Effect of Dynamic DNA Methylation and Histone Acetylation on *cPouV* Expression in Differentiation of Chick Embryonic Germ Cells

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As a crucial pluripotency-related factor, the epigenetic regulation of *Oct4* has been studied intensively in mammals. However, its dynamic changes of DNA methylation and histone modification in avians remain poorly understood. In the present study, we first described the alterations of DNA methylation and histone acetylation in the promoter of chicken *PouV* (*cPouV*; the homologue of *Oct4* in avian) during chick embryonic germ (EG) cell differentiation. The epigenetic modification analysis showed that DNA methylation in the *cPouV* promoter increased obviously, while histone acetylation decreased dramatically detected by chromatin immunoprecipitation assay in the process of differentiation. Gene expression analysis detection indicated that the levels of DNA methyltransferase 3a (*Dnmt 3a*), *Dnmt 3b*, and histone deacetylase 3 (*HDAC 3*) transcripts were significantly high, whereas the relative abundance of *Dnmt 1*, histone acetyltransferase (*HAT*), and *cPouV* mRNA was significantly decreased during the conversion of EG to embryoid body-like structures (EBs), which was correlated with the increased level of methylation and reduced level of H3 acetylation. Moreover, in vitro methylation assay indicated that the reporter gene was remarkably inhibited by the methylated promoter of *cPouV*. To further understand the effect of epigenetic modifiers on *cPouV* expression, we performed an analysis of EB cells treated with trichostatin A (TSA), Aza-2'-deoxycytidine (Aza), or TSA plus Aza (TSA/Aza). We observed that the effect of TSA/Aza is more sensitive to the reactivation of *cPouV* compared with TSA or Aza, indicating that these epigenetic inhibitors can function synergistically to facilitate the reprogramming process. The present study provided evidences that a critical role for *cPouV* activation/repression by DNA methylation and/or histone modifications is involved in the pluripotency maintenance and differentiation process of chick EG.

Introduction

PLURIPOTENT STEM CELLS (PSCs) possess the unique ability to self-renew and can differentiate into all of cell lineages. The list of cell types sharing these properties includes embryonic stem (ES) cells, embryonic carcinoma cells and, most recently, induced pluripotent stem (iPS) cells [1–3]. As a potential research and therapeutic tool, pluripotency will pave its way for future applications as long as the foundational mechanisms are unraveled. It is now apparent that the pluripotency and differentiation of PSCs are regulated by complicated networks, including many pluripotency factors such as *Oct4* [4,5].

Oct4 is a POU domain homeobox gene, expressed in undifferentiated ES cells and is quickly downregulated upon

induction of differentiation [6]. Therefore, PSCs are particularly sensitive to dosage alterations in *Oct4*. It has been reported that the loss of *Oct4* function results in differentiation into trophoblast cells, and a 50% increase or decrease in the level of *Oct4* causes differentiation into cells expressing markers of endoderm and mesoderm or trophoblast, respectively [7]. These evidences indicated that precise levels of *Oct4* must be sustained for the maintenance of pluripotency. Recent progress from iPS cells gave us more insight into the regulating role of *Oct4* in cell reprogramming. To date, the fact is that no experimental reprogramming platform had been able to reverse a somatic cell to a pluripotent state without overexpression of *Oct4* [8,9]. These previous studies suggested that *Oct4* is not simply a reprogramming factor, but a gatekeeper into pluripotency.

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As its central role in the maintaining of pluripotent ES cells and other pluripotent cells, the regulatory characteristics of the *Oct4* expression has been studied extensively [10]. It is now well known that epigenetic mechanisms, particularly DNA methylation and histone modification, play important roles in the control of gene expression [11]. Similarly, differentiation and reprogramming studies have also unraveled a few epigenetic modifications associated with the expression state of *Oct4* [12]. In mammals, *Oct4* gene expression is dependent on three upstream elements, consisting of distal enhancer, proximal enhancer, and proximal promoter (PP). In addition, these regulatory elements possess different epigenetic status in ES cells based on its pluripotency or differentiation [13].

As one type of PSCs, embryonic germ (EG) cells have been derived and established from primordial germ cells (PGCs) in many species [14]. Most work on EG cells use cells derived from mammals, especially mouse and human. There has been very little remarkable progress in nonmammalian systems. As an important model organism, chick has long been an ideal system for the study of developmental biology [15–18]. In 2007, the existence of an avian homologue of *Oct4* called chicken *PouV* (*cPouV*) was confirmed [19]. High conservation of the different loci among species was also demonstrated through genomic analysis. Even if the importance of epigenetic regulation for *Oct4* is well established in mammals, relevant information about *cPouV* is very limited in chick. Therefore, it is of great importance to understand how *cPouV* transcription is epigenetically regulated in chick EG cells.

