Epigenetic modification does not determine the time of POU5F1 transcription activation in cloned bovine embryos

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Received: 28 May 2011 / Accepted: 5 September 2011 / Published online: 22 October 2011
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Abstract

Purpose To investigate the effect of epigenetic modification on pattern, time and capacity of transcription activation of POU5F1, the key marker of pluripotency, in cloned bovine embryos.

Methods Bovine fibroblasts were stably transfected with POU5F1 promoter-driven enhanced green fluorescent protein (EGFP). This provided a visible marker to investigate the effect of post-activation treatment of cloned bovine embryos

with trichostatin A (TSA) on time and capacity of POU5F1 expression and its subsequent effect on in vitro development of cloned bovine embryos.

Results Irrespective of TSA treatment, POU5F1 expression was not detected until 8–16 cell stage, but was detected in both inner cell mass and trophectoderm at the blastocyst stage. TSA treatment significantly increased POU5F1 expression, and the yield and quality of cloned embryo development compared to control.

Capsule Epigenetic modification and cloning efficiency

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Conclusion The POU5F1 expression of cloned embryos is strictly controlled by the stage of embryo development and may not be altered by TSA-mediated changes occur in DNA-methylation and histone-acetylation of the genome.

Keywords Epigenetic modification · POU5F1 · Bovine · Cloned embryos

Introduction

During somatic cell nuclear transfer (SCNT), chromatin structure of the somatic cell which governs its state of differentiation undergoes dramatic changes, called reprogramming, and is compelled back to the embryonic stage [1]. Somatic cell reprogramming occurs within a limited time-window, from nuclear transfer to the stage of zygote genome activation [2]. However, the specific epigenetic status of somatic cell, which is contributed by DNA-hypermethylation and histone-hypoacetylation/hypermethylation, may make chromatin structure inaccessible or hard to access by the ooplasmic remodeling factors. Therefore, chemically-assisted relaxation of chromatin structure prior and/or after SCNT using the inhibitors of histone-deacetylase (HDAC) and DNA methyl-transferase (Dnmt) has been the subject of recent SCNT studies [3–6].

Trichostatin A (TSA) is synthetic anti-neoplastic drug inhibiting HDAC enzymes [7, 8]. TSA is among most widely used epigenetic drug with almost promising results [7, 8]. Since HDAC inhibitors (HDACi) have a genome-wide effect, chromatin relaxation at locations associated with pluripotency is expected to provide a chromatin conformation which is acquiescent to transcriptional activation [2, 7]. Therefore, it is important to investigate if HDACi have any effect on the time and capacity of pluripotency gene expression in cloned embryos.

POU5F1 is a well-known key regulator of pluripotency and cell differentiation which belongs to POU5F1 transcription factor family and is encoded by a gene belonging to this group [9, 10]. By combining mouse POU5F1 promoter with enhanced green fluorescent protein (EGFP), Kirchhof et al. [11] provided a valuable reporter construct for viable and non-invasive assessment of pluripotency at different stages of embryonic development. In this study, bovine fibroblast cells were stably transfected with POU5F1 promoter-driven EGFP to provide a visible marker to investigate the effect of post-activation treatment of cloned bovine embryos with TSA on time and capacity of POU5F1 expression and on developmental competence of cloned bovine embryos.

Materials and methods

Chemicals were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and media from Gibco (BRL, Grand Island, NY, USA), unless otherwise stated.

Preparation of transgenic somatic cells

Skin biopsy was taken from a Holstein bull and was used for culture of fibroblasts as described previously [12]. To confirm fibroblast phenotype, somatic cells at passage 2 were used for immunostaining against anti-vimentin (for fibroblasts) and anti-pancytokeratin (for epithelial cells), as described previously [13]. Confirmed fibroblasts were plated in 6-well culture dishes (TPP, Switzerland) containing modified Eagle medium F-12 (DMEM/F-12) plus 10% fetal calf serum (FCS) until subconfluence (2×10^5). Then, cells were transfected with 4 μ g DNA (the plasmid carrying EGFP-POU5F1 cDNA-neomycin, www.royaninstitute.org) and 10 μ l Lipofectamine 2000™ according to the manufacturer's guidelines. G418-resistant colonies were isolated, passaged and cryopreserved by standard procedure. A portion of each expanded colony was analyzed for EGFP-POU5F1 transgene using polymerase chain reaction (PCR) and among confirmed transgenic colonies, one colony with reasonable growth rate and cell morphology was selected for subsequent studies (Fig. 1b). For cell cycle synchronization, cells were cultured in medium containing 0.5% FCS for 4–5 days before being used for nuclear transfer [14].

