, facilitating enucleation. A nuclear donor cell was transferred into the perivitelline space of each enucleated oocyte and then fused electrically using a single DC pulse of 32V for 70 µs. Electrofusion was performed in a 0.28 M mannitol solution supplemented with 50 µM $CaCl_2$, 100 µM $MgSO_4$ and 0.1% BSA. Oocytes were then transferred to TCM-199 medium supplemented with 3 mg/mL BSA for 1 h to allow cell fusion.

Reconstructed oocytes were exposed to ionomycin (5 µM) for 5 min and then transferred to synthetic oviduct fluid (SOF) medium supplemented with cytochalasin B (7.5 µg/mL) and cycloheximide (10 µg/mL) for 4 h. After the activation period, embryos were cultured at 39°C in an incubator culture chamber (CBS Scientific, Del Mar, CA, USA) with saturated humidity atmosphere containing 5% CO_2, 5% O_2 and 90% N_2 in 400 µL SOF medium in four-well plates for 7 days.

To assess the effect of inhibiting histone deacetylases and RNA synthesis, the reconstructed oocytes were randomly divided into four experimental groups and cultured for the first 15 h following activation in the above conditions in SOF medium containing: (a) vehicle (Control; DMSO 0.2% final concentration); (b) 500 nM Scriptaid (Scrip), an inhibitor of histone deacetylases; (c) 100 µM 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DBR), an inhibitor of RNA synthesis or (d) 100 µM DRB and 500 nM Scriptaid (DRB + Scrip). Stock solutions of DRB and Scrip (1000× concentrated) were prepared in DMSO and stored at −80°C. Reconstructed embryos were then thoroughly washed and cultured in SOF medium. Cleavage rates were determined after 48 h of culture and blastocyst rates on day 7.

Embryo cell counting, differential cell staining and nuclear area measurement

Embryos that developed to the blastocyst stage were separated, rinsed in PBS containing 0.1% polyvinyl alcohol (PBS-PVA), fixed for 15–20 min in 4% paraformaldehyde, and then stored in PBS containing 0.3% BSA and 0.1% Triton X-100 at 4°C. Fixed embryos were incubated for 1 h at room temperature in blocking solution (3% BSA and 0.2% Tween-20 in PBS), and then maintained overnight in the presence of anti-CDX-2 mouse monoclonal primary antibody (BioGenex, Fremont, CA, USA) diluted 1:500 in blocking solution. Negative controls were incubated in the absence of primary antibody. Embryos were then washed three times for 20 min each in blocking solution and incubated for 1 h at room temperature in the presence of 1:1000 diluted Goat Anti-Mouse IgG (Alexa Fluor 555, Abcam) secondary antibody. Samples were washed three times (20 min each) in blocking solution and mounted on a slide using a drop of mowiol containing 10 µg/mL of DAPI for chromatin visualization. Slides were stored in a dark box at 4°C and examined within 48 h after preparation. Nuclei were counted in each embryo using an epifluorescence microscope (DMI 4000B, Leica, 200× magnification). Differential cell count was performed by dividing the CDX-2 positive by total number of nuclei per embryo. For nuclear area measurement, reconstructed oocytes from all experimental groups were fixed at 12 h post activation (hpa) and stained with DAPI as previously described. The nuclear area was measured using the ImageJ Software.

Assessment of RNA synthesis in somatic cells

Detection of RNA synthesis was performed using the Click-iT EU RNA Imaging kits (Invitrogen, Life Technologies). Cells treated or not with 100 µM DRB for 12 h were incubated with 1 mM EU (5-ethynyl uridine) for 2 h. Cells were then fixed in 4% paraformaldehyde and stained according to the manufacturer's instructions. Samples were mounted on microscope slides; RNA synthesis was evaluated using an epifluorescence microscope (DMI 4000B, Leica, 1000× magnification). Nuclear area after staining with 10 µM Hoechst 33342 was measured using the LAS AF software.

RNA extraction, reverse transcription and quantitative PCR

Total RNA was extracted from groups of 15 embryos on days 3 (D3), 4 (D4) or 5 (D5) of development using the PicoPure RNA Isolation Kit (Life Technologies) according to the manufacturer's instructions. RNA was treated with DNase I (Qiagen) and reverse transcribed using SuperScript VILO cDNA Synthesis Kit (Life Technologies). Real-time quantitative PCR (qPCR) reactions were performed in a CFX 384 real-time PCR detection system (BioRad) using Advanced qPCR Mastermix (Wisent Bioproducts – St-Bruno, QC, CA, USA). Primers were designed based on bovine sequences available in GenBank (Supplementary Table 1, see section on supplementary data given at the end of this article) and synthesized by IDT (Windsor, ON, CA, USA). Samples were run in duplicates and the standard curve method was used to determine the abundance of mRNA for each gene, and expression was normalized to the mean abundance of the internal control gene histone 2A (*H2A*). All reactions had efficiency between 90 and 110%, $r^2 \geq 0.98$ and slope values from −3.6 to −3.1. Dissociation curve analyses were performed to validate the specificity of the amplification products.