The aim of this study is to assess the epigenetic features in pluripotent elements of *cPouV* during differentiation of chick EG cells. First, we performed modification analysis of DNA methylation and histone acetylation in three regions of *cPouV* in the process of differentiation. An inverse correlation between *cPouV* expression and DNA methylation was observed. In contrast, histone acetylation can promote the transcription of *cPouV*. In addition, in vitro methylation assay indicated that the reporter gene was inhibited remarkably by methylated promoter of *cPouV*. To further investigate the correlation between *cPouV* expression and epigenetic patterns, differentiated cells from embryoid body-like structures (EBs) were cultured with the chromatin-modifying agents trichostatin A (TSA) and/or Aza-2'-deoxycytidine (Aza), which affect histone acetylation and DNA methylation, respectively. After the treatment, the reactivation of *cPouV* was detected, indicating that DNA demethylation and recovery of histone acetylation are involved in the dynamic expression of *cPouV*. Our results provide evidences that the expression of *cPouV* is tightly associated with epigenetic regulation in chicken pluripotent EG cells.

Materials and Methods

Isolation and maintenance of EG cells in culture

EG cells were derived and maintained as our previous study with minor modifications [20]. In brief, fertilized eggs were obtained from Shouguang black chickens (*Gallus domesticus*). Gonadal PGCs were prepared by isolating embryonic gonads at stage 28 and dissociating the gonad tissue in 0.25% trypsin–0.05% ethylene diamine tetraacetic acid (EDTA) by gentle pipetting. For primary culture, the isolated PGCs from four gonads were seeded in one well of the 24-well culture plate and incubated in a CO₂ incubator at 37°C

until the PGCs had colonized. For the generation of EG cells, gonadal PGCs were cultured in 24-well plates with EG cell culture media consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% chicken serum (Gibco), 1 mM sodium pyruvate, 2 mM L-glutamine, 5.5×10^{-5} M-mercaptoethanol, 10 mg/mL of streptomycin, 100 units/mL of penicillin, 5 ng/mL of human stem cell factor (Sigma), 10 units/mL of murine leukemia inhibitory factor (mLIF; Sigma), 20 ng/mL of bovine basic fibroblast growth factor (bFGF; Sigma), and 10 ng/mL of human insulin-like growth factor-I (Sigma). For subculture, the colonies of chicken EG cells were agitated by gentle pipetting without trypsin–EDTA treatment and harvested from the plate. These seeded cells were centrifuged at 500g for 5 min and divided into a fresh 24-well plate simultaneously with mouse embryonic fibroblasts, which were mitotically inactivated. The EG cell colonies were passaged at an interval of 5–6 days on average.

Characterization of EG cells

The chicken EG cells from third passages were fixed to the plate in 1% glutaraldehyde for 5 min and rinsed with 1× phosphate-buffered saline (PBS) twice. For periodic acid-Schiff (PAS) staining, the EG cells were immersed in the periodic acid solution (Sigma) for 5 min at room temperature. After washing with 1× PBS, the fixed EG cells were immersed in the Schiff's solution (Sigma) for 15 min at room temperature and washed twice with 1× PBS. For alkaline phosphatase (AP) staining, fixed cells were immersed in a filtered AP staining solution [2 mg naphthol AS-MX phosphate, 200 µL *N,N*-dimethylformamide, 9.8 mL of 0.1 M Tris (pH 8.2), and 10 mg Fast Red TR salt] for 30 min, and then rinsed three times with 1× PBS. To detect expression of stem cell markers, EG cell colonies were examined for expression of the stage-specific embryonic antigen-1 (SSEA-1), and SSEA-3 by reaction with streptavidin-horseradish peroxidase, 3,3'-diaminobenzidine, and hydrogen peroxide (Sigma) via the biotinylated anti-mouse secondary antibody (1:150; Chemicon) [21].

Formation of EBs

For the formation of EBs, suspension culture was performed. Briefly, EG cells were passaged to a 0.1% gelatin-coated plate to eliminate possible contamination by fibroblasts. After 4–6 days in culture, colonies were gently dislodged from the plate with the aid of a micropipette and were disaggregated by incubation in 0.25% trypsin–EDTA for 10–15 s at 37°C. Dissociated cells were cultured in the EB cell culture medium containing the DMEM, 20% FBS, and 5.5×10^{-5} M-mercaptoethanol on a 35-mm nonadhesive Petri dish (Falcon). Suspension cultures were monitored daily for EB formation, with the medium changed every other day. During this period, the cells aggregated to form EBs. Subsequently, part of EB cells were collected for molecular detection and the left were treated with epigenetic reagents described as following.

