Exploration of the temporal-spatial expression pattern and DNA methylation-related regulation of the duck telomerase reverse transcriptase gene

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ABSTRACT Telomerase reverse transcriptase (TERT) is a catalytic subunit of telomerase that adds TTAGGG repeats to the 3′-overhang of telomeres. In the present study, we detected that the duck TERT (dTERT) gene was highly expressed in small intestine and kidney, followed by heart, leg muscle, spleen, pancreas, gonad, and liver at neonatal stage. From embryonic to neonatal stage, the highest dTERT mRNA in liver appeared at stage E19 (19 days at embryonic stage), while for the leg muscle the maximum expression occurred at E26. We also measured the relative telomerase activity (RTA) and relative telomere length (RTL) in the examined tissues and found that the changed tendency of RTA and RTL was not very consistent with that of TERT. In silico analysis revealed that there were three CpG islands (S1, S2, and S3) within the 5′ regulatory region of the dTERT gene. Bisulfite sequencing PCR (BSP) assay showed that liver (D7, 7 days after birth) which expressed significantly lower dTERT mRNA had an obviously higher methylation level of S1 compared with small intestine (D7) or liver (E19). Quantitative real-time PCR analysis revealed that the expression of DNA methyltransferase DNMT1 in liver (D7) was significantly higher than that in small intestine (D7) or in liver (E19). In vitro, dTERT expression was upregulated and the methylation status of S1 decreased in both duck embryonic fibroblasts and small intestinal epithelial cells following treatment with the demethylation reagent, 5-aza-2′-deoxycytidine (5-aza-dC), further suggesting that dTERT is epigenetically regulated by DNA methylation. This work lays a solid foundation for further study of TERT function and regulation in avian species.

Key words: duck, TERT, DNA methylation, telomerase activity, telomere length

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INTRODUCTION

Telomerase reverse transcriptase (**TERT**), the catalytic subunit of telomerase, which adds TTAGGG repeats to the 3′-overhang of telomeres using telomerase RNA component (**TERC**) as an internal template (Mitchell et al., 2010), has been widely studied in many species, especially in human beings (Horikawa et al., 1999; Poole et al., 2001; Lewis and Tollefsbol, 2016; Zhang et al., 2016).

The human telomerase reverse transcriptase (**hTERT**) mRNA is repressed in most adult somatic tissues and cells, but highly expressed in early embryonic tissues with gradual reduction during development (Wright et al., 1996; Ulaner et al., 1998; Forsyth et al., 2002; Liu et al., 2004b; Cheng et al., 2013). However, hTERT expression can be detected in stem and germinal cells, as well as in ovary, testis, bone marrow, skin, and other self-renewing tissues, which all contain multipotent adult stem cells and/or germinal cells (Liu et al., 2004a; Zhang et al., 2016). In addition, about 90% of tumor tissues and cancer cells display high telomerase activity and hTERT expression (Meyerson, 2000; Granger et al., 2002). As inappropriate regulation of hTERT is often associated with harmful developmental consequences, numerous studies have focused on revealing the regulation mechanisms of hTERT expression.

Past research reported that both transcriptional factors and epigenetic alterations like histone modifications and DNA methylation were involved in the control of hTERT transcription (Zhu et al.,

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2010; Lewis and Tollefsbol, 2016; Ramlee et al., 2016; Zhang et al., 2016). DNA methylation is established through the DNA methyltransferases (**DNMT**: DNMT1, DNMT3A, and DNMT3B), which are capable of adding a methyl group to the fifth carbon atom of the cytosine residues within cytosine-phosphate-guanine (CpG) and non-CpG dinucleotides sites (Reik and Dean, 2001; Chen and Li, 2004). In general, DNA methylation is associated with gene repression; however, it can also act as an activator by preventing repressors from binding to their target DNA (Jones and Takai, 2001). Therefore, the actual role of DNA methylation in the regulation of hTERT expression is controversial. In the previous studies, some groups reported that methylation of the hTERT promoter led to a silence of the gene and treatment of cells with demethylating reagent resulted in an increase in hTERT transcription (Lopatina et al., 2003; Shin et al., 2003; Liu et al., 2004a,b). While others demonstrated that the hypermethylation in the regulatory region of hTERT was associated with increased hTERT expression in most cancer cells (Renaud et al., 2007; Meeran et al., 2008), and demethylation in several telomerase-positive tumor cell lines caused reduced hTERT expression and telomerase activity (Guilleret and Benhattar, 2003). The function of DNA methylation in the regulation of TERT activity became more confusing when several groups reported that there was no significant correlation between the methylation of the hTERT promoter and subsequent expression of the gene (Devereux et al., 1999; Dessain et al., 2000).

Unlike hTERT, the mouse TERT (**mTERT**) expression is found in most adult tissues and organs, and mouse cells have a higher frequency to undergo spontaneous immortalization in culture condition owing to the constitutive telomerase expression (Prowse and Greider, 1995; Rangarajan and Weinberg, 2003). Whereas, the domestic chicken contains many common characteristics as human in telomere biology, including similar telomerase and TERT repression, as well as telomeric shortening in vivo and in vitro (Forsyth et al., 2002; Delany and Daniels, 2004; Swanberg et al., 2004; Me and Se, 2005; O'Hare and Delany, 2005; Swanberg et al., 2010). Moreover, it is reported that several transcription factor binding sites in the promoter of chicken TERT (**chTERT**) were similar to those verified in the promoter of hTERT (Delany and Daniels, 2004), suggesting that bird shares similar genetic mechanisms as human in TERT regulation. However, to date there has been no report on the TERT study in duck which is another important avian species.

In the present study, we investigated the temporal-spatial distribution of duck TERT (dTERT) as well as the related telomerase activity and telomere length at early developmental stages. As the dTERT gene promoter has been cloned in our laboratory recently (unpublished data), the CpG island was predicted by an online tool MethPrimer (Li and Dahiya, 2002), and bisulfite sequencing PCR (**BSP**) assay was adopted to examine the correlation between methylation levels and dTERT expressions. We also studied the expression of DNA methylation-related genes (DNMT1, DNMT3A, and DNMT3B) in selected tissues. Finally, duck embryonic fibroblasts (**DEFs**) and small intestinal epithelial cells (**DIECs**) were treated with the methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-aza-dC) in vitro to further investigate the involvement of DNA methylation in dTERT regulation. This work provided solid data for understanding the tissue distribution and DNA methylation-related regulation of TERT in poultry.

