PRMT5 enhances generation of induced pluripotent stem cells from dairy goat embryonic fibroblasts *via* down-regulation of p53

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Abstract

Objectives: Protein arginine methyltransferase 5 (PRMT5), is thought to play a role in epigenetic reprogramming of mouse germ cells. However, up to now there has been little information concerning its expression profile and effects on generation of induced pluripotent stem cells (iPSCs) from somatic cells, in livestock. Here, we have explored PRMT5 expression profiles in dairy goats and its consequences to derivation of iPSCs from dairy goat embryonic fibroblasts (GEFs).

Materials and methods: We investigated effects of PRMT5 on iPS-like cells production *in vitro*. alkaline phosphatase (AP) staining, QRT-PCR and western blotting analysis of expression of related markers were used to evaluate efficiency of generation of iPSCs derived from GEFs.

Results: These showed *PRMT5* to be a conservative gene widely expressed in various tissues and different-aged testes. *PRMT5* overexpression in combination with OCT3/4, SOX2, KLF4 and C-MYC (OSKM) significantly increased number of AP positive iPS-like colony-derived GEFs compared to OSKM alone, in our dairy goats. Moreover, our results demonstrated that *PRMT5* overexpression stimulated GEF proliferation and down-regulated *p53*, *p21* (a target gene of p53) and

the apoptotic marker caspase 3, to enhance somatic cell reprogramming.

Conclusion: This study provides an efficient model for future studies on mechanisms underlying goat somatic cell reprogramming and differentiation.

Introduction

Embryonic stem (ES) cells, derived from the inner cell mass of mammalian blastocysts, have the ability to proliferate infinitely while maintaining pluripotency, and the ability to differentiate into cells of all three germ layers (1). Goat ES cells have a very important use to produce transgenic animals of agricultural significance and can aid as research models for cell proliferation and cell differentiation *in vitro*; however, there is a lack of stable goat ES cell lines. One way to circumvent this issue is to generate pluripotent cells directly from somatic ones. Mouse induced pluripotent stem cells (iPSCs) have been generated from somatic cells upon forced expression of transcription factors OCT4, KLF4, SOX2 and C-MYC (OSKM), and it has been definitively shown that iPSCs share similar characteristics and morphology with ES cells (2,3). Apart from potential clinical applications, iPS cell generation also provides an excellent model system for investigating mechanisms underlying cell reprogramming *in vitro* via identification of alternative or additional factors involved in the processes (4). Different factor combinations, including OCT4, SOX2, KLF4, C-MYC, LIN28, NANOG and NR5A2, have been used to reprogram cells of many species, including humans, rat, pig, sheep, horse, rabbit, monkey, goat and cattle (3,5–16). However, low efficiency of iPSC generation is a handicap for mechanistic studies and high-throughput screening, and *bona fide* colony isolation is time consuming and costly (17). Efficiency of alkaline phosphatase positive (AP⁺) colony formation with the four

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Yamanaka factors (OSKM) in mouse fibroblasts is approximately only 1% of the starting population, but even only 1 in 10 of these colonies is sufficiently reprogrammed to be chimaera competent (17,18).

Due to this limitation, most studies have focused on the immediate response of somatic cells to factor expression. Protein arginine methyltransferases (PRMT5) is thought to play a role in epigenetic reprogramming in germ cells (19,20) and PRMTs play important roles in numbers of cellular processes, including signalling, gene regulation and transport of proteins and nucleic acids, to affect growth, differentiation, proliferation and development (21). PRMT5 is a particularly interesting target because it is highly expressed in blood, breast, colon and stomach cancers and promotes cell survival in the presence of DNA-damaging agents (22). Recently, it has been shown that PRMT5 combined with KLF4 and OCT3/4 reprogrammed mouse somatic cells to a state indistinguishable from ES cells, in terms of their gene expression profile, DNA methylation status, capacity to differentiate into the three germ layers and germline transmission (4). However, there has been lack of information concerning PRMT5 expression profile and its effects on somatic cell reprogramming in livestock (23,24). Dairy goats have a relatively short period of gestation and provide milk, meat, fur and other valuable products; also they can be used as animal models for biomedical research in production of peptides, using transgenic animals (25,26).

