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Expression of 2A peptide mediated tri-fluorescent protein genes were regulated by epigenetics in transgenic sheep

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ABSTRACT

A number of gene therapy applications and basic research would benefit from vectors expressing multiple genes. In this study, we constructed 2A peptide based tricistronic lentiviral vector and generated transgenic lambs by injecting lentivirus carrying the tricistronic vector into perivitelline space of zygotes. Of 7 lambs born, 2 lambs (#6 and #7) carried the transgene. However, no fluorescent proteins were identified in transgenic sheep. To investigate why the transgene was silenced in transgenic sheep, we analyzed the methylation status of transgene. The methylation level of CMV promoter was 76.25% in #6, and 64.7% in #7. In the coding region of three fluorescent protein genes, methylation levels were extremely high, with the average level of 98.3% in #6 and 98.4% in #7 respectively. Furthermore, the ratio of GFP^+ cells were increased significantly when the fibroblasts derived from the transgenic sheep were treated with 5-azaC and/ or TSA. Our results showed that 2A peptide based tricistronic construct was subjected to hypermethylation in transgenic sheep. Moreover, the silencing could be relieved by treating with methytransferase inhibitor and/or deacetylase inhibitor.

1. Introduction

Transgenic animals play a critical role in biomedical research and agricultural industry [1,2]. However, many potential transgenic applications require vectors to express multiple proteins simultaneously. Till now, a number of strategies have been used to express multiple genes in one vector, including mRNA splicing, internal promoters, internal ribosomal entry sites (IRESes), fusion proteins and cleavage factors [3]. Among them, 2A mediated polycistronic vector has been proved to be preferred to expression of multiple genes simultaneously [4].

2A peptide, known as 'cis-acting hydrolase element' (CHYSEL), consists of 18 amino acids and shares a highly conserved motif D(V/I)EXNPGP [5]. It was originally identified and characterized in picornaviruses. 2A peptide could efficiently mediate 'ribosomal skipping' at the terminal of 2A proline and subsequent 2B glycine, resulting in co-translational cleavage of the polyprotein between Glycine and Proline [6]. Multiple genes mediated by 2A or 2A like peptide cassette could express discrete proteins derived from a single open reading frame (ORF) [7]. Update, 2A peptide has been applied in variety of eukaryotes, ranging from yeast to plant and mammal [4,5,8,9]. However, except transgenic pigs [9], 2A peptide cassette hasn't been reported to be applied in other transgenic farm animals.

Lentiviral transgene delivery system offers high efficient means in producing transgenic animals [10–12]. Our previous study documented that lentiviral transgenesis achieved high efficiency and widespread expression of eGFP in transgenic sheep [12]. Combining the high efficiency of lentiviral transgenesis and 2A mediated polycistronic co-expression approach, it would facilitate generation of transgenic animals modified by multiple genes.

Epigenetic modifications have been frequently observed in lentiviral transgenic animals. DNA methylation, as one of the important mechanisms of transgene silencing has been addressed in mice [13], pig [14,15], and sheep [12]. The methylation pattern in transgenic animals was probably established by de novo methylation, which happened in CpG islands adjacent to promoters, and made these regions in hypermethylated state [16]. Extensive hypermethylation leading to transgene silencing has been reported in transgenic mice and pigs [13,14].

In this study, a 2A peptide-based tricistronic lentiviral vector which mediated expression of three fluorescent protein genes was constructed and used to generate transgenic sheep. The

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expression and methylation status of three fluorescent protein genes and the CMV promoter (CMVp) were investigated in transgenic sheep. Our results indicated that the CMVp and fluorescent protein genes of 2A peptide based lentiviral tricistronic construct were subjected to hypermethylation in transgenic sheep and lead to silencing of transgene expression. Furthermore, the silencing could be relieved by mediation of epigenetic state with 5-azaC and/or TSA treatment.