MATERIALS AND METHODS

Ethics Statement

All animal procedures were performed in strict accordance with the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China) and the protocol was approved by Animal Ethics Committee of Zhejiang University (Hangzhou, China).

Prediction of CpG islands in the 5′-regulatory region

A CpG island is a segment of DNA with high GC and CpG dinucleotide contents. The CpG island of the duck TERT 5′-regulatory region was predicted by a bioinformatics software, MethPrimer (http://www.urogene.org/methprimer/index1.html) in this study.

Sample Collection, Cell Culture, and Treatment

More than 300 Shaoxing duck eggs from the same batch were incubated at 38°C and 70% relative humidity. Six embryos at each embryonic stage (E13 (13 days at embryonic stage), E19, E26) were randomly selected to collect liver and leg muscle samples. At neonatal stage (D7 (7 days after birth)), 6 male ducklings were also randomly selected to collect various tissues (heart, liver, spleen, kidney, small intestine, leg muscle, gonad, and pancreas). All samples were separated and frozen immediately in liquid nitrogen and then stored at −80°C for RNA, DNA, and protein extraction.

DEFs and DIECs were isolated and preserved in our lab previously. DEFs were grown in Dulbecco's Modified Eagle's medium (DMEM; GIBCO) with 10% fetal bovine serum (FBS; GIBCO), and 1% penicillin/streptomycin (GIBCO). While DIECs were cultured in DMEM/F12 (GIBCO) containing 5% FBS, 1% penicillin/streptomycin (Solarbio), 10 mM HEPES (Solarbio), 10 ng/ml Epidermal Growth Factor (EGF; Sigma), and 1% Insulin, Transferrin, Selenium Solution (ITS-G; GIBCO). Both cell types were

maintained at 37°C in a humidified 5% CO_2 incubator. Cells were treated with 5-aza-2′-deoxycytidine (5-aza-dC; 5, 10, 15 μM; Meilun) for 48 h. Untreated cells were used as the control group. DMSO concentrations were below 0.1% for all assays. All cells were harvested with Trizol Reagent (Takara) after treatments.

Quantitative Real-time PCR

Total RNA was extracted with Trizol Reagent. cDNA was synthesized with a HiScript II Q RT SuperMix for quantitative PCR (**qPCR**) (+gDNA wiper) reagent kit (Vazyme) according to the manufacture's protocol. Following reverse transcription, qPCR amplification was carried out with the gene specific primers listed in Table 1. Briefly, each qPCR reaction contained 10 μL of ChamQ Universal SYBR qPCR Master Mix (2×) (Vazyme), 0.5 μL of forward primer (10 μM), 0.5 μL of reverse primer (10 μM), 1.0 μL of template cDNA, and 8.0 μL of double-distilled water. The qPCR amplification was conducted in a LightCycler 96 Real-Time PCR System (Roche). The cycling conditions consisted of an initial single cycle of 95°C for 5 min, followed by 45 cycles of 95°C for 10 s, 60°C for 30 s, then a single cycle of 95°C for 10 s, 65°C for 60 s, 97°C for 1 s. All samples were examined in triplicate and the results were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Relative Telomerase Activity Detection by Real-time Quantitative Telomerase Repeat Amplification Assay

The conventional real-time quantitative telomerase repeat amplification assay (**RQ-TRAP**) assay was performed with some modifications (Hiyama et al., 1995; Wege et al., 2003; Jeon et al., 2011). Briefly, 40 to 100 mg tissues or 1×10^5 to 1×10^6 harvested cells were either quick frozen in liquid nitrogen and stored at –80°C for future analysis or immediately lysed in 200 μL of ice cold CHAPS Lysis Buffer [0.5% CHAPS, 10 mM Tris-HC1 (pH 7.5), 1 mM $MgC1_2$, 1 mM EGTA, 5 mMβ-mercaptoethanol, 0.1 mM AEBSF, 10% glycerol, 200 units/mL RNase inhibitor]. Tissues in CHAPS Lysis Buffer were homogenized with a mechanical homogenizer until a uniform consistency was achieved. After being incubated on ice for 30 min, the lysate was centrifuged at 16,000× g for 20 min at 4°C to remove debris. The protein concentration of each sample was measured by the BCA Protein Assay Kit (Meilun) and 5 μg of total protein was analyzed by RQ-TRAP. Primer sequences were listed in Table 1. Each reaction contained 10 μL of ChamQ Universal SYBR qPCR Master Mix (2×) (Vazyme), 0.8 μL of primer TS (10 μM), 0.4 μL of primer ACX (10 μM), and was adjusted to 20 μL of total volume with RNase-free water. The assay run consisted of 30-min incubation at 30°C, followed by 10-min incubation at 95°C and 40 cycles of 95°C for 10 s, 60°C for 30 s, then a single cycle of 95°C for 10 s, 65°C for 60 s, 97°C for 1 s. The qPCR amplification was conducted in the LightCycler 96 Real-Time PCR System (Roche). In addition to sample measurements, each run also included measurements of telomerase-positive control 293T cells and telomerase-negative control 293T cells inactivated by incubation at 85°C for 10 min. The telomerase activity was expressed relative to that of 293T cells.

Relative Telomere Length Measurement by Quantitative Real-time PCR

Telomere qPCR was performed as described in previous studies (Cawthon, 2002; Nasir et al., 2009; Reichert et al., 2017) with the following modifications. Genomic DNA was extracted from tissues according to the manufacturer's protocol using the Wizard Genomic DNA Purification Kit (Promega). Primers used to amplify the telomere and the single copy reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each sample are listed in Table 1. 20 μL of final volume per reaction containing 10 μL of ChamQ Universal SYBR qPCR Master Mix (2×) (Vazyme), 1 μL of each telomere primer (Tel1b, Tel2b, 10 μM) or 0.4 μL of each TelGAPDH primer (10 μM), 2 μL of template DNA (10 ng/μL), and double-distilled water. Telomere and GAPDH real-time amplifications were performed on two different plates in the LightCycler 96 Real-Time PCR System (Roche). Telomere qPCR conditions were 10 min at 95°C followed by 30 cycles of 10 s at 95°C and 30 s at 58°C. GAPDH qPCR started with 10 min at 95°C followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. Relative telomere length (**RTL**) was determined by the T/S ratio of the telomere product amplification (T), to the internal single copy reference gene GAPDH (S) (Cawthon, 2002).