Table 1 Development of SCNT embryos from different treatments.

Treatment	Cultured embryos	Cleaved (%)	Blastocysts (%)*
Control	162	117 (72.2 ± 3.3)	21 (17.9 ± 2.0)^{b}
DRB	161	119 (73.9 ± 4.0)	26 (21.8 ± 2.2)^{a,b}
Scrip	168	128 (76.2 ± 2.7)	34 (26.6 ± 2.1)^{a}
DRB + Scrip	159	119 (74.8 ± 2.9)	34 (28.6 ± 3.6)^{a}

Different superscripts indicate significant differences between treatments, $P < 0.05$.
*Percentages of embryos per cleaved.

Statistical analysis

Data of cleavage, embryo development, number of nuclei, nuclear swelling and gene expression were analyzed by the LSMeans Student *t-test* using the JMP software (SAS Institute Inc., Cary, NC, USA). Data were tested for normal distribution using the Shapiro–Wilk test and normalized when necessary. Results are presented as means ± standard error of the mean (S.E.M.). $P < 0.05$ was considered statistically significant.

Results

Effect of inhibiting RNA synthesis and histone deacetylases on embryo development

In the first experiment, embryos were treated with DRB and Scrip alone or combined (DRB + Scrip) for 15 h starting after activation. A total of 650 SCNT embryos were produced in eight replicates. Cleavage rates were not different between Control, DRB, Scrip and DRB + Scrip groups (Table 1). However, development of cleaved embryos to the blastocyst stage was significantly increased ($P < 0.05$) by 37.4% in the DRB + Scrip and 32.7% in the Scrip groups compared with Control. Blastocyst development in the DRB group was not statistically different from the other groups (Table 1). Interestingly, the total number of nuclei in blastocysts was significantly increased ($P < 0.05$) by 35.6, 48.4 and 34.5% in the DRB + Scrip (101.7 ± 9.1) compared with Control (75 ± 8.9), DRB (68.5 ± 3.7) and Scrip (75.6 ± 8.4) groups, respectively (Fig. 1). The ratio of ICM/total cell number in blastocysts was not affected by treatment (Fig. 1A and B).

Effect of inhibiting RNA synthesis and histone deacetylases on nuclear decondensation

The nuclear area (*pixels*^{2}), determined at 12 hpa, was significantly increased ($P < 0.05$) by 91.2 and 99.2% in embryos treated with DRB (6019.8 ± 567.5) or DRB + Scrip (6270.5 ± 1073.3) compared with the Control group (3147.8 ± 668.1), respectively. Nuclear area in embryos treated with Scrip (4896.8 ± 964.4) was not statistically different from the other groups (Fig. 2).

Effect of inhibiting RNA synthesis and histone deacetylases on gene expression

Embryos were collected on D3, D4 or D5 of development to determine the relative mRNA level of several genes encoding histone lysine demethylases (KDMs) of H3K4, H3K9 and H3K27, as well as DNA methyltransferase 1 (*DNMT1*) and *XIST*. Because treatment with DRB alone did not affect embryo development and cell number, this treatment was not included in the experiments of mRNA analyses, which was performed with embryos from Control, Scrip and DRB + Scrip groups.

Relative mRNA abundance of H3K4 demethylases

The relative mRNA abundance of genes encoding KDMs of H3K4 changed at the different developmental stages of SCNT embryos from all the treatments (Fig. 3). While the relative mRNA abundance of three KDMs (*KDM1A*, *KDM5A* and *KDM5C*) increased from D3 to D5 of development in Control embryos, mRNA of four KDMs was altered (*KDM1A*, *KDM2B* and *KDM5C* increased and KDM1B decreased) between D3 and D5 in Scrip-treated embryos. Interestingly, the mRNA abundance of all six KDMs analyzed changed (*KDM1A*, *KDM2B*, *KDM5A*, *KDM5B* and *KDM5C* increased, and *KDM1B* decreased) from D3 to D5 of development in embryos treated with DRB + Scrip. Regarding the effect of treatment within developmental stages, significant differences in mRNA abundance were found between treatments, but only at day 3 of embryo development (Fig. 3). While treatment with Scrip increased mRNA levels of *KDM1A* compared with Control, treatment with DRB + Scrip decreased mRNA abundance of *KDM1A*, *KDM5A*, *KDM5B* and *KDM5C* compared with Control and Scrip treatments, and increased *KDM1B* mRNA compared with Control group.