In this study, first we explored the expression profile of PRMT5 in the dairy goat and found that overexpression of PRMT5, in combination with OSKM, significantly increased numbers of alkaline phosphatase positive (AP+) cells and iPS-like colony formation, derived from dairy goat fibroblasts *via* down-regulation of p53.

Materials and methods

Animals and cell lines

ICR mice were used and maintained in a controlled environment at 20–25 °C, 12/12 h light/dark cycle and 50–70% humidity. All experiments requiring use of ani-

mals were approved by the Shaanxi Centre of Stem Cell Engineering & Technology, Northwest A&F University. HEK293T cells were maintained in our laboratory. MEF feeder cells were isolated and prepared according to previous reports (27,28).

Quantitative real-time – polymerase chain reaction

Quantitative real-time – polymerase chain reaction (QRT-PCR) was used for detection of expression levels of genes in this experiment. Total RNA from dairy tissues and cells was extracted using Trizol reagent (TaKaRa, Biotech. Co., Ltd, Dalian, China). Single-strand cDNAs were prepared from 1 µg RNA using a reverse transcription kit (TaKaRa, Biotech. Co. Ltd), and *PRMT5* gene expression was analysed. QRT-PCR primers used are listed in Table 1.

Quantitative real-time – polymerase chain reactions were set up in 25 µl reaction mixtures containing 12.5 µl SYBR® Premix (BORI, Hangzhou, China), 0.5 µl sense primer, 0.5 µl antisense primer, 11 µl distilled water and 0.5 µl template. QRT-PCR conditions were initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 10 s, annealing temperature of 58 °C for 30 s and 72 °C for 30 s, with final extension at 72 °C for 10 min.

Generation of pCDH-PRMT5-EGFP vector

PRMT5 gene CDS region was cloned by PCR from 3-month-old Guanzhong dairy goat testicular tissue, and amplified using primers incorporating restriction sites for XbaI (forward primer: 5'-TCTCGAGCGCCGC-TACCGCCAGCCACCA-3') and BamHI (reverse primer: 5'-AGGATCCCTGCACCTTCTGTGCTACAGG-3'). Resulting products of the polymerase chain reaction were purified using TIANGEN gel purification kit (TIANGEN Biotech (Beijing) Co., Ltd.) according to the manufacturer's instructions, cloned into pMD-18T vector (TaKaRa, Biotech. Co. Ltd) and confirmed by PCR using the same primer sets as described above. Next, purified *PRMT5* fragment was constructed into the pCDH-EGFP vector. Constructed vector pCDH-PRMT5-

Table 1. The primer sequences for QRT-PCR

Gene	Forward	Reverse
β-actin	5'-ACGGCATCACCAACT-3'	5'-AGGAAGGAAGGCTGGAAGAG-3'
Prmt5	5'-GCTTCTGGGCTCATTTGCTG-3'	5'-TGCAGCCGTACCACATAAGG-3'
P53	5'-GGGAATCTTCTGGGACGG-3'	5'-CTTCTTGGTCTTCGGGTAGC -3'
P21	5'-AGGGCAVGTCTCAGGAGGA-3'	5'-CAGTCTGCGTTTGGAGTGCTAG-3'

EGFP plasmid was identified by digestion with XbaI/BamHI restriction enzymes and sequencing.

Cell culture and viral transduction

Dairy goat embryonic fibroblast cells (GEFs) were isolated from a 60-day embryonic foetus. Muscles and skin were digested with 1 mg/ml collagenase I at 37 °C for 15–30 min. After centrifugation at 350 g for 5 min, specimens were incubated in dissociation solution consisting of a mixture of 0.2% (v/w) trypsin (Invitrogen, Carlsbad, CA, USA) and 1.4 mg/ml DNase (Invitrogen), for 10 min at 37 °C. Samples were again centrifuged at 350 g for 5 min, and the cell suspension in culture medium was transferred to a 100 mm diameter culture plate. GEF cells used in this experiment were cultured in DMEM/F12 (Invitrogen), supplemented with 10% foetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mM L-glutamine (Invitrogen), 1% non-essential amino acids (NEAA; Invitrogen), 0.1 mM β -mercaptoethanol (Sigma, St. Louis, MO, USA) and 100 mg/ml penicillin/streptomycin.