BSP Assay

Genomic DNA was extracted from tissues and cells, and approximately 1 μg DNA of each sample was treated with sodium bisulfite using the EpiTect Fast DNA Bisulfite Kit (QIAGEN). The modified DNA samples were purified and diluted in 15 μL elution buffer for subsequent PCR amplification. PCR primers were designed based on the locations of the CpG islands (Table 1). The PCR reaction was conducted in a 30 μL volume containing 3 μL of 10X Taq buffer, 1 μL of dNTP (2.5 mM each), 1 μL of Taq polymerase (Takara), 1 μL of each primer (10 μM), 2 μL of bisulfite-treated DNA, and 21 μL of double-distilled water. The cycling conditions were as follows: an initial denaturation at 95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for

Table 1. Sequences of Primers.

Primer name	Sequence (5′-3′)	Primer usage	Product length (bp)
β-actin-F	TCGCTGACAGGATGCAGAAG	Real-time PCR	225
β-actin-R	TGGGTGTTGGTAACAGTCCG		
dTERT-F	TCACTTCCAAATCATGCCGC		238
dTERT-R	CGCTGGGGCTGATTCTACATT		
DNMT1-F	TGGGTTACCAGTGCACCTTC		219
DNMT1-R	GGAAGGGACCGGAATACGTC		
DNMT3A-F	GATGCCAAAGAGGTGTCTGCG		187
DNMT3A-R	CCTGCTTGATGGAGTTGGAGC		
DNMT3B-F	AATTACCCAGCCCAGGAAGC		151
DNMT3B-R	CCTCTACAGTCCGCGTTTGT		
TS	AATCCGTCGGAGCAGAGTT	Relative Telomerase Activity Real-time PCR	Uncertain
ACX	GCGCGGCTTACCCTTACCCTTACCCTAACC		
Tel1b	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT	Relative Telomere Length Real-time PCR	Uncertain
Tel2b	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT		
TelGAPDH-F	AACCAGCCAAGTATGATGACAT		52
TelGAPDH-R	CCATCAGCAGCAGCCTTCA		
S1-F	GTGTTGATTGTAGTTTAGGTAATTTT	Bisulfite Sequencing PCR	313
S1-R	ACACATCATCATAACCATTCTCTCC		
S2-F	ATTGGGGAGTTGGATAGTAG		226
S2-R	CTCCCCCRAATAAAAAACTA		
S3-F	TGTTGGTGGTATTGGTAGTATTG		291
S3-R	CCCCATACCTACTCCAAACTA		

40 s, and then a final extension at 72°C for 5 min. The PCR products were separated by electrophoresis using a 2.5% agarose gel and purified using a Gel Extraction Kit (Generay), then cloned into the pTG19-T Vector (Generay). A minimum of 10 colonies were selected for sequencing. Methylation data from BSP were analyzed using an online analyzer QUMA (Kumaki et al., 2008) to generate black-and-white circle diagrams that display the percentage of methylation at each CpG island.

Statistical Analysis

Differences between groups were analyzed with the unpaired, two-tailed Student *t*-test or one-way ANOVA followed by Tukey's multiple comparison tests (V22.0, SPSS Inc., IBM Corp, Armonk, NY, USA). The results were expressed as mean value with standard deviation (SD) of at least three independent experiments. $P < 0.05$ was considered statistically significant.

RESULTS

Temporal-spatial Expression of dTERT Gene

Real-time PCR was conducted to determine the duck TERT mRNA expression pattern; β-actin was employed as endogenous control for each sample. The results showed that dTERT mRNA was expressed in all the eight tested tissues (heart, liver, spleen, kidney, small intestine, leg muscle, gonad, and pancreas) at 7 days old (Figure 1A). Particularly, high expression levels of TERT were detected in small intestine and kidney, followed by heart, leg muscle, spleen, pancreas, and low levels were observed in gonad and liver.

We also found that the duck TERT mRNA expression showed different patterns in liver and leg muscle from embryonic to neonatal stages (E13, E19, E26, D7). During the liver development, gene expression of dTERT firstly increased from stage E13 to E19, then decreased sharply from E19 to E26 and reached the lowest level at stage D7 (Figure 1B). Whereas in the leg muscle, an obvious increase of dTERT expression was seen from stage E19 to E26, followed by a decrease from E26 to D7, the highest expression was observed at stage E26, which is just one or two days before hatching, and the other three stages had no significant differences in dTERT expression (Figure 1C).

Relative Telomerase Activity and Relative Telomere Length in Duck Tissues

We determined the relative telomerase activity (**RTA**) and relative telomere length (**RTL**) using a real-time qPCR protocol. At neonatal stage (D7), RTA was detected in all of the tested tissues, among which small intestine showed a significantly high level of telomerase activity. As for other organs, the activity was moderate in kidney, heart and pancreas, but quite low in liver and leg muscle (Figure 1D). The levels of RTA in liver and leg muscle both decreased markedly as embryogenesis progressed, from high at E13 to extremely low at D7 (Figure 1E and F).