2. Materials and methods

2.1. Construction of tricistronic lentiviral vector containing 2A peptide

2A linker, the cassette containing Thosea asigna virus 2A peptide bridge (T2A) and G-S-G flexible linker, was synthesized by Sangon Biotech. After digestion with BspEI and Hind III A linker was inserted into the multiple cloning sites of ptdTomato-C1 and pzsYellow1-C1 vector (Clontech, Mountain View, CA) respectively. The resulted plasmids were then amplified by PCR as templates for generation of tdTomato-2A and zsYellow1–2A fragments with addition of restriction sites by primers P1 and P2 (P1 primer, forward: CTAGCTAGCACTAGTCGCCACCATGGTGAG, reverse: CCGCTC GAGTGGGCCAGGATTCTC; P2, forward: CGCTCGAGTTCGCCA CCATGGCCCAC, reverse: CGGGATCCACTGGGCCAGGATTCTCCTC) respectively. The amplicons were digested by NheI and XhoI for tdTomato-2A, XhoI and BamHI for zsYellow1-2A, and subcloned into pacGFP1-N1 (Clontech) orderly. Thus 2A mediated tricistronic vector ptdTomato-2A-zsYellow12A-acGFP1 (p2A-TYG) was generated. Subsequently, the tricistronic cassette 2A-TYG was cut off from p2A-TYG plasmid with SpeI and NotI, and subcloned into LentiORF™ pLEX-MCS lentiviral expression vector (Open Biosystems, Huntsville, AL). The new construct was designated as pLEX-2A-TYG (Fig. 1A) and used to express three fluorescent protein genes. All restriction enzymes used in this study were purchased from Fermentas, Thermo, Rockford, IL, USA.

2.2. Verification of the expression of three fluorescent protein genes in CHO cells

Chinese hamster ovary (CHO) cells were cultured at 37 °C with 5% CO_2 in DMEM supplemented with nutrient F12 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS). Cells were seeded in six-well plates one day prior to transfection. For transfection, the cells were transfected with 3 μg of the plasmid and 6 μl Lipofectamine 2000 (Invitrogen). Forty-eight hours post-transfection, cells were examined under confocal microscope (Leica, Germany) and harvested for western blotting. Fluorescent images were taken using a 10×objective. TdTomato, zsYellow1, and acGFP1 fluorescence were excited at 554 nm, 531 nm and 475 nm wavelengths, respectively. Differential interference