To produce pseudotyped lentivirus particles, a monolayer of HEK293T cells in a 100 mm diameter dish was cotransfected with three plasmids, pXPAX2 (7 μ g), pMD2.G (7 μ g) and pCDH-PRMT5-EGFP, or the four human reprogramming factors, OCT4, SOX2, KLF4 and C-MYC, along with individual additional candidate factors, which were amplified by RT-PCR and cloned into pCDH-EGFP using the calcium-phosphate method (Profection Mammalian Transfection System; Promega, Madison, WI, USA). After overnight incubation, cells were washed and incubated in fresh H-DMEM (Invitrogen). Supernatant containing lentivirus of the four factors – OSKM in combination with pCDH-PRMT5-EGFP, or OSKM, alone were harvested after 48 h and 72 h post-transfection. Using the supernatant containing lentivirus, transduction of *PRMT5* was performed in GEF cells, and medium was changed as normal medium after 6–8 h (26).

Generation of dairy goat iPSCs using lentiviral vector

To generate dairy goat iPSCs, GEF cells were cultured in DMEM/F12 (Invitrogen). After 2–3 days culture, cells were remained in excellent condition. Lentiviral transduction was performed for 5–6 h, and fresh DMEM/F12 was added. Two to three days after transduction, cells were gradually replaced with iPSC medium. The dairy goat iPSCs in this study were cultured in knockout DMEM (Invitrogen), supplemented with 17% KSR (Invitrogen), 3% FBS (HyClone), 0.1 mM non-essential amino acids (Invitrogen), 1 mM L-glutamine (Invitrogen), 1/100 N2 (Invitrogen), 1/50 B27 (Invitrogen), 50 μ g/ml ascorbic

acid (Sigma), 2.5 μ M PD99023 (Sigma), 2.5 μ M CHIR-99021 (Stemgent, Bergisch Gladbach, Germany), 10 ng/ml recombinant human leukaemia inhibitor factor (LIF; Invitrogen), 10 ng/ml bFGF (Millipore, Billerica, MA, USA), 0.1 mM β -mercaptoethanol (Sigma), and 50 units/50 mg/ml penicillin/streptomycin, at 37 °C and 5% CO₂ in a humidified atmosphere. Culture medium was initially changed every 2 days, subsequently being changed every day. After formation of cell colonies, colonies were picked and seeded with inactivated mouse embryonic fibroblast (MEF) feeder cells (22,29).

Alkaline phosphatase staining

To determine numbers of iPSC-like cell colonies, specimens on culture plates were fixed in 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature, and washed three times in PBS. Fixed cells were washed three times in PBS and stained with naphthol AS-MX phosphate (200 μ g/ml; Sigma) and fast red TR salt (1 mg/ml; Sigma) in 100 mM Tris buffer, pH 8.2–8.4, for 10–30 min at room temperature, and washed in PBS to terminate staining (28,30).

Immunofluorescence analysis

Cell samples were fixed in 4% PFA in PBS for 15 min and washed three times in PBS, treated with 0.2% Triton X-100 in PBS for 10 min at room temperature, and then washed three times in PBS. After blocking with 1% BSA in PBS for 30 min, cells were incubated with primary antibodies against PRMT5 (1:100; Millipore), OCT4 (1:500; Chemicon, Temecula, CA, USA), SSEA1 (1:200; Chemicon), SSEA4 (1:200; Chemicon), C-MYC (1:200; Chemicon), NSE (1:200; Chemicon), VASA (1:200; Sino Biological Inc., Beijing, China) and cardiac α -ACTIN (1:500; Sigma) overnight at 4 °C. Cells were then washed three times in PBS, and appropriate secondary antibodies were applied for incubation for 1 h at room temperature, in the dark. After washing in PBS, cells were finally exposed to Hoechst33342 in PBS to counterstain nuclei, for 2 min.