RTL was also evaluated in tissues at neonatal stage, as well as in liver and leg muscle at different developmental stages. As shown in Figure 1G, the longest telomere length was shown in leg muscle, followed by

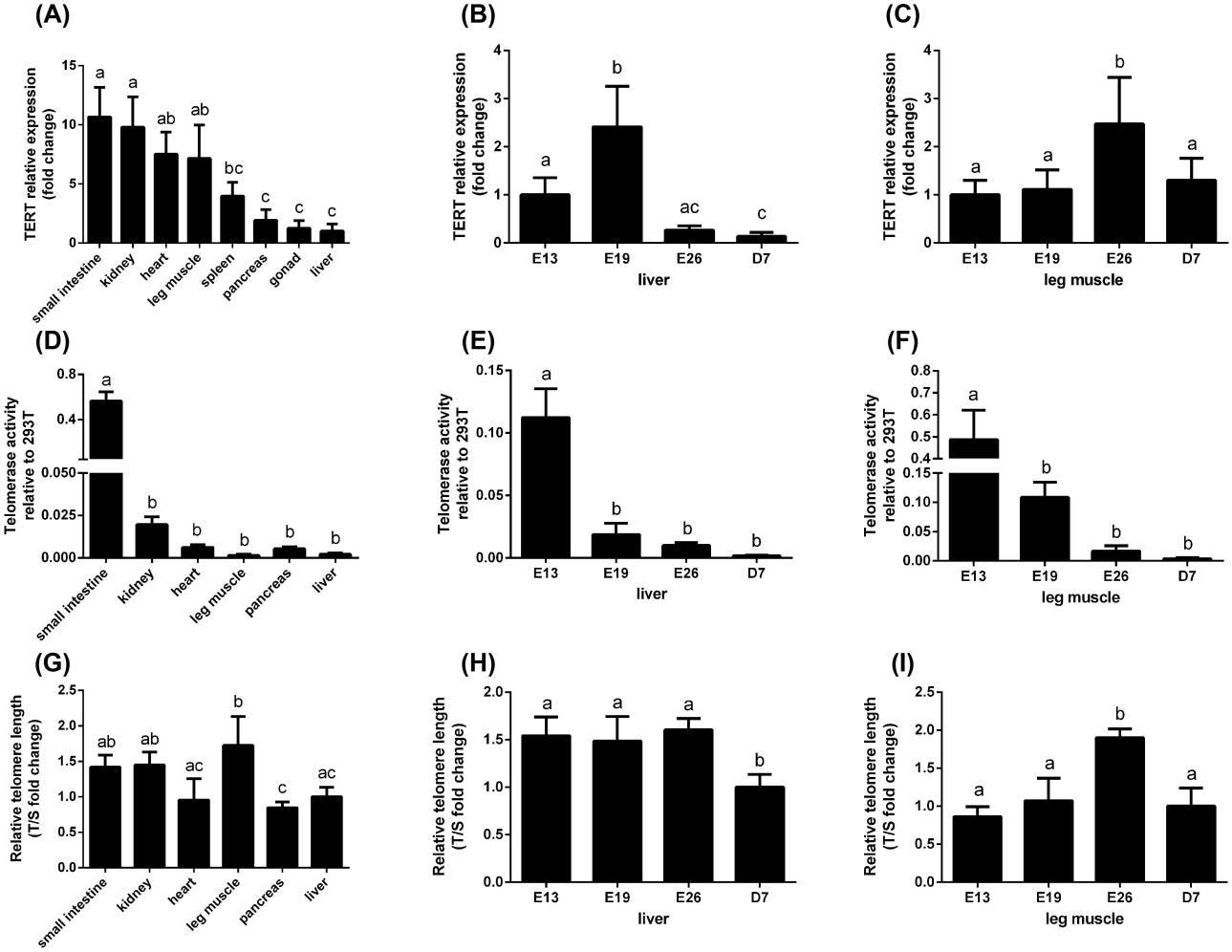

Figure 1. Detection of telomerase reverse transcriptase (TERT) expression, relative telomerase activity, and relative telomere length in duck tissues during early developmental stages. (A) Tissue distribution of duck TERT (dTERT) at neonatal stage. (B) Expression pattern of dTERT in liver from embryonic to neonatal stages. (C) Expression pattern of dTERT in leg muscle from embryonic to neonatal stages. (D) Relative telomerase activity in duck tissues at neonatal stage. (E) Relative telomerase activity in duck liver from embryonic to neonatal stages. (F) Relative telomerase activity in duck leg muscle from embryonic to neonatal stages. (G) Relative telomere length in duck tissues at neonatal stage. (H) Relative telomere length in duck liver from embryonic to neonatal stages. (I) Relative telomere length in duck leg muscle from embryonic to neonatal stages. dTERT mRNA expression, relative telomerase activity (RTA) and relative telomere length (RTL) were all assessed by real-time PCR and normalized to that of β-actin, RTA of 293T cells and GAPDH, respectively. Data were calculated as fold change over the respective control. The controls are liver (A), liver (E13) (B), and leg muscle (E13) (C), respectively, for dTERT expression detection; and liver (G), liver (D7) (H), leg muscle (D7) (I), respectively, for RTL measurement. The data were presented as means with SD (n = 4∼6). Bars with different letters differed significantly ($P < 0.05$).

kidney, small intestine, liver, heart, and pancreas. From embryonic to neonatal stages, the RTL in liver remained steadily high from E13 to E26 and decreased obviously at D7 (Figure 1H). In leg muscle, there was an increase from E13 to E26 and a significant reduction at D7 in RTL (Figure 1I).

Prediction of the CpG Islands

The CpG island of the dTERT promoter was predicted using the online software MethPrimer (http://www.urogene.org/methprimer/index1.html). As shown in Figure 2, there were 3 CpG islands in the 5′-flanking region of dTERT that contained 19, 16, 31 CG dinucleotide sites, respectively.

BSP Analysis

BSP assay was employed to identify whether the TERT expression was affected by DNA methylation in duck. Based upon the temporal-spatial expression of dTERT, we examined and compared the methylation status of 3 regions (S1, S2, S3; Figure 2) that contain abundant CpG sites from spatial and temporal perspectives, i.e., liver and small intestine at the same stage D7, and liver at different stages E19 and D7, which exhibited notable differences in dTERT expression (Figure 1A and B). As seen in Figure 3A, the methylation density of S1 was as high as 83.2% in liver (D7) which was accompanied by a low dTERT expression. In contrast, the methylation level of S1 was lower in small intestine (D7; 68.9%) and in liver (E19; 70.0%) together