Characterization of EB differentiation in vitro

The progeny of EB cells were characterized by cytochemistry staining. For neurogenic (ectodermal) or hepatogenic (endodermal) differentiation, the progeny cells were

fixed with 4% paraformaldehyde. The specific markers for astrocytes or hepatocytes were detected by primary antibodies, including the mouse anti-glial fibrillary acidic protein (GFAP; Beyotime) or mouse alpha-1-fetoprotein (AFP; Beyotime) for 40 min, respectively. After three time washes in PBS, cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Boster) for 1 h at 37°C. After washing, the immunoreaction was detected by a fluorescence microscope. For the evaluation of adipogenic (mesodermal) differentiation, the differentiated cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Then, adipogenesis was assessed by Oil Red (Shenggong Biotechnology) staining.

Treatment of EBs with epigenetic reagents

To examine the correlation of epigenetic changes in *cPouV* promoter and its expression, part of obtained EB cells aforementioned were treated with Aza (0, 1, 5, and 10 μ M), TSA (0, 100, 200, and 400 nM), or a combination of Aza (5 μ M) and TSA (200 nM) in the EB cell culture medium for 48 h. The Aza and TSA were dissolved in dimethyl sulfoxide and prepared as a 1,000-fold concentrated stock solution. These stock solutions were added to the culture medium at a 1:1,000 dilution according to the experimental procedure. After 2 days, cells were harvested for RNA extraction.

Bisulfite modification and sequencing of genomic DNA

Isolation and purification of genomic DNA was performed through phenol/chloroform extraction and ethanol precipitation processes. Bisulfite conversion was conducted using the CpGenome Fast DNA Modification Kit (Chemicon-Millipore), as indicated by the manufacturer. Briefly, unmethylated cytosines in DNA were converted into uracil via the heat-denaturation of DNA and with a specifically designed C-T conversion reagent. After desulfonation, clean and elution, the bisulfite-modified DNA was then immedi-

ately utilized for polymerase chain reaction (PCR) or stored at or below -20°C . The primers of bisulfite sequencing PCR were listed in Table 1. The PCRs were conducted in a Eppendorf 96 Mastercycler Gradient 5331 (Germany) in accordance with the following protocol: 95°C for 5 min, 32 cycles of 95°C for 20 s, 53°C – 58°C for 40 s, 72°C for 30 s, followed by an extension at 72°C for 10 min, and soaking at 4°C . Following electrophoresis on a 1.5% agarose gel, the remaining PCR products were cloned into bacteria (DH5 α) by a pGEM T-Easy Vector System I (Promega), and 10 clones selected randomly were sequenced.

Plasmid construction, in vitro methylation, and luciferase reporter gene assay

The *cPouV* promoter region (-101 to $-1,102$) and the region (-504 to $-1,102$) were amplified from chick liver genomic DNA. The primers used for amplification are as follows: for pGL3-Basic-0.6 ($-504/-1,102$), 5'-CCCCGCGGCCGCC GCCTT-3' (sense) and 5'-TCGCTCGGCCCGCTTCCCGT-3' (antisense); for pGL3-Basic-1.0 ($-101/-1,102$), 5'-CCCCGCG GCCGCCGCCTT-3' (sense) and 5'-TTGTGTGAGCGGG TCGGC-3' (antisense). After ligated into the pGEM-T Easy vector, these DNA fragments (0.6 and 1.0 kb) derived from the *cPouV* promoter were inserted into *Hind*III and *Kpn*I sites in the promoter-less luciferase reporter pGL3-Basic (Promega) to create the plasmids pGL3-Basic-0.6 and pGL3-Basic-1.0, respectively. All inserted fragments were amplified by PCR using Pfu DNA polymerase (Stratagene). The resulting plasmids were sequenced to confirm the accuracy of the inserts.

In vitro methylation of pGL3-Basic-0.6 and pGL3-Basic-1.0 was performed using *M.Sss*I (New England Biolabs). Forty-five units of enzyme were incubated with 45 mg of plasmid DNA in the presence of 160 mM S-adenosylmethionine (SAM) overnight. After 3 h of incubation, fresh SAM (160 mM) was added to ensure complete methylation. Methylation status of the plasmid after in vitro methylation was tested by digestion with *Msp*I and *Hpa*II (New England Biolabs).