Western blotting

Total cell extracts were prepared from the PRMT5-overexpressing group compared to the control group, and proteins were extracted in 1X SDS–PAGE sample loading buffer. Total cell proteins were resolved by SDS–PAGE, transferred to 0.22 mm PVDF membrane for around 55 min in 80 V, and probed with β -actin (1:1000; Bioss, Beijing, China), p53 (1:1000; Bioss, Beijing, China), CCDN1 (1:1000; Sino Biological Inc., Beijing, China) and caspase 3 (1:1000; Bioworld, Shanghai, China). Horseradish perox-

idase-conjugated anti-rabbit antibody was used as a secondary antibody (1:1000; Bioworld). Detection was performed using Thermo Scientific Pierce ECL Western blot substrate (Thermo Scientific) (31,32).

Statistical analysis

Data are presented as mean and SEM. Differences in expression of specific markers were evaluated using Student's *t*-test (Excel, Microsoft Corporation). Results of different treatments were considered significantly different when $P < 0.05$ was obtained (32).

Results

PRMT5 was expressed in different tissues of dairy goat and co-localized with OCT4/p53

QRT-PCR analysis confirmed *PRMT5* expression in different tissues; expression levels in heart, liver, kidney and testis were relative higher than in spleen, ovary and

lung (Fig. 1a). Levels of *PRMT5* varied between different aged testes (Fig. 1b). *PRMT5* protein was also detected in the testis and ovary of dairy goats at 90 dpp (Fig. 1c). In addition, *PRMT5* protein co-localized with pluripotentiality marker, OCT4, as analysed using immunofluorescence (Fig. 1d).

Construction of pCDH-CMV-PRMT5-EF1-GreenPuro lentiviral vector

The *PRMT5* CDS region (1920 bp) was cloned from Guanzhong dairy goat testicular tissues (Fig. 2a-I) then recombined into the pMD18-T vector for sequencing. Correctly sequenced PCR product was excised from pMD18-T (Fig. 2a-II) then recombined with pCDH-CMV-EF1-GreenPuro vector and identified by double digestion, using Xba1/BamHI restriction enzymes (Fig. 2a-III). A schematic of the successful construction vector is shown in Fig. 2b. Furthermore, structure of the dairy goat *PRMT5* protein was predicted using Swiss Institute of Bioinformatics online software analysis.

Figure 1. Expression and subcellular location of dairy goat *PRMT5*. (a) Quantitative PCR detection of *PRMT5* expression in different tissues; (b) quantitative PCR detection of *PRMT5* expression among different aged testes; (c) immunohistochemical detection of localisation of *PRMT5* in testis and ovary; (d) immunofluorescence analysis of the localisation of OCT4 and *PRMT5* in the ovary (scale bar = 100 μ m).

Figure 2. Construction and analysis of pCDH-CMV-Prmt5-EF1-GreenPuro vector. (a) (I) *PRMT5* sequence was amplified using PCR, (II) restriction fragment of *PRMT5* from positive T vector, (III), digestion confirms the pCDH-CMV-Prmt5-EF1-GreenPuro vector; (b) schematic of the recombinant vector; (c) comparison of the *PRMT5* protein structure between dairy goat and *Bos*; (d) evolutionary analysis of *PRMT5* gene.

These results showed that goat and *Bos* *PRMT5* protein had the same structure (Fig. 2c). We found that dairy goat *PRMT5* had high sequence homology compared to corresponding regions in *Bos taurus*, *Callithrix jacchus*, *Mus musculus*, *Canis familiaris* and *Pan troglodytes*. Next, we constructed the phylogenetic tree using MEGA4.1 software, which indicated that *PRMT5* of goat and cattle is homologous and conserved (Fig. 2d).