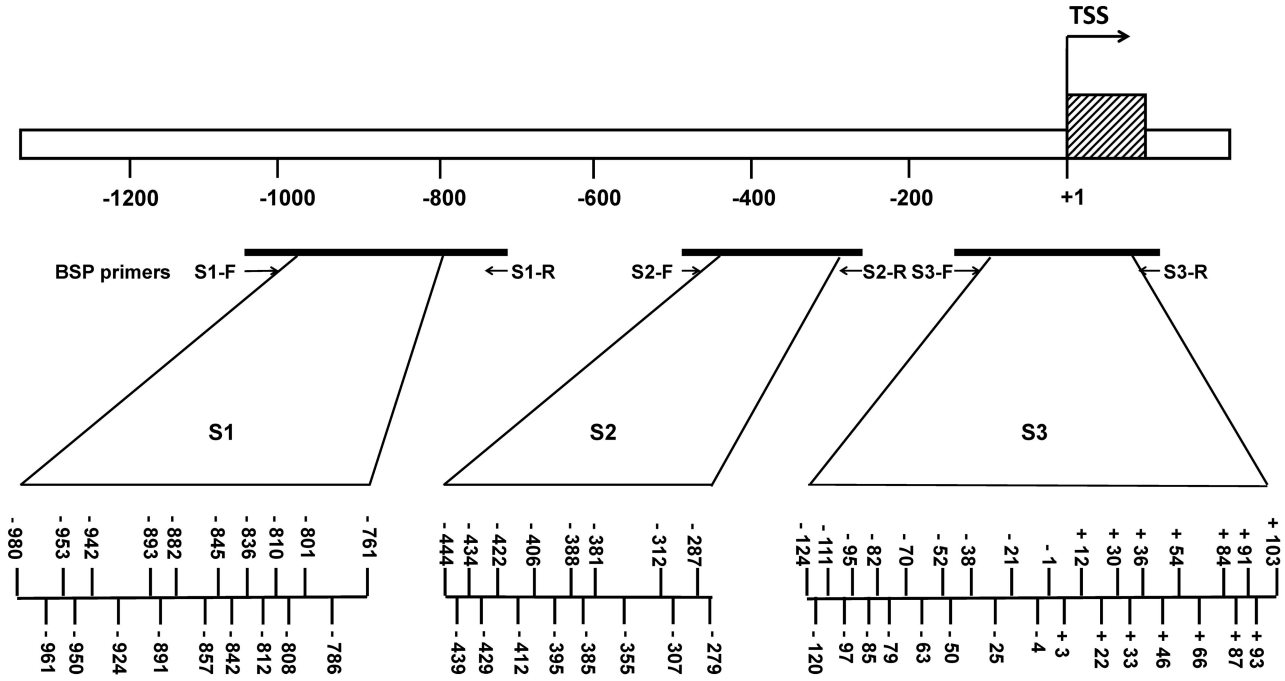

Figure 2. Schematic representation of CpG islands (S1, S2, S3) predicted by MethPrimer. Primer pairs used for BSP were indicated with arrows. Each horizontal line and corresponding number represents the position of CG dinucleotide site.

with an up-regulation of dTERT expression. However, the DNA methylation of S2 and S3 were not found in all the 3 samples (Figure 3B and C). Based on these data, the expression of dTERT might be negatively regulated by DNA methylation of region S1 in duck.

Comparison of DNA Methylation Related Genes Expression

To explore the potential mechanisms leading to the DNA methylation differences, we compared the transcriptional levels of DNA methylation related genes (DNMT1, DNMT3A, DNMT3B) between liver (D7) and small intestine (D7), as well as between liver (D7) and liver (E19), respectively. Quantitative real-time PCR results revealed that the expression of DNMT1 in liver (D7) was significantly higher than that in small intestine (D7) or in liver (E19). While for the DNMT3B, both small intestine (D7) and liver (E19) exhibited higher expression than liver (D7). In the case of DNMT3A, its mRNA level in liver (D7) was distinctly higher than that in small intestine (D7), but significantly lower than that in liver (E19) (Figure 4). These pieces of evidence suggest that DNMT1, but not DNMT3A or DNMT3B, might play a core role in the methylation of dTERT promoter.

Effect of Epigenetic Reagent on the dTERT Expression in Vitro

5-aza-dC has been widely used to study DNA methylation of gene because of its specific inhibition of the DNMT activity. To further verify the involvement of DNA methylation in the regulation of dTERT expression, DEFs and DIECs were treated with 5-aza-dC in different concentrations (5, 10, 15 μM) in vitro for 48 h. As shown in Figure 5, the dTERT mRNA levels were upregulated 2.2- to 2.6-folds in DEFs and 1.8- to 2.5-folds in DIECs, respectively. To further confirm the demethylation effect of 5-aza-dC, BSP analysis was performed on both treated cell lines. Overall methylation of the region S1 in dTERT promoter was significantly reduced in DEFs (78.8 to 68.4%) and DIECs (79.1 to 68.4%) treated with 10 μM 5-aza-dC (Figure 6). These results indicate that the transcription of TERT might be repressed by DNA methylation in duck.

DISCUSSION

The overall goal of this research was to characterize the tissue distribution and examine the possibility of DNA methylation involved in the regulation of TERT gene expression in duck.

Firstly, we investigated the temporal-spatial patterns of TERT expression at early development stages in duck. The TERT gene expression was ubiquitously present in all the examined tissues of 7-day-old ducks. Specifically, higher mRNA levels were detected in small intestine and kidney, while relatively low levels were found in gonad and liver, which is not very consistent with previous report on human and chicken that a subset of highly proliferating and self-renewal tissues like immune and reproductive organs always exhibited high TERT activity (Forsyth et al., 2002; Swanberg et al., 2010; Zhang et al., 2016). We inferred that the