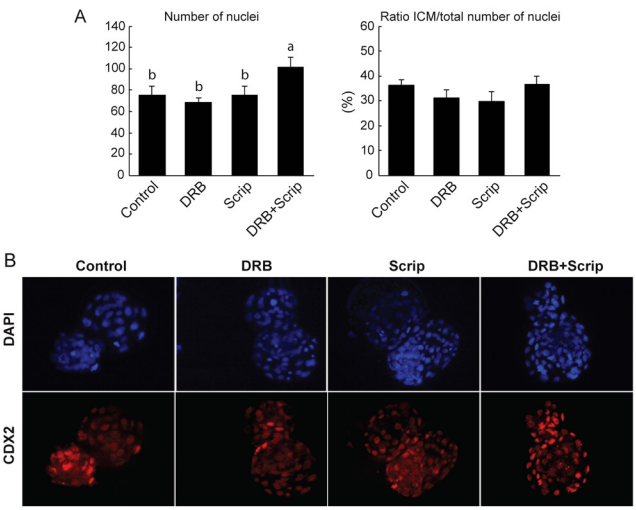

Figure 1 Number of nuclei in SCNT-derived embryos from Control, DRB, Scrip and DRB + Scrip-treated embryos. (A) Total number of nuclei on D7 blastocysts and ICM/total number of nuclei ratio. (B) Representative pictures of CDX2 immunofluorescence staining for differential cell count in D7 blastocysts. Results are presented as means ± S.E.M., and $P < 0.05$ was considered statistically significant.

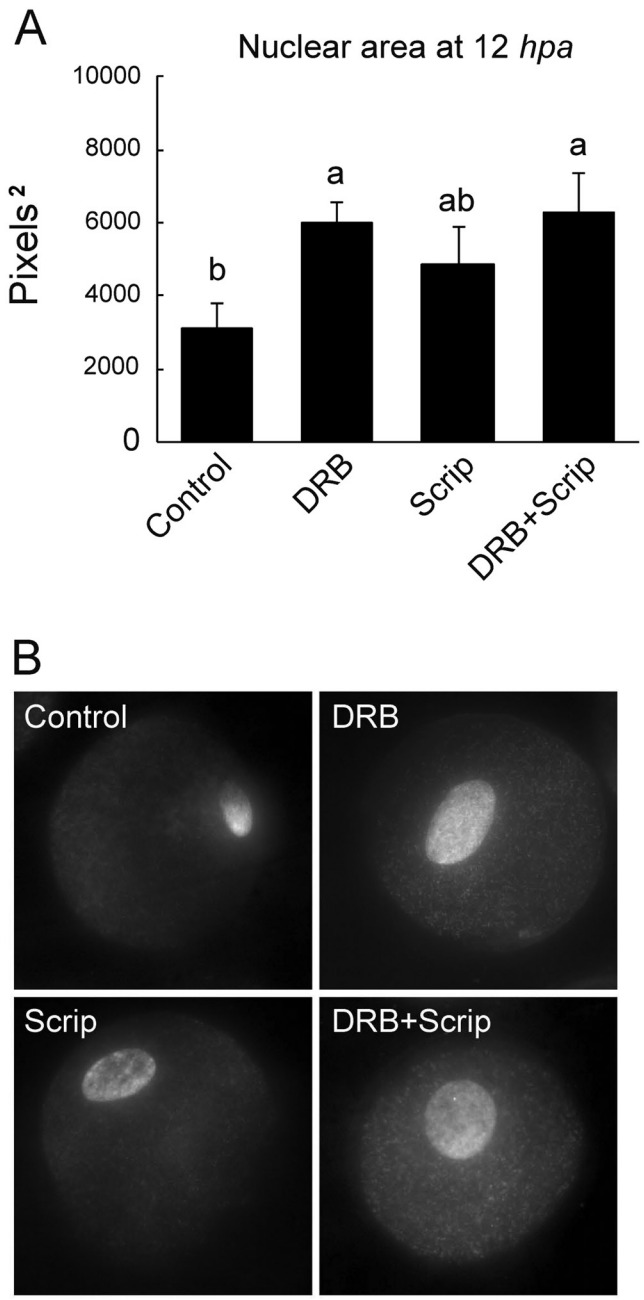

Figure 2 Nuclear area at 12 hpa in 1-cell SCNT embryos from different experimental groups (A) and representative pictures showing nuclear swelling (B). Results are presented as means ± S.E.M., and $P < 0.05$ was considered statistically significant.