Somatic cell nuclear transfer (SCNT)

Abattoir-derived bovine ovaries were used as the source of oocyte. The process of oocyte in vitro maturation was as described previously [15]. In brief, cumulus-oocyte complexes were aspirated of antral follicles (2–8 mm) and cultured in tissue culture medium 199 (TCM199) containing 10% FCS, FSH (10 μ g/ml), LH (10 μ g/ml),

Fig. 1 **a** in vitro culture and characterization of bovine dermal somatic cells: somatic cell outgrowth from skin biopsy (i), cultured somatic cells at passage 2 (ii), immunostaining showing that somatic cells were positive for antivimentin (iii: green signals indicate intermediate filaments and yellow signals indicate nuclei), and negative for antipancytokeratin (iv: red signals indicate nuclei of the cells), and therefore it was indicated that the cultured cells were fibroblast cells. **b** a pure colony of transgenic fibroblast cells (containing EGFP-POU5F1-neomycin construct) that was developed in presence of G-418 (i) along with PCR test indicating the presence of 714 bp band of the transgene in the developed colonies. **c** Images and **(d)** statistical analysis showing the effect of TSA treatment on the intensity of EGFP-POU5F1 fluorescence at three different stages of cloned embryos (<8, 8–16 -cell, and blastocyst); ($n=10$, number of the embryos were analyzed). Bars with different letters differ significantly at $P<0.05$. Unless specified, scale bars represent 100 μ m

estradiol-17 β (1 μ g/ml), cysteamine (0.1 mM) at 39°C, 5% CO₂ and humidified air for 18–20 h. Matured oocytes were denuded by vortexing for 3–5 min in hepes-buffered TCM199 (HTCM199) supplemented with 300 IU/ml hyaluronidase. For SCNT, the high throughput zona-free SCNT method of Oback et al. [16] was used. In brief, denuded oocytes were treated with pronase (5 mg/ml, 1 min) to remove zona. Oocyte were then incubated with 5 μ g/ml H33342 for five min, transferred on the microscope stage (Olympus; IX71, Japan) equipped with Narishige micromanipulators (Olympus, Japan). At 100 $\times$ magnification, zona-free oocytes were enucleated using a blunt (10–15 μ m inner-diameter) micropipettes under constant UV exposure. For renucleation, oocytes were adhered to individual transgenic fibroblasts in medium containing 10 mg/ml phytohemagglutinin. The couplets were then electrofused [a sinusoidal electric current (7 V/cm) for 10 s followed by two direct currents (1.75 kV/cm for 30 μ sec and 1 s delay)] in 290 mOsm fusion buffer free of Ca²⁺ and Mg²⁺. The reconstructed oocytes were activated using ionomycin (5 μ M, 5 min) followed by incubation with 2 mM 6-dimethyl aminopurine for 4 h [17]. Activated reconstructs were cultured in presence (SCNT-TSA) or absence (SCNT) of 50 nM TSA for 12 h and then cultured up to 8 days as described by Vajta et al. [18]. Accordingly, embryos in groups of five to seven were cultured in wells drained in 20 μ l of modified synthetic oviductal fluid (mSOF) under mineral oil at 39.0°C, 5% CO₂, 5% O₂ and humidified air. As an external control, in vitro fertilization (IVF), was carried out as described previously [19].