Generation of dairy goat induced pluripotent stem-like cells using lentivirus vector

Schematic diagram of generation of dairy goat iPSCs is summarized in Fig. 3a. Lentiviral vectors containing four human reprogramming factors OCT4/SOX2/KLF4/C-MYC (OSKM) were used. AP-positive cells appeared after 7 days and gradually formed larger and compact colonies (Fig. 3a). ES-like colonies were formed and selected at 30–40 days after transduction and transferred to a fresh plate with MEF feeders (Fig. 3a). Colonies reprogrammed with OSKM were characterized by well-defined borders; all features were very similar to iPSC colonies and goat ES cells (23). Within 3–4 passages, typical iPSC-like cell colonies were identified, using RT-PCR and immunofluorescence analysis. RT-PCR results showed that these cells were positive for SOX2, KLF4, OCT4, C-MYC and NANOG (Fig. 3b). Immunofluorescence results also

confirmed that the cells were positive for the following pluripotentiality markers: SSEA1, SSEA4, C-MYC and OCT4 (Fig. 3c).

To test potential of the iPSC-like cell colonies, cells were cultured in suspension for 2 days. They spontaneously differentiated into typical embryoid bodies (Fig. 3d) and were then transferred into petri dishes containing normal cell culture medium without LIF and bFGF for 7 days. Differentiated cells expressed specific markers of the different germ layers, including α -ACTIN (marker of mesoderm), NSE (marker of ectoderm), VASA (germ cell marker) and PDX1 (endoderm marker) (Fig. 3e).

PRMT5 enhanced generation of dairy goat iPSCs

We investigated effects of *PRMT5* on derivation of iPSCs from dairy goat fibroblasts, and numbers of AP positive colonies, at D7 and D14 in the *PRMT5*/OCT4/SOX2/KLF4/C-MYC (POSKM) transduced group and discovered they were significantly higher than in OSKM (Fig. 4a). Moreover, most dairy goat iPSC-like colonies were GFP positive, as analysed using immunofluorescence microscopy. These findings indicated that *PRMT5* transduction clearly increased the ability to form iPSC-like colonies derived from GEFs (Fig. 4b). Moreover, the results showed that *PRMT5* enhanced generation of goat iPSCs derived from GEFs.

Figure 3. Characterization of dairy goat iPSCs. (a) Induced goat iPSC-like colonies were positive for AP staining; (b) RT-PCR analysis showed that goat iPSC-like cells were positive for SOX2, KLF4, OCT4, C-MYC and NANOG; (c) immunofluorescence analysis of dairy goat iPSCs at passage 2; pluripotent relative markers (OCT4, C-MYC, SSEA1, SSEA4) were expressed. Nuclei were counterstained with Hoechst 33342. (d) Goat iPSCs formed embryoid bodies (EBs). (e) Immunofluorescent staining showed that the EBs derived from goat iPSCs differentiated into three germ layer cell types, including the NSE, α -ACTIN, PDX1 and VASA-positive cells. Goat iPSCs differentiated into EBs.

PRMT5 enhanced somatic cell reprogramming by mediating down-regulation of p53

Expression of PRMT5 was significantly increased in GEFs with overexpressed PRMT5 (Fig. 5a,d). In addition, overexpression of PRMT5 resulted in more rapid cell proliferation compared to the control group (Fig. 5b,c). FACS cell cycle analysis showed that there were more cells in S-phase in overexpressed PRMT5 group compared to controls (Fig. 5c). Furthermore, we confirmed that PRMT5 enhanced somatic cell reprogramming *via* regulation of p53. PRMT5 overexpression in GEFs resulted in down-regulation of expression of p53 and *p21* (target gene of p53) (Fig. 5d). In addition, Western blotting detection also demonstrated that p53, CCND1 and caspase 3 protein expression was reduced by PRMT5 (Fig. 5e).

Discussion

Previous studies have shown that PRMT5 is important for establishment of ES cell lines derived from blastocysts, and is indispensable for embryonic development (33). In addition, PRMT5 exhibits remarkable effects on reprogramming of mouse embryonic fibroblasts into iPSCs in which PRMT5, KLF4, and OCT3/4 have been introduced in combination (4). However, there has been little information on reprogramming of somatic cells and effects of PRMT5 in livestock. In this study, we first examined effects of *PRMT5* gene on goat iPSC generation and found that it is expressed at high levels in iPSC-like cells. Furthermore, addition of PRMT5 in combination with the four typical OSKM pluripotentiality transcription factors clearly increased numbers of AP positive colonies generated, thus sustaining the notion