Relative mRNA abundance of H3K9 demethylases

The relative mRNA expression of H3K9 KDMs changed from D3 to D5 of embryo development, but the number of differently expressed genes was affected by treatment (Fig. 4). In the Control group, the relative mRNA abundance of *KDM3B*, *KDM4A* and *KDM4B* increased between D3 and D5. In the Scrip-treated embryos, mRNA of *KDM3B*, *KDM4A* and *KDM4B* was more abundant in D5 embryos, but there was a decrease in the *KDM3A* mRNA at D5 compared with D3, which was likely a consequence of the upregulation of this transcript on D3 embryos. As observed for the KDMs of H3K4, the mRNA abundance of most KDMs of H3K9 (*KDM3A*, *KDM3B*, *KDM3C*, *KDM4A* and *KDM4C*) increased from D3 to D5 of embryo development in embryos treated with DBR + Scrip. Differences in mRNA expression were also found between treatments within embryo developmental stages, but only on D3 (Fig. 4). Scrip treatment resulted in a significant increase in the mRNA abundance of *KDM3A* and *KDM4C* compared to control embryos. Treatment with DRB + Scrip decreased mRNA abundance of *KDM3A*, *KDM4A* and *KDM4C* compared to DRB + Scrip and *KDM3A* compared to Control embryos (Fig. 4).

Relative mRNA abundance of H3K27 demethylases

The relative mRNA expression of KDMs of H3K27 revealed an increase in *KDM6A* levels between D4 and D5 of development in embryos treated with Scrip or DRB + Scrip, but not in Control embryos. The relative mRNA levels of *KDM7A* decreased between D3 and D4 in Control and Scrip-treated embryos, but not in the DRB + Scrip-treated embryos (Fig. 5). Regarding the effect of treatment within developmental stages, DRB + Scrip treatment decreased *KDM7A* mRNA in D3 compared with Control and Scrip embryos, and increased *KDM6A* mRNA in D4 embryos compared with Control embryos. The relative levels of *KDM6A* mRNA were increased in D5 embryos treated with Scrip compared with Control and DRB + Scrip-treated embryos (Fig. 5).

Relative mRNA abundance of DNMT1 and XIST

The relative mRNA levels of *DNMT1* increased between D4 and D5 of development in Scrip treated embryos but not in Control and DRB + Scrip-treated embryos. DRB + Scrip treatment decreased *DNMT1* mRNA levels on D3 embryos compared with Control and Scrip treatments, but the expression was increased by Scrip treatment on D4 embryos compared with DRB + Scrip and on D5 embryos compared with both Control and DRB + Scrip treatments (Fig. 6). The relative mRNA expression of *XIST* increased between D3 and D5 of development in Scrip and DRB + Scrip-treated embryos but not in Control embryos. The expression was lower in DRB + Scrip-treated embryos on D4 compared to Scrip alone (Fig. 6) but was not different from Control group in the same day.

Discussion

There is increasing interest in creating animals by SCNT for different reasons including animal production and conservation, but its overall low efficiency limits wider applications (Keefer 2015). Insufficient nuclear

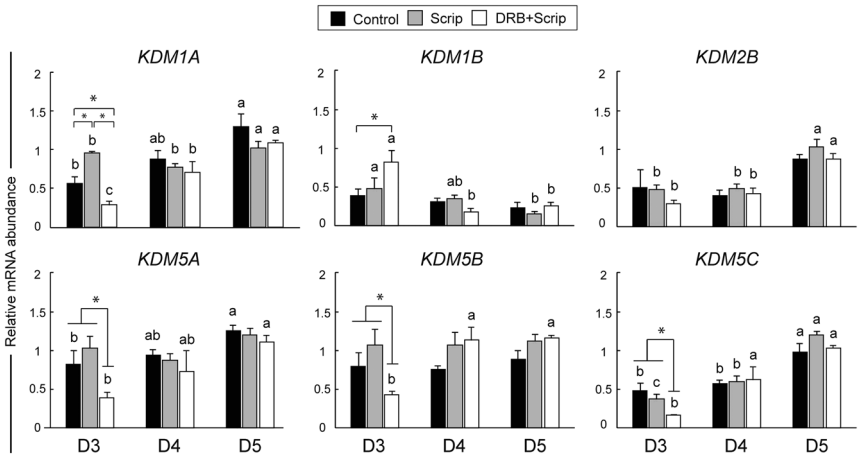

Figure 3 Relative mRNA expression of H3K4 lysine demethylases on days 3, 4 and 5 of development in SCNT embryos from Control (black bars), Scrip (gray bars) and DRB + Scrip (white bars) treatments. Results are presented as means ± S.E.M., and $P < 0.05$ was considered statistically significant. Different letters indicate statistical significance in the same treatment between days, and asterisks indicate statistical significance between treatments in the same day.