EGFP- POU5F1 fluorescence

Cloned embryos were checked for EGFP fluorescence as described by Wuensch et al. [10]. In brief, embryos were washed in phosphate buffer saline (PBS) and fixed in 4% (w/v) paraformaldehyde (PF) for 30 min at room temperature (RT). Fixed embryos were washed with PBS plus 1 mg/ml poly vinyl alcohol (PVA), mounted on the microscopic slide, and observed using a fluorescent microscope (Olympus, BX51, Japan). Under 450–490 nm excitation, the resulted fluorescence emission of each embryo was detected using a 515–569 nm filter at 400 $\times$ magnification. Immediately after exposure, a digital image of each blastocyst was taken with a high sensitive camera (DP-72, Olympus, Japan) operated on DP2-BSW software. At the blastocyst stage, the median pixel intensity of 10–15 blastomeres in each blastocyst was detected using Image J. software (National Institute of Mental Health, Bethesda, Maryland, USA). To avoid autofluorescence, the software was firstly zeroed against IVF hatched embryos.

Immunofluorescence staining

Cloned and IVF blastocysts were used for detection of 5-methyl cytosine (5-MC) and acetylated histone H3 on lysine 9 (H3K9-Ac) as described by Jafari et al. [19] and Beaujean et al. [20]. In brief, blastocysts were fixed with 4% PF for 30 min and permeabilized with 0.5% Triton X-100 for 15 min. For DNA-methylation, fixed embryos were further treated with 4 N HCl for 60 min at RT. After washing with PBS, embryos were treated with 3% bovine serum albumin (BSA/PBS) for 60 min at RT to preclude unspecific binding of primary antibody. Then, embryos were incubated with primary antibodies [either mouse monoclonal anti-5-methyl cytosine for DNA-methylation (Eurogentec, BI-MECY-0100) or mouse monoclonal anti-H3K9 for histone-acetylation at lysine 9] for 1 h at 37°C. Embryos were then washed thrice with PBS/PVA and incubated with goat anti-mouse IgG-TRITC for 60 min at 37°C in dark. Stained embryos were mounted and imaged as described for EGFP fluorescence except for excitation (557 nm) and emission (576 nm). The median pixel intensity of 10–15 nuclei in each blastocyst was detected using Image J. software (National Institute of Mental Health, Bethesda, Maryland, USA) (Fig. 2a). Appropriate controls were included to check the autofluorescence of the first and second antibodies.

Differential staining of the blastocysts

Total cell number (TCN), the proportion of cells allocated in inner cell mass (ICM) and trophectoderm (TE) of cloned and IVF blastocysts were determined as described by Moulavi et al. [21].

Quantitative analysis of transcripts by real time RT-PCR

Total RNA of embryos was extracted using RNeasy Micro Kit (Qiagen®, Germany) and used for first strand cDNA synthesis with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas-Germany). cDNA synthesis reaction performed at 42°C for 1 h and contained 1 μ l random hexamer primer, 1 μ l RNase inhibitor, 4 μ l 5 $\times$ reaction buffer, 2 μ l dNTP and 1 μ l M-MuLV reverse transcriptase that adjusted to 20 μ l using DEPC-treated water. Real time RT-PCR was performed using a Rotor Gene 6000 (Corbet®). Each reaction mixture contained 2 μ l cDNA, 10 μ l SYBR Premix Ex Taq II (TaKaRa, Japan) and 1 μ l of forward and reverse primers (5 μ M) that adjusted to 20 μ l using dH₂O. The pattern of the transcripts for POU5F1, VEGF and BCL2 were analyzed using quantitative real time RT-PCR. Primer sequences, annealing temperatures and the size of amplified products are shown in (Table 1).

Fig. 2 **a** Images and **b** statistical analysis showing the effect of TSA treatment on fluorescence intensities of DNA-methylation and H3K9-acetylation in cloned blastocysts developed in presence (SCNT-TSA) or absence (SCNT) of TSA in comparison with IVF ($n=10$, 10 and 8 number of embryos which were analyzed in SCNT-TSA, SCNT and IVF groups, respectively). Bars with different letters differ significantly at $P<0.05$. Scale bar represents 100 μm

Statistical analysis

Percentages data of cloned embryo development were modeled to the binomial model of parameters by ArcSin

transformation. The transformed data, along with crude data of DNA methylation/histone H3K9 acetylation, EGFP fluorescent intensity, and RT-PCR expression of POU5F1, VEGF and BCL2 genes were analyzed by one-way