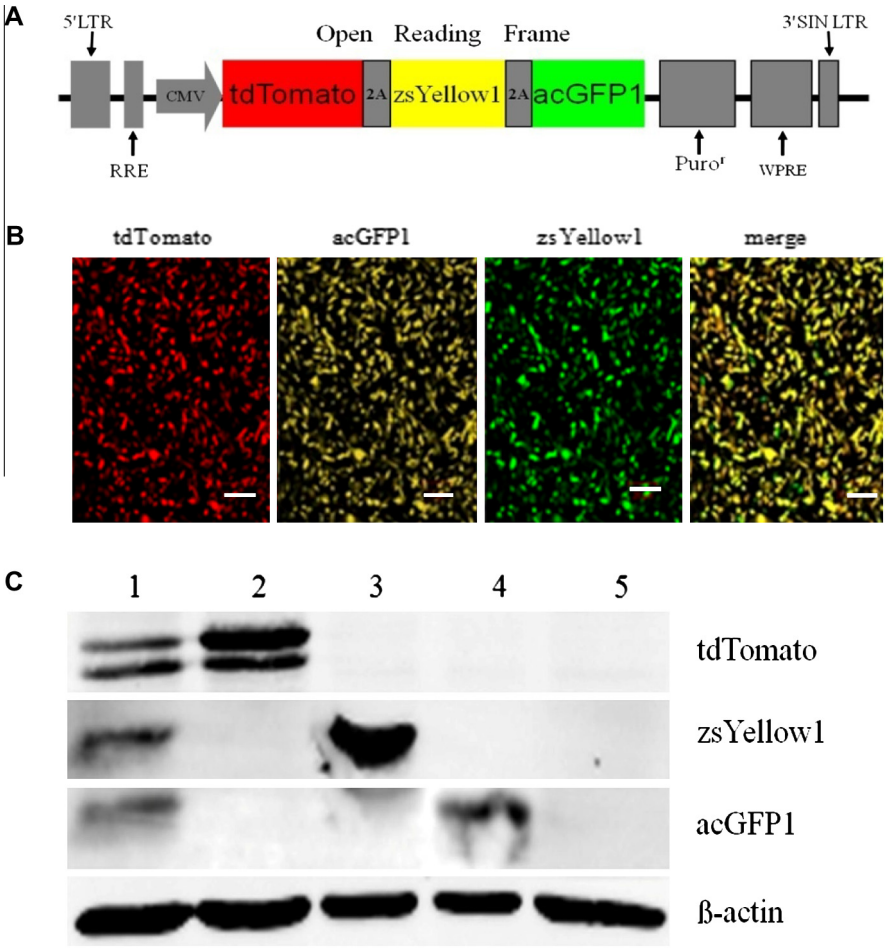

Fig. 1. Expression of three fluorescent protein genes in CHO cells. (A) Schematic of the pLEX-2A-TYG construct. LTR, long terminal repeat; RRE, rev-responsive element; WPRE, woodchuck hepatitis virus. (B) Fluorescent image of tdTomato, zsYellow1 and acGFP1 in CHO cells was imaged under confocal microscope. The scale bar represents 20 μm lengths. (C) Western blotting analysis of the expression of tricistronic construct. The expressions of three fluorescent proteins of tricistronic construct were detected by antibodies of tdTomato, zsYellow1 and acGFP1. Expression of ptdTomato, pzsYellow1 and pacGFP1 was designated as control for evaluation of the expression of tricistronic construct. Non-transfected CHO cell was set as negative control and β-actin was set as loading control. 1. p2A-TYG; 2. ptdTomato; 3. pzsYellow1; 4. pacGFP1; 5. non-transfected CHO cells control.

contrast (DIC) images were collected simultaneously. Western blotting was carried out to assay the expression of tdTomato, zsYellow1 and acGFP1 in CHO cells using their corresponding antibodies (Clontech).

2.3. Preparation of lentiviral particles and generation of transgenic sheep

Recombinant lentiviral particles were produced essentially as previously described [12,17]. Transgenic sheep were generated via injecting lentiviral particles into the perivitelline space of the zygotes [12]. In brief, embryos were obtained from Xinjiang Merino Sheep which were approximately 2 years old and weighed at least 50 kg. Superovulation was carried out within sheep breeding season from September to November and started 3 d before oestrus induced by intramuscular injection of follicle stimulating hormone (FSH, Sigma–Aldrich). FSH was injected once per 12 h lasting for 3 d. Briefly, twice injection of 40 IU FSH was performed on the first day, 30 IU on the second day and 20 IU on the last day. After 12 h of oestrus, the donors were mated with rams and repeated mating another 12 h later. At 60 h, zygotes from mated donors were collected by flushing the umbrella of oviducts with warm phosphate buffered saline containing 2% FBS. Then they were removed from the PBS and cultured in SOF medium with 3 mg/mL BSA at 38 °C in 5% CO_2.

For lentivirus injection, around 50–100 pl of concentrated lentivirus with 3×10^9 IU/ml titer were injected into perivitelline space of zygotes using a micromanipulator (ECLipse TE2000-U, Nikon). For embryo transfer, recipients were synchronized by the same treatment as donor ewes. Embryos injected with lentivirus were transferred to recipient ewes with mid-line laparotomy under general anaesthesia. This study has been approved by the Committee of Animal Research Security and Ethics, Xinjiang Academy of Animal Science.

Table 1
Primers for identification of transgenic lambs.

Name	PCR primers	Product size
tdTomato	Forward: 5′-GTGACCGTGACCCAGGACTC-3′ Reverse: 5′-GGGCCAGGATTCTCCTCGAC-3′	482 bp
zsYellow1	Forward: 5′-CCCACAGCAAGCACGGCCTG-3′ Reverse: 5′-GGCCGTCGGCGGGGAAGTTC-3′	402 bp
acGFP1	Forward: 5′-TTCGAGGGCGATACCCTG-3′ Reverse: 5′-CAGCTCATCCATGCCGTG-3′	458 bp

2.4. Identification of the transgene integration

Transgene integration was screened by PCR with genomic DNA extracted from lamb tail using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). The primers used to amplify three fluorescent protein genes were listed in Table 1. PCR was performed with 600 ng genomic DNA in the condition of 95 °C denaturation for 2 min followed by 35 cycles of 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 40 s, ended at a final extension at 72 °C for 7 min.