reprogramming and epigenetic dysregulation are pointed as the main causes of SCNT cloning inefficiency (Firas *et al.* 2014, Niemann 2016, Sepulveda-Rincon *et al.* 2016). Although the oocyte cytoplasm contains the developmental programming resources to convert sperm and oocyte nuclei into totipotent state cells (Santos & Dean 2004), it seems that the oocyte reprogramming capacity to transform differentiated somatic cells into totipotent cells is mostly insufficient (Sepulveda-Rincon *et al.* 2016). Among the tested approaches to enhance oocyte's capacity to promote somatic cell reprogramming, modulation of histone acetylation by inhibiting deacetylase enzymes was the first treatment shown to effectively improve development of SCNT embryos (Kishigami *et al.* 2006). Indeed, several inhibitors of histone deacetylates have been tested and improved development of SCNT embryos (Ogura *et al.* 2013), but the mechanism by which this action is promoted remains elusive. It is believed that hyperacetylation of histones induced by HDACi treatments have genome wide effects (Wang *et al.* 2007, Yamanaka *et al.* 2009), including demethylation of DNA (Liang *et al.* 2015) and histones (Wang *et al.* 2011, Miyamoto *et al.* 2017), which would modulate gene expression in SCNT embryos (Van Thuan *et al.* 2009, Bui *et al.* 2010, Bui *et al.* 2011, Inoue *et al.* 2015). In addition, HDACi treatment was shown to enhance DNA damage repair in SCNT embryos (Bohrer *et al.* 2014). It is well established that increased histone acetylation reduces chromatin compaction, which is correlated with an active transcriptional state (Bannister & Kouzarides 2011). Based on this, cells exposed to HDACi would be more transcriptionally active than non-treated cells. In the context of SCNT embryos, this would contradict normal embryo physiology since HDACi treatment has been applied to enhance nuclear reprograming during the first 10–15 h after nuclear transfer, a developmental stage when embryos have little to no transcriptional activity. Indeed, in the case of cattle, major activation of the embryo genome occurs after 3–4 days of development during transition from eight- to sixteen-cell stage (Graf *et al.* 2014*a*). In embryos produced by fertilization, minor EGA occurring before this stage is also critical for regulation of normal development. However, in SCNT embryos, if the transferred nucleus continues to transcribe before chromatin reprogramming, the transcripts produced would be those expressed in the somatic cell, which are not necessary or may even have detrimental consequences on early embryo development. Based on these premises, we

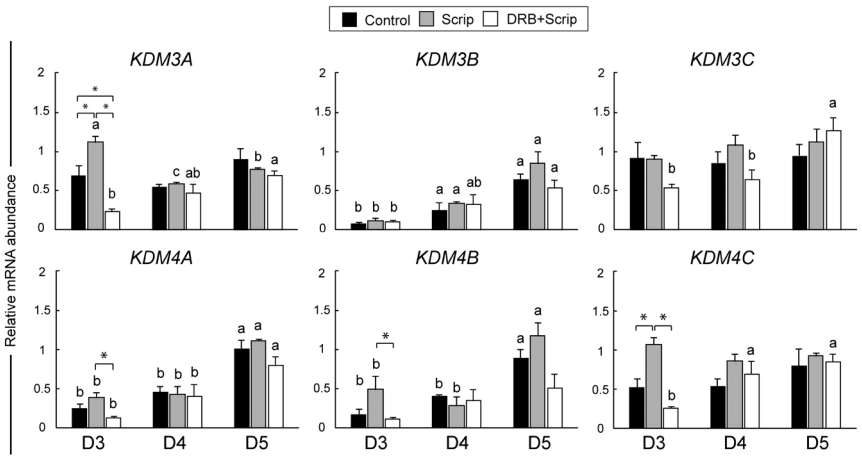

Figure 4 Relative mRNA expression of H3K9 lysine demethylases on days 3, 4 and 5 of development in SCNT embryos from Control (black bars), Scrip (gray bars) and DRB + Scrip (white bars) treatments. Results are presented as means ± S.E.M., and $P < 0.05$ was considered statistically significant. Different letters indicate statistical significance in the same treatment between days, and asterisks indicate statistical significance between treatments in the same day.