Figure 4. Generation of dairy goat iPSCs using four defined factors (OSKM) and OSKM combined with PRMT5 (OSKMP). (a) Reprogramming efficiencies at 7 days in two groups (OSKM and OSKMP) were determined. Inset shows that overexpression PRMT5 enlarged AP positive cells. AP positive colonies in each 24-well dish were quantified and plotted in the right panel, and number of AP positive colonies at D7 and D14 in OSKMP (PRMT5) was significantly higher than that in OSKM (Control) group, $*P < 0.05$. (b) Most iPS-like clusters were GFP (PRMT5) positive.

that PRMT5 up-regulated somatic reprogramming of our dairy goat cells. GEFs overexpressing PRMT5 combined with OSKM exhibited lower levels of *p53* and *p21* mRNA and protein compared to GEFs lacking PRMT5, thereby promoting reprogramming efficiency.

Reducing signals to p53 by expressing a mutated version of one of its negative regulators, by deletion or knocking down *p53* or its target gene, *p21* (also known as *CDKN1A*), or by antagonizing reprogramming-induced apoptosis, has been shown to increase reprogramming efficiency in mouse fibroblasts (34). This finding indicates that methylation of arginine residues is an underlying mechanism of control during the p53 response (35). p53 knockdown heterozygous MEFs also exhibited three-factor higher reprogramming efficiency than wild-type MEFs. These data indicated that the p53 pathway is one determinant of reprogramming

efficiency (34). It has been shown that PRMT5 protein (as a co-factor in a DNA damage responsive co-activator complex that interacts with p53), was responsible for p53 methylation (35). We first determined whether PRMT5, individually or in combination with the reprogramming factors, OSKM, activated the p53 pathway in dairy goat GEFs. Our results support the hypothesis that PRMT5 promoted dairy goat induced pluripotent stem cell generation *via* the p53 pathway, at least in part *via* repression of p53. These results suggest that PRMT5 regulates somatic cell reprogramming by down-regulation of p53/p21, thereby promoting cell proliferation and expression of KLF4 and C-MYC, and inhibiting apoptosis (Fig. 6). Previous studies have demonstrated that the p53–p21 pathway serves as a barrier, not only in tumorigenicity, but also in murine and human iPS cell generation (29,36,37).

Figure 5. PRMT5 promoted GEF cell proliferation. (a) Overexpression of PRMT5 in GEF cells. Immunostaining of PRMT5 (Red). Nuclei were stained with Hoechst 33342. (b) Overexpression of PRMT5 resulted in more rapid cell proliferation compared to control group. (c) Cell cycle analyses using FACS showed that there were 31.52% and 38.76% S-phase cells in control and PRMT5-overexpressing group, respectively. (d) QRT-PCR showed that expression of *p53* and *p21* was lower in PRMT5-overexpressing group compared to the control group, $*P < 0.05$. (e) Western blotting analyses showed that p53, CCDN1 and caspase 3 were reduced in the PRMT5-overexpressing group.

p53 protein functions mainly as a transcription factor that regulates important cellular processes, such as cell proliferation, cell cycle arrest, DNA repair and apoptosis, in response to stress signals (38). Unlike in the mouse, Gkoutela *et al.* (39) revealed that PRMT5 had no role in hESC pluripotency, and depletion of PRMT5 had no effect on expression of OCT4, NANOG or SOX2, and did not prevent teratoma formation. Instead, PRMT5 seemed to function in hESCs to regulate proliferation in the self-renewal state by regulating the fraction of cells in G1 phase of the cell cycle, and by increasing expression of

G1 cell cycle inhibitor p57. These data indicated a distinct role for PRMT5 in hESCs and identified p57 as a new target (39). Our study showed that PRMT5 promoted GEF reprogramming into iPS-like cells *via* regulation of cell proliferation and inhibition of apoptosis *via* the p53–p21 pathway.

In conclusion, this study showed *PRMT5* to be a conserved gene and that its overexpression could stimulate GEF proliferation and down-regulate p53, p21 and caspase 3, to enhance GEF reprogramming into iPSCs, in combination with OSKM. This investigation provides

an efficient method for study of the mechanisms of goat somatic cell reprogramming.

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