Table 1 Sequence specific primers used in this study

Gene	Primer sequence (5'-3')	Length (bp)	Annealing temperature (°C)	GenBank accession number
Beta-actin	F: TCGCCCGAGTCCACACAG R: ACCTCAACCCGCTCCCAAG	200	62	BT030480
POU5F1	F: AGAAGGGCAAACGATCAAGC R: AGGGAATGGGACCGAAGAGT	173	66	NM_174580
VEGF	F: TTCATTTTCAAGCCGTCCTG R: CCTATGTGCTGGCTTTGGTG	143	60	NM_174216
BCL2	F: AGCATCACGGAGGAGGTAGAC R: CTGGATGAGGGGGTGTCTTC	161	64	AF515848
EGFP	F: ATGGTGGCAAGGGCGAGGAG R: ATTACTTGTACAGCTCGTCCATG	714	63	U55763

ANOVA model of SPSS-17. Differences were compared by LSD post-hoc test. Differences were considered as significant at $P < 0.05$.

Results

EGFP-POU5F1 fluorescence

Irrespective of TSA treatment, EGFP fluorescence was not detected in cloned embryos at 2, 4, and 8 -cell stages of in vitro development. At 8–16 cell embryos, low EGFP fluorescence intensities were detected in both SCNT (3.02 ± 0.5) and SCNT-TSA (3.8 ± 0.48) embryos with no significant difference (Fig. 1c and d). At the blastocyst stage, high EGFP expression was detected in ICM and TE (Fig. 1c) of both SCNT and SCNT-TSA blastocysts. However, the mean EGFP intensity of cloned SCNT-TSA blastocysts (39.3 ± 3.4) was significantly greater than SCNT blastocysts (19.4 ± 2.1) (Fig. 1d).

DNA-methylation and histone-acetylation

As shown in Fig. 2b, the mean levels of DNA-methylation in IVF, SCNT-TSA and SCNT blastocysts were 17.9 ± 3.0 , 39.7 ± 3.5 , and 57.2 ± 3.4 , respectively, which were significantly different between the three groups. Further, the mean intensities of H3K9-acetylation were 78.3 ± 3.7 , 58.6 ± 2.4 , and 49.3 ± 3.3 in IVF, SCNT-TSA and SCNT groups, respectively. Similar to DNA-methylation, histone-acetylation values also represented significant differences between the three groups.

Embryo development

As shown in Fig. 3a, there was no significant difference between cleavage rates of the three groups. However, 12 h treatment with TSA significantly increased cloned blastocyst development ($45.5 \pm 4.9\%$) in comparison with control SCNT and IVF groups (32.6 ± 3.2 and $31.8 \pm 2.7\%$, respectively).

Differential staining

As shown in Fig. 3b, differential staining did not reveal any significant difference between total cell numbers (TCN) of the blastocysts developed in the three groups. However, embryos in SCNT-TSA group showed significantly higher proportion of ICM/TCN (35.1 ± 2.7) in comparison with the respective rates of SCNT and IVF groups (27.16 ± 1.1 , and 27.3 ± 1.3 , respectively). In contrast, the proportion of TE/TCN was significantly higher in SCNT and IVF compared to SCNT-TSA group (72.8 ± 1.2 , 72.7 ± 1.3 , and 64.9 ± 2.7 , respectively).

Quantitative analysis of transcripts by real time RT-PCR

As shown in Fig. 3c, the relative expression of POU5F1 gene transcripts in SCNT-TSA blastocysts (6.5 ± 0.7) was significantly greater than SCNT (2.2 ± 0.7) and IVF (1.0) groups. The relative expression of VEGF in SCNT-TSA group (3.4 ± 0.9) was insignificantly higher than IVF (1.0) but significantly greater than SCNT (0.8 ± 0.1). The relative expressions of BCL2 in IVF, SCNT, and TSA-SCNT groups were 1.0, 3.4 ± 1.8 , and 2.9 ± 0.6 , respectively, which indicated non-significant difference between them.