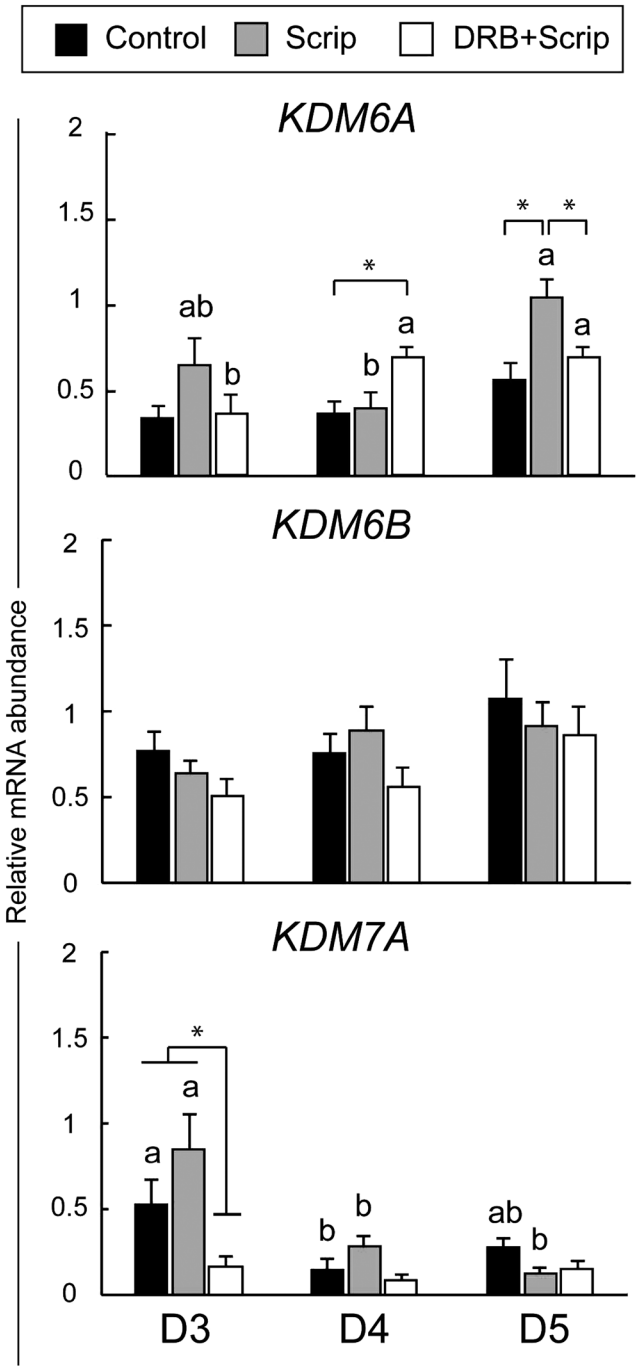

Figure 5 Relative mRNA expression of H3K27 lysine demethylases on days 3, 4 and 5 of development in SCNT embryos from Control (black bars), Scrip (gray bars) and DRB + Scrip (white bars) treatments. Results are presented as means ± S.E.M., and $P < 0.05$ was considered statistically significant. Different letters indicate statistical significance in the same treatment between days, and asterisks indicate statistical significance between treatments in the same day.

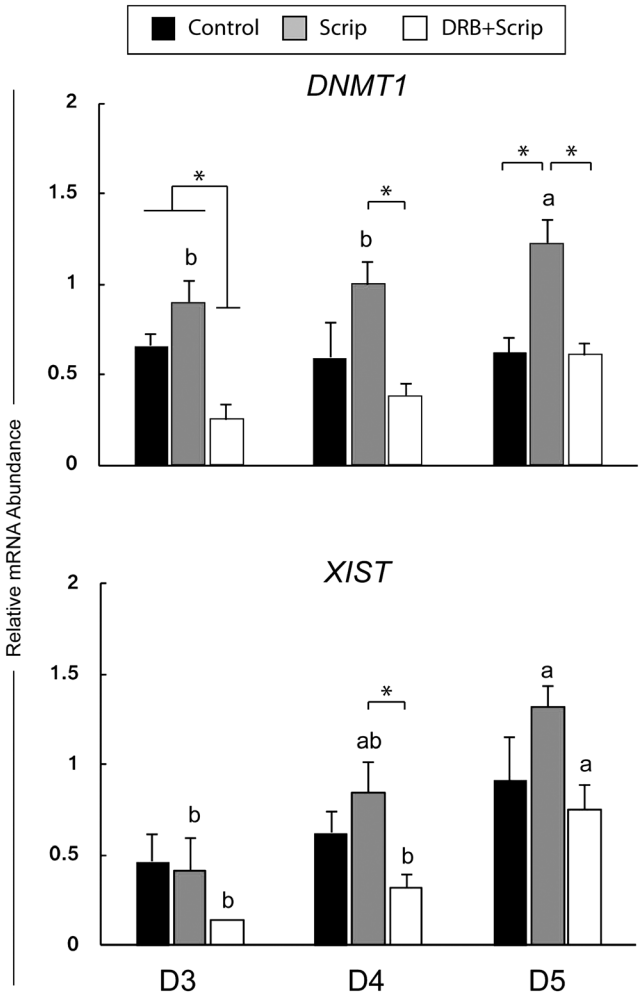

Figure 6 Relative mRNA expression of *DNMT1* and *XIST* on days 3, 4 and 5 of development in SCNT embryos from Control (black bars), Scrip (gray bars) and DRB + Scrip (white bars) treatments. Results are presented as means ± S.E.M., and $P < 0.05$ was considered statistically significant. Different letters indicate statistical significance in the same treatment between days, and asterisks indicate statistical significance between treatments in the same day.

hypothesized that HDACi treatment in association with a transcriptional inhibitor (DRB) would compose a more physiological approach to promote cell reprogramming in SCNT embryos. This treatment would, at the same time, reduce chromatin compaction and facilitate nuclear access to reprogramming factors from the oocyte cytoplasm, but preclude that this state promotes transcription of genes from the transferred and yet non-reprogramed somatic nucleus.