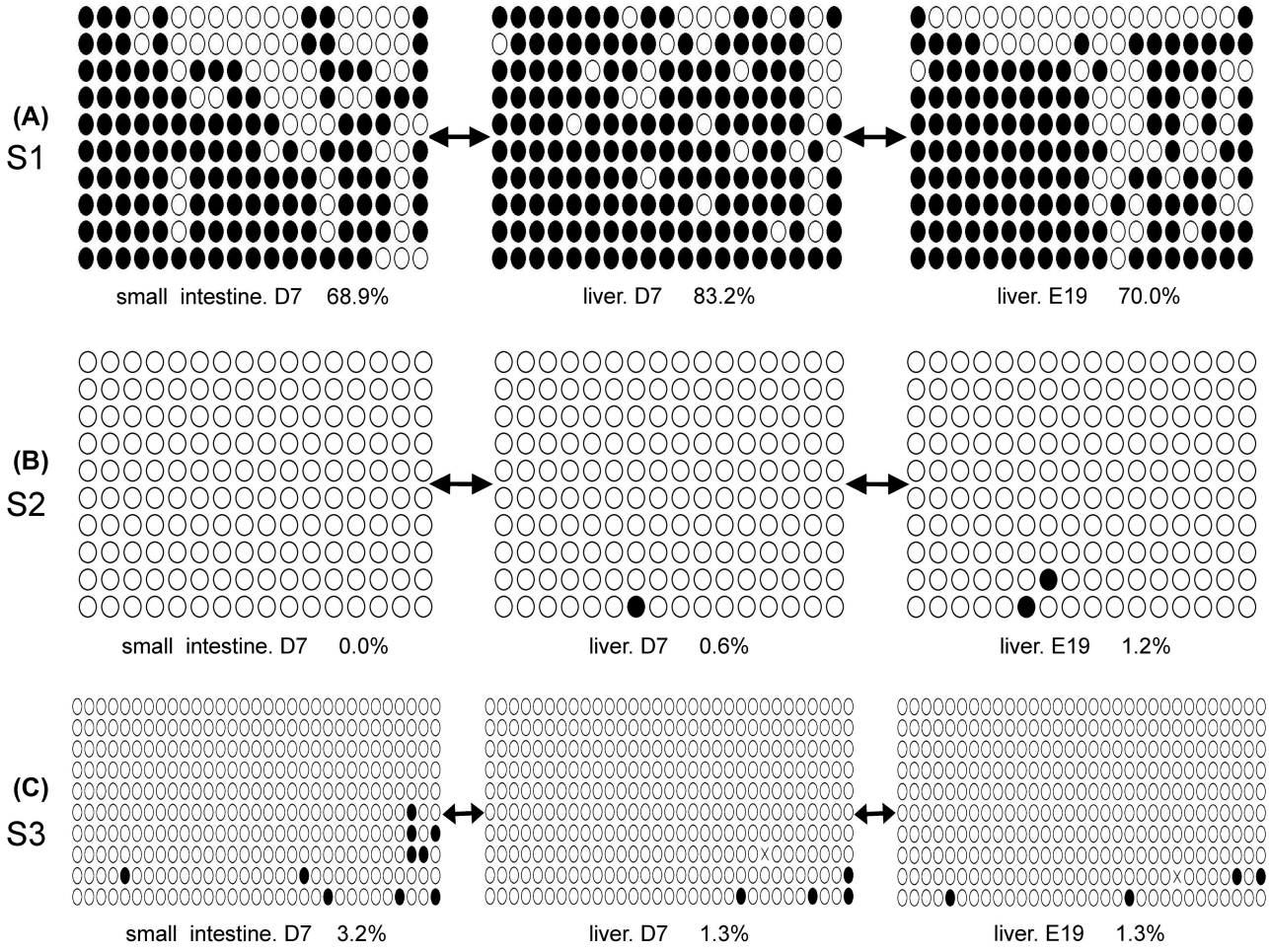

Figure 3. Bisulfite sequencing analysis of methylation status in 3 CpG islands (S1, S2, S3) of indicated tissues. Each CpG is indicated by a circle; each column represents the methylation status according to the sequences of 10 bacterial clones of the PCR product. Global percentage of methylated cytosines is shown as %. ○, unmethylated CpG; •, methylated CpG.

difference in TERT expression may result from the different developmental stages of the tissues we selected as well as the differences among species. We further examined the expression of TERT in liver and leg muscle from ducks at different developmental stages (E13, E19, E26, D7). In liver, dTERT transcription was firstly increased and then declined, with the highest level at stage E19 and lowest at stage D7. Whereas in leg muscle dTERT mRNA was most abundant at stage E26 but relatively low at other stages. Thus, the temporal expression patterns of dTERT in different tissues also varied. It has been reported that TERT overexpression was correlated with increased proliferative and regenerative potential of tissues and cells (Forsyth et al., 2002; Swanberg et al., 2010; Zhang et al., 2016); therefore, the tissue-specific expression patterns of dTERT are likely to be ascribed to the development characteristics and growth rate of the tissue.

TERT is an important component of telomerase and is suggested to be the rate-limiting factor for telomerase activity in human (Poole et al., 2001). Therefore, we examined the RTA in the corresponding TERT distributed tissues to determine the relationship between TERT expression and telomerase activity in duck. We found that the RTA was significantly high in proliferative tissues (small intestine), and decreased progressively from embryonic to postnatal stage in liver and leg muscle, which was consistent with the reports on human and chicken (Taylor and Delany, 2000; Forsyth et al., 2002). However, we noticed that the change trends of RTA were not very consistent with that of dTERT either in different tissues at neonatal stage or in liver and leg muscle throughout the early developmental stages. Considering the fact that both TERT and TERC are required for telomerase activity, we conjectured that in avian system the two main telomerase components might be equally important for maintaining telomerase activity, which might be different from the simple TERT-dependent model of telomerase activity developed for human. This assumption is supported by studies in chicken that the expression of TERT and TERC are tissue and cell specific, and both components are correlated with telomerase activity (Swanberg et al., 2004; Me and Se, 2005; O'Hare