Discussion

TSA and EGFP-POU5F1 activation

The results of this study indicated that until reaching 8–16 cell stage, the cloned bovine embryos may be transcriptionally inactive for POU5F1 gene. Importantly, post-activation treatment with TSA could not change the biologic clock of the cloned embryos. This indicates that transcription activation of POU5F1 gene is precisely regulated by the stage of embryo development and may not be changed by TSA-mediated alterations of DNA-methylation and histone-acetylation in the genome.

In this study, the fourth cell cycle of cloned embryo development was coincided with the early expression of POU5F1 which was evident with very low EGFP fluorescence in cloned embryos with no significant effect of TSA on fluorescence intensity (Fig. 1c and d). In agreement with our results, Wuensch et al. [10] observed that cloned bovine embryos were not active for POU5F1 before 8–16 cell stage. Cloned blastocyst development was coincided with a sharp increase in fluorescence intensity and interestingly, the EGFP fluorescence was observed in both ICM and TE (Fig. 1c). Importantly, TSA treatment significantly increased EGFP expression under the control of POU5F1 reporter construct compared to control SCNT. Therefore, it was indicated that initial treatment of cloned embryos with HDACi may have long acting effects on gene expression which may not be determined until advanced stages of embryonic development. Kirchhof et al. [11] also demonstrated stark differences in the pattern of POU5F1 expression between the blastocysts of different species. They found that while POU5F1 expression was restricted to ICM of murine blastocysts, its expression in porcine and bovine IVF embryos was detectable in both ICM and TE. Therefore, the present study indicated that cloned bovine embryos may follow the same pattern of EGFP-POU5F1 gene expression to fertilized bovine embryos.

Fig. 3 a In vitro development of bovine cloned embryos developed in presence (SCNT-TSA) or absence (SCNT) of TSA in comparison with IVF. **b** Comparison between TCN, ICM:TE and TE:TCN of the blastocysts developed in presence (SCNT-TSA) or absence (SCNT) of TSA in comparison with IVF ($n=6$, 8 and 10 number of embryos which were analyzed in SCNT-TSA, SCNT and IVF groups, respectively). (C) Real-time quantification of mRNA abundance in cloned embryos developed in presence (SCNT-TSA) or absence (SCNT) of TSA in comparison with IVF ($n=21$, number of embryos which were analyzed in each group in three replicates). Bars with at least one common letter do not differ significantly at $P<0.05$

TSA and epigenetic marks

The results of Fig. 2a and b, in agreement with the other infield studies [2, 22–24] indicated that cloned blastocysts were generally hypermethylated (DNA) and hypoacetylated (histones) in comparison to IVF blastocysts. Further, it was indicated that treatment with TSA modified the epigenetic status of the reconstructed embryos, though the acetylation levels of embryos were yet significantly lower than fertilized embryos. Importantly,

direct TSA-mediated increase in acetylation levels of embryo was concomitant with indirect decrease in methylation. Accordingly, Cedar et al. [25] demonstrated that DNA-methylation and histone-acetylation are inter-associated pathways, and showed that this crosstalk has important implications in normal development, somatic cell reprogramming and tumorigenesis.

Although majority of the studies have been focused on epigenetic characteristics of cloned embryos at the blastocyst stage, assessment of epigenetic marks during

earlier stages of in vitro embryo development also has been the matter of a number of studies. For example, Li et al. [26] and Wang et al. [27] in mice and Martinez-Diaz et al. [28] in porcine observed that after TSA treatment of the reconstituted embryos, there was a significant increase in histone-acetylation of early embryos compared to control cloned embryos. Therefore, one may conclude that although TSA-mediated alterations of epigenetic marks at early stages of cloned embryo development is possible; these alterations may not change the biologic clock for transcription activation of some important genes such as POU5F1.