Findings from this study demonstrate that development of bovine SCNT embryos to the blastocyst stage was increased by 32.7 and 37.4% in Scrip and DRB + Scrip treatments compared to non-treated Control embryos, respectively. However, blastocyst cell number was increased approximately 35% in embryos treated with DRB + Scrip compared to Scrip. This indicates that HDACi improves embryo development, but inhibiting RNA synthesis during HDACi treatment improves both development and quality of SCNT embryos (Table 2). Although a recent study revealed that exposure to

Table 2 Results summary.

	DRB	SCRIP	DRB + SCRIP
Embryo development	—	↑	↑
Number of nuclei	—	—	↑
Nuclear swelling	↑	—	↑
Genes with significant changes in mRNA from D3 to D5			
KDMs of H3K4	n.a	1 ↓ 3 ↑	1 ↓ 5 ↑
KDMs of H3K9	n.a	1 ↓ 3 ↑	0 ↓ 5 ↑
KDMs of H3K27	n.a	1 ↓ 1 ↑	0 ↓ 1 ↑
DNMT1/*XIST*	n.a	0 ↓ 2 ↑	0 ↓ 1 ↑
Total	n.a	3 ↓ 9 ↑	1 ↓ 12 ↑
Genes with significant changes in mRNA levels compared to control			
D3	n.a	0 ↓ 3 ↑	7 ↓ 1 ↑
D4	n.a	0 ↓ 0 ↑	0 ↓ 1 ↑
D5	n.a	0 ↓ 2 ↑	0 ↓ 0 ↑
Total		0 ↓ 5 ↑	7 ↓ 2 ↑

DRB between 4 and 20 h after fertilization arrested mouse embryo development at two-cell stage, which confirms the importance of the minor EGA (Abe *et al.* 2018), our findings indicate that inhibiting transcription for 15 h after nuclear transfer did not compromised minor EGA in SCNT bovine embryos. To understand if this treatment enhanced nuclear reprogramming, we first assessed nuclear swelling in SCNT embryos at 12 hpa. Interestingly, exposure to DRB alone or combined with Scrip significantly increased nuclear area, which suggests that inhibiting RNA synthesis enhances chromatin remodeling in one-cell stage SCNT embryos. Furthermore, despite of effectively inhibiting transcriptional activity, the effect of DRB on nuclear swelling was not observed when nuclear donor cells (fibroblasts) were cultured in the presence of DRB for 12 h (Supplementary Fig. 1). This indicates that DRB effects in promoting nuclear swelling in SCNT embryos depends on interactions with reprogramming factors present in the oocyte cytoplasm. This may include exchange of nuclear proteins such as the embryonic form of the linker histone H1 (Bordignon *et al.* 1999). The action of DRB as transcription inhibitor involves two protein kinases, the positive transcription elongation factor (P-TEFb) and the DRB sensitivity inducible factor (DSIF), whereas DSIF negatively regulates transcription elongation and P-TEFb reverts this negative effect (Bensaude 2011). In the presence of DRB, the P-TEFb is inhibited then DSIF continues to repress transcription (Yamaguchi *et al.* 1998). One possibility is that inhibition of P-TEFb may facilitate nuclear protein displacement. Further studies should determine how the inhibition of transcriptional activity promotes nuclear swelling and protein exchanges in SCNT embryos.