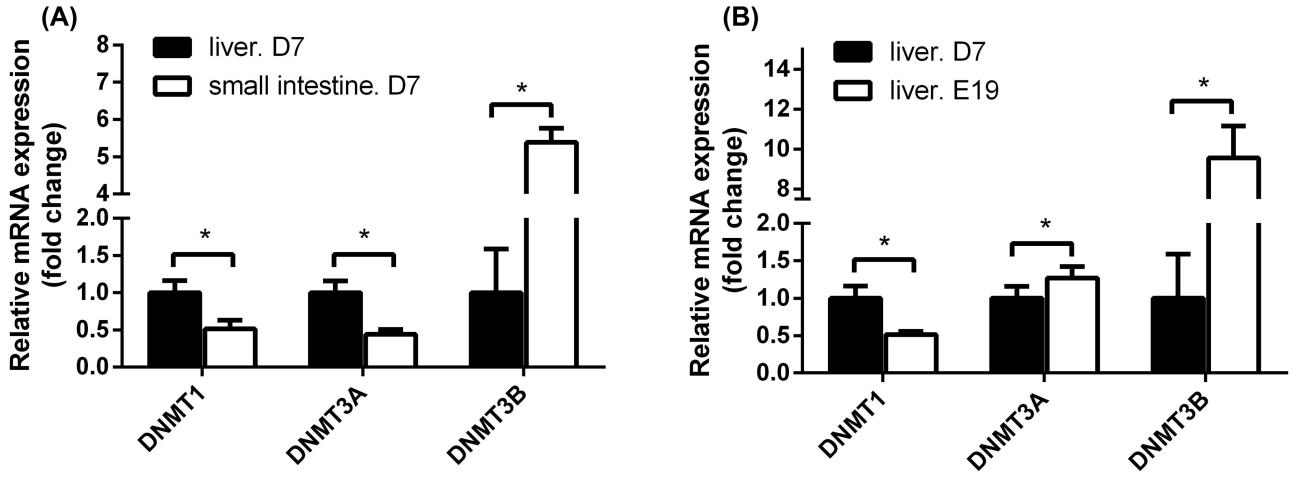

Figure 4. Comparison of relative mRNA levels of DNA methyltransferases DNMT1, DNMT3A and DNMT3B in indicated tissues. (A) liver (D7) vs. small intestine (D7); (B) liver (D7) vs. liver (E19). Each gene expression was assessed by real-time PCR and normalized to that of β-actin. Data were calculated as fold change over the control group (liver (D7)) and presented as means with SD (n = 6; $^{*}P < 0.05$).

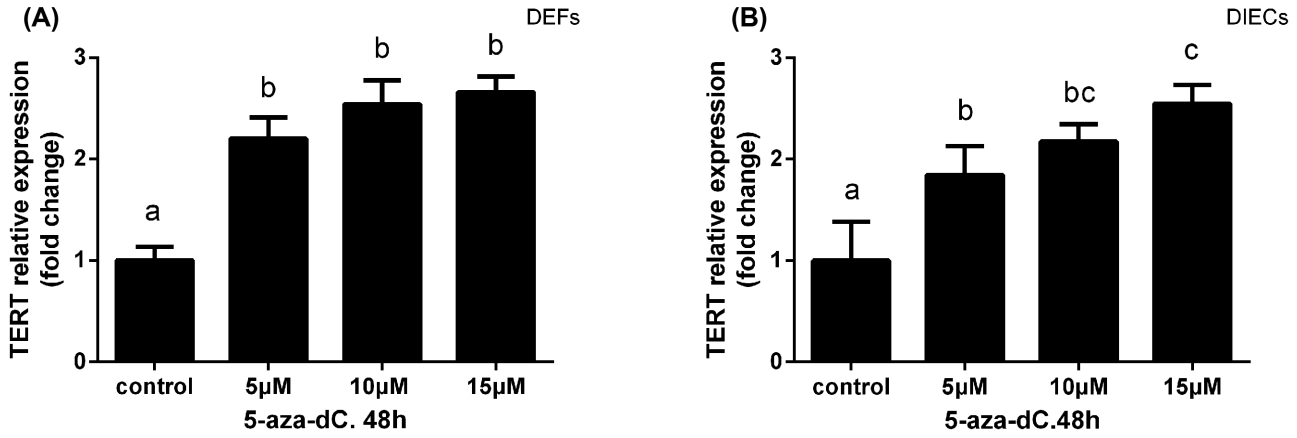

Figure 5. Upregulation of duck telomerase reverse transcriptase (dTERT) expression by the methyltransferase inhibitor, 5-aza-2′-deoxycytidine (5-aza-dC). Duck embryonic fibroblasts (DEFs) (A) and duck small intestinal epithelial cells (DIECs) (B) were treated with 5-aza-dC (5, 10, 15 μM) for 48 h. dTERT mRNA expressions were assessed by real-time PCR and normalized to that of β-actin. Data were calculated as fold change relative to the respective control and expressed as means with SD (n = 3). Bars with different letters differed significantly ($P < 0.05$).

and Delany, 2005). Further study is needed to define the relative contribution of each component in avian telomerase activity.

As telomerase lengthens telomere repeat sequences, we also detected the RTL in the examined tissues. During embryogenesis, the RTL kept steady or had a gradual increase in liver and leg muscle. We inferred that although the telomerase activity decreased progressively in these organs before hatching, it might be still high enough to elongate or compensate for the loss of telomeric repeats. While at postnatal stage, when the activity was present at trace level, the RTL reduced significantly in liver and leg muscle, which is consistent with human and chicken that the telomerase activity diminishes and the telomere shortens in most somatic tissues shortly after embryogenesis (Swanberg et al., 2010). At nenotal stage, the difference of RTL between most tissues was not significant. It is reported that in chicken the length of telomeric DNA became shortened with aging in most tissues, while in proliferative tissues with high telomerase activity such as intestine and gonad, telomeres were much longer than those in liver, brain, heart and kidney(Taylor and Delany, 2000; Sobn et al., 2006). Thus, we speculated that the obvious differences in telomere length between tissues would appear with aging.

Our previous study revealed that the 5′-regulatory region of duck TERT was CG-rich (unpublished data), which indicates that its transcriptional activity might be dependent on the methylation level of the CpG island (Bird and Wolffe, 1999). Thus, we sought to identify any associated CpG islands located in the promoter of the gene. Our in silico analysis revealed the presence of 3 putative CpG islands (S1, S2, and S3) in the 5′ end of the dTERT gene locus, which is similar to hTERT (Kyo et al., 2008). In order to explore whether the expression of dTERT is DNA methylation dependent, BSP assay was employed to measure the methylation status of the 3 CpG abundant regions in tissues which had significant differences in dTERT expression.