TSA and embryo development

The results of this study showed that the treatment of SCNT embryos for 12 h after activation with 50 nM TSA significantly increased the capability of embryos to develop to the blastocyst stage. These results are in agreement with the reported beneficial effects of TSA in pig [29], and porcine [2, 9, 30], but are contradictory to Lager et al. [3] who found no effect of TSA on cattle SCNT efficiency. Importantly, TSA treatment significantly increased blastocyst production over IVF. Although this great increase in blastocyst yield is promising, the increase in blastocyst production is not necessarily indicative of greater developmental competence in bovine clones. Indeed, it has been stated that “cloned blastocyst rates can reach nearly 80% and sometimes surpass IVF rates, while the proportion of developmentally competent embryos is greater following IVF” [31]. Importantly, by treatment of both nuclei donor cells and the resulted cloned embryos with a combination of TSA and 5-aza-2'-Deoxycytidine (an inhibitor of DNA methyl transferase), Wang et al. [31] observed significant improvement in both in vitro and in vivo embryo development of cloned embryos. Their results further confirm the need for assisted epigenetic modification to improve reprogramming ability of the recipient oocyte, which may have important consequences on subsequent development of the cloned embryos.

TSA treatment of cloned embryos significantly increased the proportion of ICM/TCN compared to control SCNT and IVF. It may suggest that HDAC inhibition may change cell allocation in the resultant blastocysts. Accordingly, Hansis et al. [32] demonstrated that differential expression of POU5F1 in individual human blastomeres directs cells toward either ICM or trophoctoderm lineage. However, while the increase in ICM number has been related to higher quality of embryos in different studies [21], the relevant of ICM increase in cloned embryos with final quality of the embryos needs further investigation.

TSA and gene expression

The relative expression of POU5F1 in SCNT-TSA embryos was significantly higher than control SCNT and IVF-derived embryos which is in agreement with the fluorescence analysis of EGFP-POU5F1 (Fig. 1c and d). In contrast, Lager et al. [3] and Cervera et al. [9] observed similar pattern of POU5F1 expression between IVF, SCNT and cloned embryos treated with TSA for 13 and 26 h, respectively. Importantly, after treatment of cloned embryos with 50 nM TSA, Lager et al. [3] observed that the expression of Nanog, a hallmark of pluripotency, was significantly higher in TSA-SCNT blastocyst compared to IVF. In another study, using treatment of cattle cloned embryos with S-adenosylhomocystein (SAH), a non-toxic inhibitor of DNA methyl transferase, Jarafri et al. [19] observed that the expression of POU5F1 in SAH-treated cloned embryos were significantly higher than IVF. Therefore, higher expression of pluripotency gene in cloned blastocysts with higher methylation status than IVF is a dogma that was also observed by other studies. It seems that the higher expression of POU5F1 in TSA-treated cloned embryos is more a reflection of aberrative gene expression rather than higher quality of these embryos. These data emphasizes the lack of reliable in vitro marker of developmental competence, and the importance of live offspring production as the only true gauge for final cloning efficiency.

Concluding remarks

Using EGFP-POU5F1 transgenic fibroblasts, this study showed that pluripotency gene expression of cloned embryos is strictly controlled by the stage of embryo development and may not be altered by changes in the levels of DNA-methylation and histone-acetylation. Further, it was indicated that POU5F1 expression in cloned bovine embryos is not restricted to ICM and can be observed in both ICM and TE of the cloned blastocysts. It was also found that the increase in the degree of POU5F1 expression in cloned embryos, which here induced by TSA treatment, had a regulatory role on cell fate determination, as TSA-treated cloned embryos had significantly greater ICM/TCN proportion compared to un-treated cloned embryos. To make a final conclusion about the impact of TSA on cloning efficiency, further studies on full-term development of the resultant cloned embryos should be performed.

Acknowledgements This study was funded by a grant from the Royan Institute of IRI. All the study was carried out in Royan Institute, IRI. The authors would like to thank Mrs. Hosseini for ovum pick up assistance. Authors also sincerely thank Mrs Mansouri for statistical analysis and Mr. Heidari, and Khajeh for preparation the ovaries.

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Authors contribution

Conceived and designed the experiments: SMH, FJ, MHN. Performed the experiments: SMH, MH, MF, FJ, PA, SO, HA, SJ. Analyzed the data: SMH, MHN. Contributed reagents/materials/analysis tools: MHN, HG, AHS, ADV. Wrote the paper: SMH, MHN.