TABLE 1. BISULFITE SEQUENCING AND CHROMATIN IMMUNOPRECIPITATION PRIMERS USED IN THIS STUDY

Gene	Primer sequences	Sequence coverage relative to TSS (nucleotide No.)	Product size (bp)
Bisulfite sequencing primers			
cPouV	F-TAGATTTTGGTTGGGGGAATTA	+135 to +269	135
	R-TCCTTAACAACTACTCCAATCCT		
	F-GGTGTGTGTTTGGGGGTTTGG	-129 to -269	141
	R-ACCCAAACAAAAAACAC		
	F-TAGAGAAAGGGGATTAAGTTTA	-1,545 to -1,700	156
	R-AAAACTACCTCCATACTACC		
ChIP primers			
cPouV	F-CCTGCTGCGAATGTGTAAATG	+21 to +276	256
	R-CTTGAGGTCCTTGGCAAAT		
	F-GTTCCCGTACTCTGCGAA	-66 to -492	427
	R-CGTATCTGCCTGGGATGAA		
	F-GAAAGGGGATCAAGTTCACGCC	-1,443 to -1,696	254
	R-CGTCCGGGCTGAACATGGTG		
GAPDH	F-GGATTGGCCGTATTGG	+79 to +428	360
	R-GCTGAGGGAGCTGAGATG		

TSS, transcriptional start site; cPouV, chicken *PouV*; ChIP, chromatin immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

For the reporter gene assay, HEK293T cells were plated in six-well plates and grown to 70% confluence. Subsequently, the firefly luciferase reporter plasmid pGL3-Basic-0.6 or pGL3-Basic-1.0 (100 ng) was cotransfected with the pU6B-Renilla reporter (5 ng) using Lipofectamine 2000 (Invitrogen) overnight. Cells after transfection were lysed in PBS containing 0.5% NP40 and mammalian protease inhibitors at 24 and 48 h. The lysate was cleared by centrifugation and the luciferase reporter activities were assayed using the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activities were normalized for analyses using Renilla luciferase activities.

Quantitative real-time reverse transcription PCR

Total RNA was extracted from undifferentiated EG cells and EB cells with the Trizol reagent (Invitrogen). The cDNA was synthesized from total RNA using a AMV First-Strand cDNA synthesis kit with oligo(dT)₁₈ primers according to the manufacturer's instructions (TaKaRa). PCR was subsequently carried out on 1 μ L cDNA template for 30–35 cycles with primers as listed in Table 2. The relative expression of epigenetic-related genes [DNA methyltransferase (*Dnmt*) 1, *Dnmt* 3a, *Dnmt* 3b, histone acetyltransferase (*HAT*), and histone deacetylase 3 (*HDAC* 3)] was detected. The β -actin was used as the internal control. SYBR green fluorescence was measured at the end of each extension step. Melting curve analysis was undertaken to determine the specificity of the PCR products. All reactions were performed in triplicate, and the relative expression level of genes in EG cells was normalized as 1.

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) experiments were performed using a Chromatin Immunoprecipitation Assay Kit (Upstate) according to the manufacturer's recommendations. In brief, 1×10^5 cells were treated with 1% formaldehyde for 10 min to make the crosslink of histone and DNA. After crosslinking, cells were collected, resuspended in the sodium dodecyl sulfate lysis buffer, and sonicated to shear the DNA. Subsequently, the lysates were centrifuged to clear debris, and the supernatants were collected and diluted with

a 10-fold ChIP dilution buffer. The diluted supernatants were then incubated with either anti-acetyl histone H3 antibody (Upstate) overnight at 4°C. After incubation, extensive washing, and elution, the histone-DNA crosslinks were reversed by heating at 65°C for 4 h. Precipitated DNAs were recovered by phenol/chloroform extraction and ethanol precipitation, and then stored at –20°C until used for PCR. PCR amplification was performed on DNA recovered from the ChIP and the total chromatin input, using primers listed in Table 1. Five microliters of immunoprecipitated DNA (from a total of 50 μ L) was quantified by real-time PCR using SYBR Green master mix (AB Applied Biosystems) on a 7500 Real-Time PCR System (AB Applied Biosystems). The PCR condition was 95°C for 3 min; and 35 cycles of 95°C for 30 s, 58°C–60°C for 45 s, 72°C for 30 s, followed by an extension at 72°C for 10 min, and soaking at 4°C. Data were expressed as fold enrichment of DNA associated with different immunoprecipitated regions relative to a 1/100 dilution of input chromatin. The relative fold change in EG cells was normalized as 1.