This study has next evaluated if DRB + Scrip treatment affected gene transcription in SCNT embryos. For successful nuclear reprogramming in SCNT embryos, gene expression from the transferred somatic cell must be repressed and expression of totipotency-related genes activated at the proper stages of embryo development (Sepulveda-Rincon *et al.* 2016). This process is mainly regulated by epigenetic changes in histones and DNA (Niemann 2016). Indeed, it has been shown that the methylation status of lysine residues in the histone H3, including lysine 4 (Liu *et al.* 2016*a*,*b*, Zhang *et al.* 2016*a*), lysine 9 (Matoba *et al.* 2014, Chung *et al.* 2015, Liu *et al.* 2018*b*) and lysine 27 (Bogliotti & Ross 2012, Xie *et al.* 2016, Liu *et al.* 2016*b*, Chung *et al.* 2017), regulates EGA transition, embryo cell differentiation and development in IVF and SCNT embryos. The methylation status on those lysine residues is regulated by lysine methyltransferases (KMTs) and demethylases (KDMs) enzymes (Hyun *et al.* 2017). Recent studies by our group and others have investigated the expression profile of genes encoding those enzymes in early developing embryos (Shen *et al.* 2017, Glanzner *et al.* 2018). It was observed that mRNA expression of several KDMs was dysregulated and earlier transcribed in bovine and porcine SCNT compared to IVF embryos, and differently expressed in embryos having lower developmental competence (Glanzner *et al.* 2017, 2018). Other studies also reported early transcriptional activity in bovine SCNT compared to IVF embryos, as assessed by 5-EU staining for newly synthesized mRNA (Liu *et al.* 2018*a*). Findings from those studies suggest that gene transcription from the transplanted somatic nuclei is defectively reprogrammed during early development of SCNT embryos. Results reported in this study revealed that co-exposure to DRB and Scrip for 15 h after nuclear transfer improved reprogramming of genes encoding important epigenetic regulators as evidenced by the reduced mRNA levels compared to Controls, especially on D3 of development (Table 2). Indeed, embryos treated with DRB and Scrip recapitulate more accurately gene expression profiles observed in IVF embryos (Glanzner *et al.* 2018). As hypothesized, we observed that treatment with Scrip alone, in addition to improving embryo development, increased early mRNA expression of KDMs (*KDM1A*, *KDM3A*, *KDM4C* and *KDM6A*), *DNMT1* and *XIST* compared to SCNT Control embryos. On the other hand, treatment with both DRB + Scrip prevented early mRNA expression of all those six genes, as well as other KDMs (*KDM4A*, *KDM4B*, *KDM5A*, *KDM5B*, *KDM5C* and *KDM7A*) compared to embryos treated with Scrip alone. Treatment with DRB + Scrip also resulted in lower mRNA expression of KDMs (*KDM1A*, *KDM3A*, *KDM5A*, *KDM5B*, *KDM5C* and *KDM7A*) and *DNMT1* genes compared to SCNT Control embryos. Interestingly, for most of those genes, lower transcript levels in the DRB + Scrip-treated compared with Scrip-treated and control embryos were observed on D3 of development (Table 2). These findings are in agreement with those of our previous study showing that mRNA levels of several KDMs were increased on D3 of development in SCNT compared to IVF embryos (Glanzner *et al.* 2018). This suggests that the temporal expression of genes encoding important epigenetic regulators in SCNT embryos

was better recapitulated when RNA synthesis was inhibited during HDACi treatment compared to HDACi alone and control treatments. It is worth highlighting that previous studies demonstrated high correlations between mRNA expression levels of KDMs with their target histone lysine methylation levels, as assessed by immunofluorescence or Chip-Seq (Matoba *et al.* 2014, Chung *et al.* 2015, Dahl *et al.* 2016, Liu *et al.* 2016*a*). Therefore, the observed variations in mRNA abundance of KDMs in early developing SCNT embryos are very likely reflecting changes in their epigenetic marks targeted by the encoded KDMs.

Although further studies are required to determine the consequences of preventing early gene expression by inhibiting RNA synthesis on cell differentiation and full-term development of SCNT embryos, the fact that some of the KDMs affected by DRB treatment (e.g. *KDM4A*, *KDM4B*, *KDM4C*, *KDM5A*, *KDM5B*, *KDM5C*) have critical consequences for embryo development and cell reprogramming (Matoba *et al.* 2014, Dahl *et al.* 2016, Huang *et al.* 2016, Liu *et al.* 2016*a*) suggests that this may be the reason for the increased total cell number observed in the blastocysts that developed from the DRB + Scrip treatment. This is further supported by the fact that the mRNA expression of *DNMT1*, which is known to be a barrier for cell reprograming (Zhang *et al.* 2016*b*, Song *et al.* 2017), was also decreased in embryos treated with DRB + Scrip compared with Control and Script-treated embryos. In addition, the fact that KDMs have critical roles in the regulation of transcriptional activity, DNA repair and cell differentiation, its proper temporal expression is likely an important indicator of cell reprogramming and function in SCNT embryos.

In conclusion, findings from this study revealed that development of bovine SCNT embryos to the blastocyst stage was improved by treatment with either HDACi alone or combined with inhibition of transcriptional activity. However, better quality SCNT blastocysts having significantly higher number of nuclei were obtained only when embryos were treated with HDACi associated with inhibition of transcriptional activity. This effect was correlated with a better modulation and prevention of early mRNA expression of *KDMs*, *DNMT1* and *XIST*, which are key epigenetic regulators affecting transcription, cell reprogramming and differentiation, and embryo development.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-18-0366.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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