2.5. Bisulfite sequencing analysis

Genomic DNA extracted from tail tissues of transgenic sheep were treated by Epitect Bisulfite Kit (Qiagen) followed manual instruction. CpG islands were predicted using Methyl Primer Express® Software v1.0 (Applied Biosysterms). The CMVp and three fluorescent protein genes were amplified using primers listed in Table 2 with PCR Master Mix kit (Promega). All PCR products were purified and cloned into pGEM-T vector (Promega) for sequencing. For each sample, ten clones were picked.

2.6. 5-azaC/TSA treatment and flow cytometry analysis of cells derived from transgenic sheep

Primary skin fibroblasts of transgenic lambs were isolated from the tail tissues [18]. The cells were expanded and seeded in six-well culture dishes. 24 h later, cells were treated with 80 μM methytransferase inhibitor (5-azacytidine, 5-azaC, Sigma, St Louis, MO), 80 nM deacetylase inhibitor (trichostatin A, TSA, Sigma), DMSO (Sigma) treated cells were set as control [14]. After 48 h additional culture, cells were imaged and fluorescent photos were taken under ECLIPSE TE2000-U Fluorescent Microscope (Nikon, Japan) using the C-FL Epi-Fl Filter Block N B-2A (ex 450–490 nm) for acGFP1 (ex 475 nm), G-2A (ex 510–560 nm) for tdTomato (ex 554 nm) and zsYellow1 (ex 531 nm). The percentages of GFP^+ cells were determined by Fluorescence Activated Cell Sorting (FACS) analysis (Becton–Dickinson, Rutherford, USA). Every experiment was performed three repeats.

Table 2
Primers and PCR conditions for bisulfite sequencing.

Name	PCR primers and condition	Product size	No. of CG sites
CMV	outer forward 5′-GGGTTATTAGTTTATAGTTTATATATGG-3′ reverse 5′-GATTCACTAAACCAACTCTACTTA-3′ inner forward 5′-ATTAGTTTATAGTTTATATATGGAGTTTC-3′ reverse 5′-CAACTCTACTTATATAAACCTCCC-3′ 40 cycles of 95 °C 30 s, 50 °C, 30 s, 72 °C, 40 s	540 bp	30
tdTomato	Outer forward 5′-GAGGAGGTTATTAAAGAGTTTATG-3′ reverse 5′-ACCATATAAATAATCTTAAACTCCAC-3′ inner forward the same as outer forward reverse 5′-CTTCAACTTCAAAACCTAATAAATC -3′ 40 cycles of 95 °C, 30 s, 52 °C, 30 s, 72 °C, 40 s	492 bp	40
zsYellow1	outer forward 5′-GGAGATGATTATGAAGTATTATATGGAG-3′ reverse 5′-TCCTTCAACAACAAATACATACTCAC-3′ inner forward 5′-GGTTTGAAGGAGGAGATGATTATG-3′ reverse the same as the outer reward 40 cycles of 95 °C, 30 s, 55 °C, 30 s, 72 °C, 40 s	489 bp	36
acGFP1	outer forward 5′-GGATTTAGTTATTATGGTGAGTAAG-3′ reverse 5′-CCTTAATACCATTCTTAACCTTATC-3′ inner forward the same as the outer forward reverse 5′-TCCATCTTATTACCCAAAATATTAC-3′ PCR condition the same as Tdtomato	441 bp	32

2.7. Western blotting

Western blotting was carried out to detect the expression of tdTomato, zsYellow1 and acGFP1 in CHO cells and transgenic lambs. For CHO cells, transfected cells were collected and treated with Pierce® RIFA buffer (Thermo) supplemented with 1% Halt™ Protease Inhibitor Cocktail (Thermo). 20 μl protein lysis was loaded for electrophoreses in 12% SDS–PAGE. Proteins were then transferred onto PVDF membrane (Thermo). Membranes were blocked by 5% non-fat powder milk in PBS and detected by incubating with tdTomato, zsYellow1 and acGFP1 antibodies. β-actin antibody was used as loading control. For skin protein extraction, Tail tissues frozen in liquid nitrogen were ground to powder and resolved in Pierce® RIFA buffer and 1% Halt™ Protease Inhibitor Cocktail overnight at 4 °C. Then the tissue mixture was centrifuged at 12,000 rpm for 10 min, and the supernatant was used for western blotting assay mentioned above. Detected proteins were imaged by Odyssey Infrared Imaging System (Li-Cur, Lincoln, NE).