Statistical analysis

Statistical analyses were performed using the SPSS 13.0 software package (SPSS Inc.). The results are expressed as mean value $\pm$ standard deviation of three independent experiments. The data from differently treated groups were compared by using the unpaired, two-tailed Student *t*-test. A *p*-value < 0.05 was considered statistically significant.

Results

Isolation and maintenance of EG cells in culture

For primary culture, gonadal PGCs from 5.5-day-old chick embryos were colonized as early as day 5. ES cell-like phenotypes, such as a large translucent nucleus, a relatively little cytoplasm, and colonies with a well-defined border were observed in most EG cells (Fig. 1A). For subculture, the colonies of EG cells were passaged at an interval of 5–6 days. These colonies were maintained for up to 15 passages and proliferated over a period of 3 months. Morphologically, almost all of the colonies were uniformly round and pack

TABLE 2. PRIMER SEQUENCES FOR TRANSCRIPTION DETECTION OF CHICK GENES

Gene	Primer sequence (5' $\rightarrow$ 3')	Accession number	Product size (bp)
<i>cPouV</i>	F-AGAACATGTGCAAGCTGAAGCCAC R-GGGCTTCACACATTGCGGAAGAA	DQ867024	188
<i>Dnmt</i> 1	F-ATGACAACATCCCCTGAGATGCCCT R-TTCCCATCGCTCTTGATGGGTTCT	NM_206952	112
<i>Dnmt</i> 3a	F-AAGTGAGAACCATCACCCTCGCT R-TGCACCACAGGATGTCTTCCTTCT	NM_001024832	99
<i>Dnmt</i> 3b	F-ACAAGAGGGTGAGCAAAGACCTGT R-GCTGCTGCTTTCCTTGATCTGCAT	NM_001024828	188
<i>HAT</i>	F-TGACGTGTCCAGGCTTTCGAGAAT R-TGAAAGGCTTCAGACATTCTTGATGTGGT	AF257739	173
<i>HDAC</i> 3	F-TGACATGTGCAGATTCCTCACTCCGA R-TCTTGTGTTCAGCTGCGTTGCTC	AF039753	197
β -actin	F-AGACATCAGGGTGTGATGGTTGGT R-TCCCAGTTGGTGACAATACCGTGT	NM_205518	125

Dnmt, DNA methyltransferase; HAT, histone acetyltransferase; HDAC, histone deacetylase.

FIG. 1. Characterization and differentiation of chicken EG cells. **(A)** EG cell colonies. **(B)** EG cell colonies stained positive for AP. **(C)** EG colonies showed PAS-positive. **(D, E)** Immunocytochemical staining of SSEA-1 and SSEA-3 of EG cells at the end of the third passage, respectively. **(F)** EBs on day 14. **(G)** Astrocyte-like cells stained with anti-GFAP antibodies. **(H)** Small lipid droplets stained positively with Oil Red during adipogenic differentiation. **(I)** Positive cells stained with anti-alpha-1-fetoprotein. Scale bars=20 μ m. EG cell, embryonic germ cell; AP, alkaline phosphatase; PAS, periodic acid-Schiff staining; SSEA, stage-specific embryonic antigen; EBs, embryoid body-like structures; GFAP, glial fibrillary acidic protein.

together in small nests. For characterization, chick PGC-derived EG cells with brown staining indicated the detection of a strong AP activity, which retained during subculture (Fig. 1B). Similarly, glycogen in the cytoplasm of chick EG cells could still be stained by PAS reaction even after several passages (Fig. 1C). In addition, chicken PGC-derived EG cells were further characterized with two monoclonal antibodies: SSEA-1 and SSEA-3. As seen in Fig. 1D and E, colonies stained strongly for both antibodies.

Formation of EBs and its differentiated potential in vitro

To explore the potential of the chick EG cells to differentiate in vitro, we tried to aggregate the chick EG cells in suspension to form EBs. In suspension, the EG cells from six or seven passages were cultured without LIF and bFGF. In addition, cells were grown on Petri dishes to prevent their adherence to the plate. Under these conditions, chick EG cells consistently aggregated and formed EBs. Figure 1F shows EB formation was detected after 2–4 days of suspension culture. Soon after, these EBs enlarged and the bodies began to accumulate fluid. Eventually a structure named cystic EB was formed after 5–10 days.