2.8. Data analysis

The statistical analysis was performed using SPSS 13.0. Data was shown as mean ± SD. One-way ANOVA was used to assess differences between groups. The LSD method was used for multiple comparisons. Error bars represent the standard error of the mean. $P < 0.05$ (two-tailed) was considered as statistically significant.

3. Results

3.1. Expression of 2A peptide-based tricistronic construct in CHO cells

Expression of 2A peptide-based three fluorescent protein genes in CHO cells were visualized by confocal imaging and assayed by western blotting. The image showed that three fluorescent proteins were visualized under confocal microscope (Fig. 1B). Western blotting revealed that the sizes of tdTomato, zsYellow1, and acGFP1 proteins were identical with those of fluorescent proteins expressed discretely (Fig. 1C). The collective data demonstrated that 2A peptide-based tricistronic construct could efficiently mediate the co-expression of tdTomato, zsYellow1 and acGFP1 fluorescent proteins.

3.2. Generation of transgenic sheep carrying pLEX-2A-TYG vector

By combining the characteristics of 2A mediated multicistronic expression and high efficiency of lentiviral transgenesis, we generated transgenic sheep with pLEX-2A-TYG lentiviral particles. Total of 37 zygotes injected with pLEX-2A-TYG lentivirals were transferred to thirty-seven recipients. Seven pregnant ewes were carried out to term and 7 lambs were born. Two lambs (#6 and #7) carrying transgene were identified by PCR screening (Fig. 2A and Suppl. Fig. 1). Unfortunately, the fluorescence was not observed in transgenic lambs even when the nose, tongue, hoof and other parts of