To further elucidate whether these embryoid bodies can differentiate into various cell types in vitro, EBs were characterized after differentiation. The progeny of EB cells were

strongly stained with antibodies for astrocyte-specific GFAP and AFP as seen in Fig. 1G and I. In addition, the results of Oil Red staining shown in Fig. 1H indicate that EBs have the ability of differentiation into adipocytes. Therefore, these results suggest that the chicken EG cells were capable of differentiation into endoderm, mesoderm, and ectoderm lineage in vitro.

*Bisulfite sequencing and ChIP analysis of *cPouV**

To determine whether *cPouV* expression was affected by epigenetic alteration during EG differentiation, the levels of DNA methylation and histone acetylation in its promoter were identified by bisulfite sequencing and ChIP analysis, respectively. We first extracted genomic DNA from both EG and EB cells and examined DNA methylation in three regions of *cPouV*, which contains abundant CpG sites (Fig. 2A). As expected, we found a significant increase of DNA methylation in these regions during differentiation as shown in Fig. 2B. On the contrary, the degree of H3 acetylation decreased during the process detected by ChIP using the acetyl-histone H3 antibody (Fig. 3A–C). With dynamic changes of DNA methylation and histone acetylation, the expression of *cPouV* decreased progressively during the differentiation (Fig. 2D). These results indicated that epigenetic patterns not only changed significantly, but also can impact on the expression of *cPouV* during the course of EG cell differentiation.

FIG. 2. CpG methylation states of *cPouV* and the expression of Dnmt 1, Dnmt 3a, Dnmt 3b, and *cPouV* in EG cells and EBs. **(A)** Schematic representation of the *cPouV* gene. Numbers depict the positions of primer pairs used for BiS-PCR relative to TSS. **(B)** Bisulfite sequencing analysis of CpG methylation on *cPouV* regulatory regions in indicated cell types. Each vertical line and corresponding number represent the position of CpG in amplicon. Each CpG is indicated by a circle; each column represents the methylation state deduced from the sequence of 10 bacterial clones of the PCR product. Global percentages of methylated cytosines are shown as %Me. ○, unmethylated CpG; ●, methylated CpG. **(C)** The relative expression of Dnmt 1, Dnmt 3a, and Dnmt 3b analyzed by real-time PCR in EG cells and EBs. The relative fold change in EG cells was normalized as 1 (* $p < 0.05$). **(D)** The relative expression of *cPouV* analyzed by real-time PCR in EG cells and EBs. The relative fold change in EG cells was normalized as 1. *cPouV*, chicken *PouV*; BiS, bisulfite sequencing; PCR, polymerase chain reaction; TSS, transcriptional start site; Dnmt, DNA methyltransferase.

In vitro methylation and luciferase reporter gene assay

To further verify the effect of DNA methylation on *cPouV* expression, the GL3-Basic-luc reporter plasmid was constructed by inserting a 0.6- or 1.0-kb genomic DNA fragment from the *cPouV* promoter in front of a luciferase reporter gene. After methylation in vitro, methylated and unmethylated plasmids were confirmed by digestion with

MspI and *HpaII*. Then, we did the luciferase assay by cotransfection of GL3-Basic-0.6 or GL3-Basic-1.0 (methylated and unmethylated plasmids) with pU6B-Renilla reporter constructs into HEK293T cells at 24 and 48 h, respectively. The results showed that methylation of a promoter in *cPouV* results in the downregulation of *cPouV* obviously comparing with the unmethylated group (Fig. 4). These observations further demonstrated that DNA methylation negatively regulated the expression of *cPouV*.

FIG. 3. Histone H3 acetylation states of *cPouV* and the expression of HAT and HDAC 3 in EG cells and EBs. **(A)** Schematic representation of the *cPouV* gene. Numbers depict the positions of primer pairs used for ChIP relative to TSS. **(B)** A representative electrophoresis photograph of ChIP analysis of histone acetylation in corresponding regions of *cPouV* in EG (upper) and EB (lower) cells. **(C)** Quantitation of PCR signals in **(B)**. Data are expressed as percent precipitated relative to input DNA. The ratio of signals at EG cells was set as 1. **(D)** The relative expression of HAT and HDAC 3 analyzed by real-time PCR in EG cells and EBs. The relative fold change in EG cells was normalized as 1 (* $p < 0.05$). HAT, histone acetyltransferase; HDAC 3, histone deacetylase 3; ChIP, chromatin immunoprecipitation; I, input; N, negative (IgG); P, positive (GAPDH); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Quantitative real-time reverse transcription PCR

To elucidate the underlying mechanisms leading to the epigenetic changes, we performed quantitative real-time RT-PCR to detect the transcriptional level changes of epigenetic-related genes, such as *Dnmt 1*, *Dnmt 3a*, *Dnmt 3b*, HAT, and HDAC 3. As anticipated, the expression of *Dnmt 3a* and *Dnmt 3b* increased remarkably during the conversion of EG cells to EBs, whereas the expression of *Dnmt 1* maintained a relatively stable level (Fig. 2C). In the case of HDAC 3, its transcription upregulated significantly during the process, compared with reduction of HAT expression (Fig. 3D).