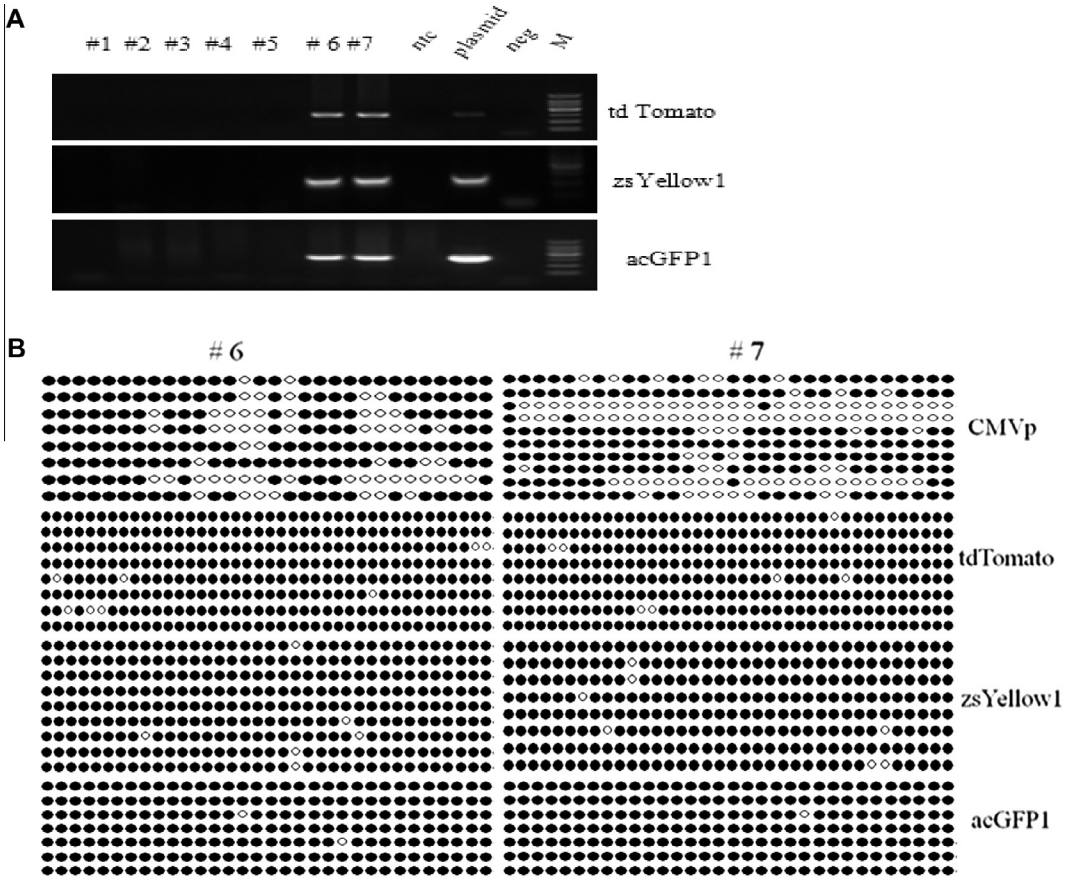

Fig. 2. Identification and analysis of transgenic sheep. (A) PCR screening of seven new born lambs numbered by #1 to #7. Two transgenic lambs (#6 and #7) were identified carrying transgene. Plasmid, pLEX-2A-TYG vector as positive control; ntc, non-transgenic sheep as mock control; neg, no DNA as negative control. M, 150 bp DNA marker. The sizes of PCR products of tdTomato, zsYellow1 and acGFP1 gene were 482 bp, 402 bp and 458 bp respectively. (B) DNA methylation analysis of the CMVp and three flurescent protein gene coding regions by bisulfite sequencing in transgenic lamb #6 and #7. Methylation levels of 487 bp region containing 30 CpGs in the CMVp, and tdTomato, 536 bp, 40 CpGs; zsYellow1, 489 bp, 36 CpGs; acGFP1, 441 bp, 32 CpGs were detected. Black circles represented methylated CpGs, white circles represented unmethylated CpGs.

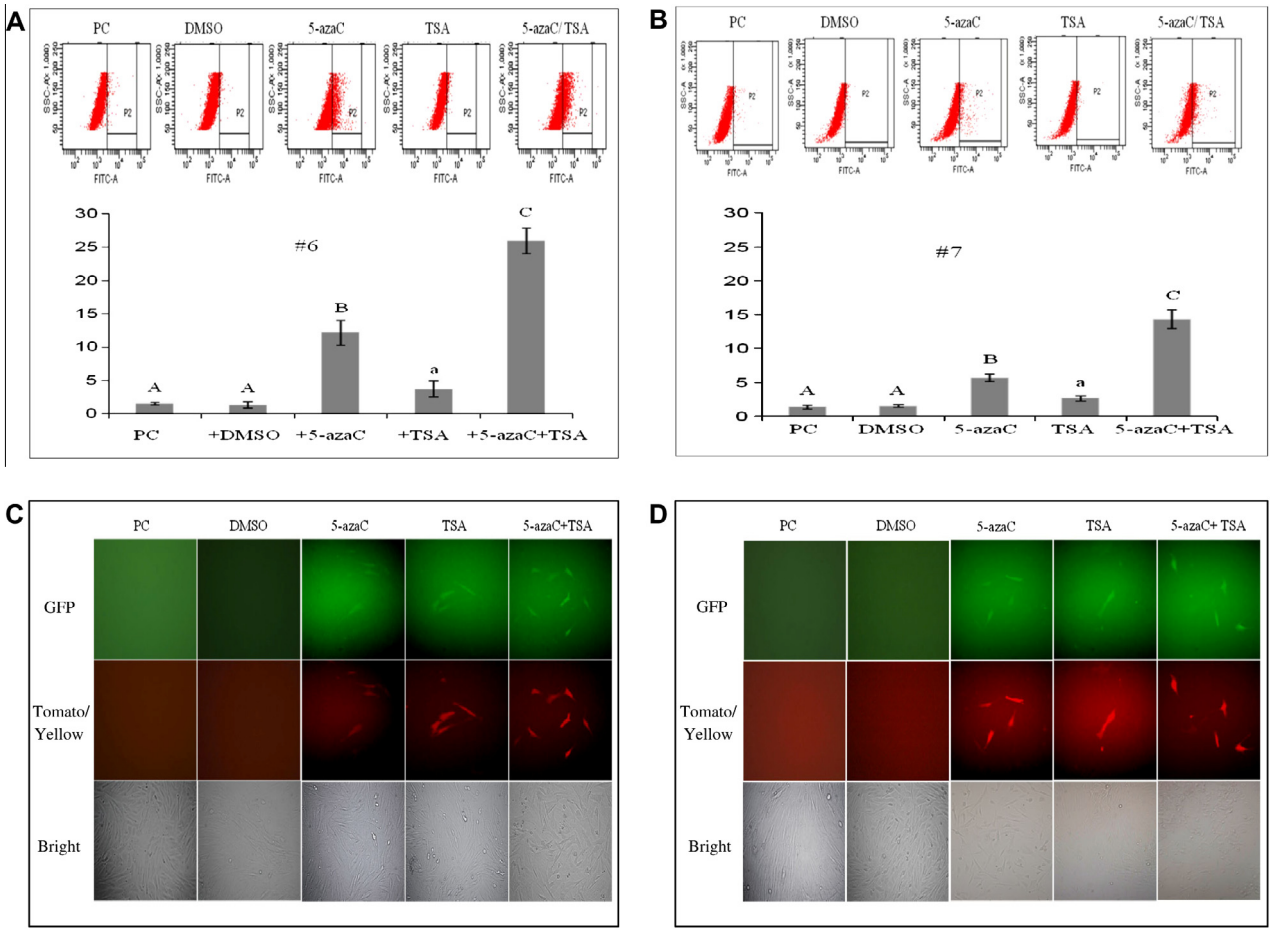

Fig. 3. Analysis of fluorescent protein gene expression of skin fibroblasts isolated from transgenic lambs. (A) Graph of analysis of GFP$^+$ fluorescent cells by flow cytometry (Upper panel) and quantification of the cells (Lower panel) of #6 fibroblast. Cells isolated from #6 were treated with 5-azaC, TSA and 5-azaC/TSA together. Parental cells (PC) and DMSO treated cells were set as control. The same letters represented no significant differences statistically. The different letters indicated the significant differences statistically in $p < 0.05$ level (small letter) or $p < 0.01$ level (capital letter). (B) Graph of analysis of GFP$^+$ fluorescent cells by flow cytometry (Upper panel) and quantification of the GFP$^+$ cells (Lower panel) of #7. Cells were treated and analyzed the same as that of #6. (C) Images of fluorescent cells of #6 under microscope. Cells were visualized under fluorescent microscope after treatment with 5-azaC, TSA, and 5-azaC/TSA together. Parental cells (PC) and DMSO treated cells were set as control. Upper panel (GFP): the cells were visualized with blue filter; Middle panel (Tomato/zsYellow1): visualized with green filter; Lower panel: visualized under bright field. All images were taken under the objective DIC 20x. (D) Images of fluorescent cells of #7 under microscope. Cells were treated and visualized the same as that of #6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the body were imaged by UV light. No fluorescent proteins were identified by western blotting either. The results indicated that the transgene was silenced in transgenic sheep.

3.3. DNA methylation status of the promoter and three fluorescent protein genes in transgenic lambs

To investigate whether the effect of methylation plays an essential role in transgene silencing, the methylation levels of CMVp and three fluorescent protein genes were examined (Fig. 2B). For CMVp, the methylation level was 76.25% ± 0.17 in #6, and 64.7% ± 0.35 in #7. For three fluorescent protein genes, the methylation levels were extremely high, with 97.5% ± 0.03 for tdTomato, 98.1 ± 0.02% for zsYellow1, 99.2 ± 0.02% for acGFP1 in #6, and 97.6 ± 0.02% for tdTomato, 97.6 ± 0.01% for zsYellow1, 99.5 ± 0.02% for acGFP1 in #7, respectively.