Effect of epigenetic reagents on the *cPouV* expression

Since the expression of *cPouV* is downregulated during EG differentiation by the increase of DNA methylation and H3 deacetylation in its promoter, it is interesting to test whether epigenetic inhibitors can reactivate the *cPouV* transcript. To identify the hypothesis, EB cells were treated with Aza or/and TSA. After the treatment of Aza alone, the expression level of *cPouV* only increased slightly. In contrast, treatment with TSA alone or TSA + Aza resulted in upregulation of *cPouV* (Fig. 5). These evidences indicated that TSA is more

FIG. 4. Luciferase reporter assay of various plasmid constructs in HEK293T cells. The firefly luciferase reporter plasmid pGL3-Basic-0.6 or pGL3-Basic-1.0 was cotransfected with pU6B-Renilla reporter using Lipofectamine 2000. At 24 and 48 h, the luciferase reporter activities were assayed using the Dual-Luciferase Reporter Assay System. The firefly luciferase activities were normalized for analyses using Renilla luciferase activities (* $p < 0.05$).

efficient for the reactivation of *cPouV* than Aza. In addition, it was observed that *cPouV* can be reactivated more significantly after the combination treatment of both Aza and TSA (Fig. 5). Based on these data, it might have a synergistic effect between DNA demethylation and histone acetylation.

Discussion

As a classic model, the chick embryo has been used to gain insight into the developmental processes for over a century. Especially in the last two decades, the advancement of stem cell biology further promises to give the chick embryo a huge new impetus as a leading system in developmental biology and many other areas [22]. Because the organization of the early chick embryo differs significantly from that of the mammals, it is impossible for chick to isolate cells equivalent to the inner cell mass of the mammalian blastocystic embryo.

FIG. 5. Effect of Aza and/or TSA on the reactivation of *cPouV* in EB cells. EB cells were treated with Aza (0, 1, 5, and 10 μ M), TSA (0, 100, 200, and 400 nM), or a combination of Aza (5 μ M) and TSA (200 nM) for 48 h. In real-time PCR, the β -actin was used as the internal control. The relative expression of *cPouV* in EG cells was normalized as 1. Means with different letters are significantly different ($p < 0.05$). Aza, 5-aza-2'-deoxycytidine; TSA, trichostatin A.

Alternatively, avian PSC lines, which are also named as EG cells, can be derived from postmigratory PGCs [23–26].

ES cells are mainly studied in mammals, initially in mouse, and increasingly in human. Despite the existence of PSCs in chick, the regulating mechanisms of pluripotency and differentiation have not been sufficiently established compared to its mammalian counterparts [27]. Available studies suggested that the epigenetic mechanism in the establishment and maintenance of the pluripotent state is an area of intense investigation in ESC biology. One of the best-known epigenetic factors is DNA methylation. It has been reported that many stemness-related genes switch from a demethylated and transcriptionally active state in embryonic cells to a fully methylated repressed state in somatic cells [28]. As another important mechanism for epigenetic regulation, histone modification consists of many types, such as acetylation, methylation, phosphorylation, and ubiquitination. Among these modifications, acetylation is the one so far more thoroughly analyzed. Histone hyperacetylation is associated with an open chromatin state and general transcriptional activity. Regarding its effect on chromatin accessibility and expression of pluripotency-related genes, it is plausible that histones H3 and H4 are both hyperacetylated in ES cells compared to differentiated cells [29].

In mammals, many studies demonstrated that pluripotency maintenance requires the proper expression of *Oct4* [30]. Undoubtedly, its appropriate expression is involved in a series of events. Among them, epigenetic mechanisms also play a critical role. According to recent studies, several regions in *Oct4* are subjected to epigenetic regulation. For instance, in vitro studies demonstrate that DNA methylation severely impairs the efficiency of reporter gene expression driven by the *Oct4* promoter in ES cells, which robustly express *Oct4* [31]. It has also been reported that hypermethylation of the *Oct4* promoter/enhancer region in mouse trophoblast cells correlated with gene silencing, whereas hypomethylation in ES cells allowed cells to maintain high levels of *Oct4* expression, thus keeping them in a pluripotent state [32]. Conversely, DNA demethylation of the *Oct4* locus has been routinely monitored in models of cell dedifferentiation, such as somatic cell nuclear transfer (SCNT) and iPS [33,34]. In addition to DNA methylation, previous studies also showed a progressive decrease of H3-Lys9 (H3K9) acetylation in the *Oct4* promoter region by ChIP analysis during differentiation [35]. On the other hand, studies about cell reprogramming provide further insights. Advances from SCNT and iPS demonstrated that the reprogramming process can be facilitated through the reactivation of *Oct4* after the treatment of chromatin-modifying agents, such as TSA, a histone deacetylase inhibitor [36].

Since both DNA methylation and histone modification can influence gene expression, it is of interest to clarify the relationship between them. Indeed, there is a heated debate about which event would be a dominant event in gene regulation. Some experiments supported that DNA methylation guide histone modifications; however, other studies show contradictory evidence [37]. Recently, high-throughput screening has provided insight into the characteristics and roles of DNA methylation. It demonstrated that promoters with a low CpG content (e.g., *Oct4*) were more likely to be methylated than those with a high CpG content (e.g., *Sox2*) in mammalian promoters [38]. More importantly, comprehensive mapping

of DNA methylation in gene promoters found that many genes are still expressed even if methylated in the PP, indicating that DNA methylation in a gene promoter may not be sufficient to silence gene transcription by itself [39]. Therefore, it has been speculated that DNA methylation may not constitute a primary mechanism of repression *per se*, but may be established following repression by other mechanisms. This is consistent with the report that dependence of DNA methylation on histone modifications has been clearly demonstrated in fungi and plants [40].

In addition to DNA methylation, epigenetic changes also include a diverse array of distinct covalent histone modifications with a pivotal role in gene expression. To date, acetylation of histones, notably histone H3, is probably the best understood type of histone modifications [41]. In contrast, histone methylation is more complex and may be present in mono-, bi-, and trimethylated forms [42]. Accumulated studies suggest that there is crosstalk between histone modifications and DNA methylation at some gene promoters. In a previous study, a TSA-induced increase of histone acetylation associated with a significant decrease in global DNA methylation was reported [43]. Similarly, in a differentiation system with retinoic acid, acetylation of H3K9 begins to be removed within 24 h after, whereas DNA methylation in the regulatory elements of *Oct4* occurs later, between 72 and 96 h after addition of drugs [44]. These findings indicated that perhaps the decreased acetylation level first affects the chromatin structure, and then the increased DNA methylation level makes the nonpluripotent state more stable. Meanwhile, evidences from histone methylation provided more insight. In mouse ES cells, deficiency in the histone H3K9 methyltransferases Suv39h1-Suv39h2 is associated with hypomethylation at a subset of repeat elements [45]. A direct link between H3 modifications and DNA methylation was originally demonstrated in neurospora, when DNA methylation was found to depend on trimethylation of histone H3 lysine 9 (H3K9me3) [46]. Upon ES cell differentiation, there is also evidence that the histone H3 lysine 9 methyltransferase G9a can determine DNA methylation in many promoters through recruitment of Dnmts to certain loci, including *Oct4* locus [47]. More recently, high-throughput molecular analysis identified that the status of DNA methylation in proximal gene promoters is highly correlated with both H3 K4 and K27 trimethylation [48]. Collectively, for the regulation of *Oct4* and other pluripotency-related genes, data available showed that histone modifications might not merely alter the chromatin structure, but might also provide unique binding surfaces for repressors and activators of transcription.

In summary, dynamic expression and involved epigenetic modification of *Oct4* have been investigated from numerous mammalian species, but never in a nonmammalian species. The present study provided the first evidences in a novel avian system that, during differentiation of chick EG cells, expression of *cPouV* decreased gradually accomplished by DNA methylation and histone deacetylation. The regulations of several epigenetic-related genes are responsible for these changes. Moreover, treatments of differentiated EBs with epigenetic modifiers promote the reactivation of *cPouV*. Our data demonstrated that dynamic epigenetic changes of poultry *cPouV* are involved in the pluripotency and differentiation of chick EG cells, just in accordance with the results

derived from that of the mammalian counterpart. Moreover, the present study also established a solid biochemical basis for genetic and epigenetic regulation of *Oct4* in stem cell pluripotency. All of these revealed that the role of *Oct4* in stem cell self-renewal, pluripotency, and somatic cell reprogramming is universal and fundamental.

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Author Disclosure Statement

The authors declare that no competing financial interests exist for this work.

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