3.4. Effects of 5-azaC and TSA on the regulation of transgene expression in fibroblasts of transgenic lambs

To further investigate whether the hypermethylation was the cause of silencing of transgene expression, primary fibroblasts derived from two transgenic lambs were isolated and cultured. By treatment with 5-azaC and/or TSA, the green and red fluorescent cells were obviously observed under microscope (Fig. 3C and D). Consistently, the ratio of GFP$^+$ cells to total cells measured by FACS was also increased significantly (Fig. 3A and B). Additionally, both the fluorescent images and FACS results showed that treatment with 5-azaC along with TSA together could increase the fluorescent cells more significantly than that of treatment with 5-azaC or TSA alone. The results indicated that silencing of tricistronic genes in transgenic sheep could be regulated by inhibitors of methytransferase and deacetylase.

4. Discussion

IRES was the first element used to achieve multiple gene co-expression independently under the control of a single promoter [19]. However, the expression of downstream gene is often much lower than that of upstream gene, it makes IRES to be unreliable to co-express multiple genes [20]. In contrast, the 2A peptide can mediate the expression of multiple gene efficiently and independently [7]. By the virtue of 2A polycistronic expression system, it has been an attractive alternative for multiple gene expression. Update, 2A has been used successfully in variety of mammalian cells.

In our study, we observed co-expression of three fluorescent protein genes simultaneously and independently in CHO cells. The fluorescent visualization and western blotting assay of the transfected CHO cells revealed that 2A peptide-based tricistronic construct worked correctly both in co-expression of three fluorescent protein genes as well as cleavage of the tricistronic products. It showed great promise in transgenic animals.

Since the generation of the first transgenic mice as model for 2A mediated polycistronic expression [20], co-expression of multiple genes in transgenic animals had been achieved in transgenic pigs [9] and chicken embryos [21]. Hereby, we reported for the first time to generate transgenic lambs carrying 2A peptide-based discretely tricistronic lentiviral vector. In previous study, we succeeded in expressing eGFP in transgenic sheep even though the widespread methylation in lentiviral transgene was found in transgenic individuals and various tissues [12]. In this study, three fluorescent protein genes mediated by 2A peptide were expressed ectopically in mammalian cells, whereas failed to express in transgenic sheep. The studies carried out to unravel the mechanism of gene silencing showed that all the cistrons and the promoter were subjected to high methylation, the silencing of three fluorescent protein genes in transgenic sheep was partially resulted from epigenetic modification.

Cytosine methylation plays an important role in transgene expression for transgenic animals. It is often the cause of transgene silencing. Though a number of genes were successfully expressed in transgenic animals produced by lentivirus, in most cases, transgene couldn't escape from epigenetic modification [22,23]. Based on our previous study in transgenic sheep, the methylation level of lentiviral transgene was increased significantly, and the increase of methylation was inversely correlated with the expression of transgene [12]. So far, no studies have been reported the methylation status of 2A mediated multicistronic cassette in transgenic animals. On the other hand, as the 2A peptide was derived from virus, we hypothesized that the tricistronic cassette might be subjected to methylation modification and cause transgene silencing. In the study, dramatic increase of CpG methylation was found both in promoter and coding sequence of the tricistronic cassette, so we deduced that the silencing of tricistronic fluorescent protein genes most possibly resulted from hypermethylation. This is the first time to investigate the co-expression of multiple genes mediated by 2A peptide and methylation status in transgenic sheep.

In previous reports, TSA and 5-azaC has been successfully used to upregulate the expression by mediation of epigenetic state in pig fibroblasts [14,15,24], bovine fibroblasts [25], and cloned bovine embryos [26]. In our study, treatment with 5-azaC and/or TSA could significantly promote transgene expression, especially combination with 5-azaC and TSA together, which showed a synergistic role of 5-azaC and TSA in induction of transgene expression.

Taken together, 2A peptide-based tricistronic cassette, which mediated the multiple gene co-expression successfully in mammalian cells, could not be successful in transgenic lambs. The silencing of transgene may attribute to hypermethylation, and it can be relieved by regulation of the epigenetic status with methytransferase inhibitor and deacetylase inhibitor. Our studies inferred that the silencing of 2A mediated tricistronic expression was associated with hypermethylation in transgenic sheep, which might be regulated by 5-azaC and TSA. Our work lays the stone for the application and fundamental study of 2A mediated multiple gene expression in transgenic animals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.009.

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