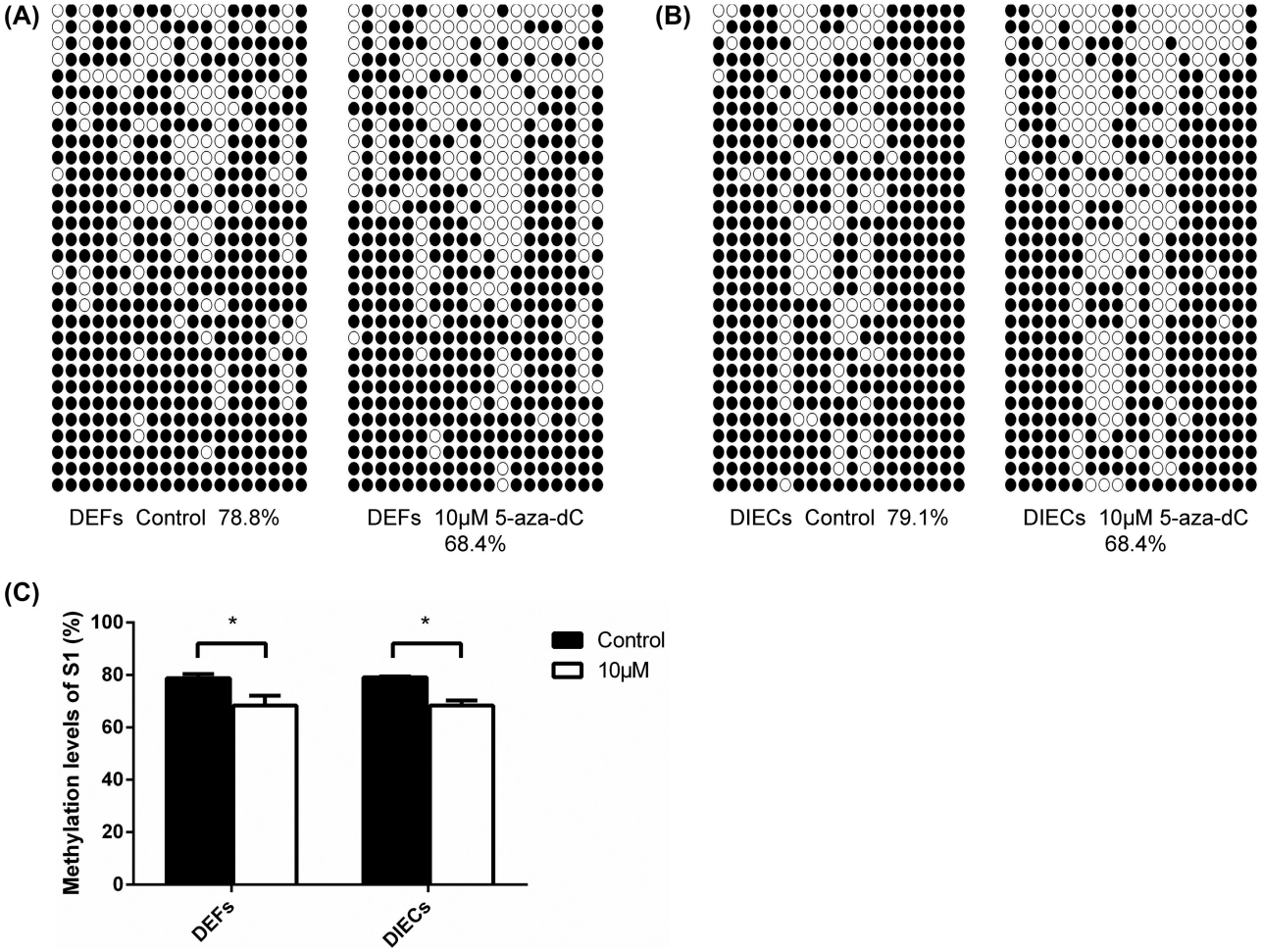

Figure 6. DNA methylation of the S1 region of duck telomerase reverse transcriptase promoter in duck embryonic fibroblasts and small intestinal epithelial cells treated with 10 μM 5-aza-2′-deoxycytidine (5-aza-dC). (A) DNA methylation patterns of S1 in duck embryonic fibroblasts (DEFs). (B) DNA methylation patterns of S1 in duck small intestinal epithelial cells (DIECs). (C) The methylation levels of S1 in two cell lines treated with 10 μM 5-aza-dC. Each circle represents a single CpG dinucleotide, and each line represents an individual bacterial clone. Global percentage of methylated cytosines is shown as %. ○, unmethylated CpG; •, methylated CpG. In the histogram, data were expressed as means with SD (n = 3; $^{*}P<0.05$).

It was found that, compared with liver (D7), both small intestine (D7) and liver (E19) exhibited obviously lower methylation levels of S1 as well as a higher dTERT expressions. Whereas S2 and S3 were not methylated in the above 3 tissues. DNA methylation is an important epigenetic phenomenon affecting gene expression without changing the DNA sequence. DNA methylation can act as an activator or repressor by preventing repressors or activators from binding to their target DNA (Jones and Takai, 2001; Wan and Bartolomei, 2008). Although many contradictory results about the role of DNA methylation in hTERT expression have been reported (Devereux et al., 1999; Dessain et al., 2000; Guilleret and Benhattar, 2003; Lopatina et al., 2003; Shin et al., 2003; Liu et al., 2004a,b; Renaud et al., 2007; Meeran et al., 2008), our data suggested that DNA methylation, particularly in region S1, may negatively regulate the transcription of the TERT in duck tissues.

DNA methylation is generally catalyzed by DNMTs. DNMT1, DNMT3A, and DNMT3B are 3 most important and well-studied methyltransferases, among which the DNMT1 enzyme has a major role in the maintenance of methylation and is responsible for restoring the methylated status of newly synthesized daughter strands, while DNMT3A and DNMT3B are involved in the de novo methylation of cytosine residues (Irvine et al., 2002; Uysal et al., 2015). We sought to explore the underlying mechanisms resulting in the differences of methylation status in the 3 selected tissues. The quantitative real-time PCR results indicate that it might be the DNMT1 that played a vital role in the methylation of dTERT promoter. In addition, in our study the demethylation reagent 5-aza-dC reactivated the expression of dTERT and reduced the methylation status of dTERT promoter in DEFs and DIECs in vitro, which is consistent with previous studies that treatment of cells with 5-aza-dC resulted in an increase in hTERT transcription (Devereux et al., 1999; Lopatina et al., 2003; Shin et al., 2003). Some studies reported that 5-aza-dC caused passive CpG demethylation through inhibition of DNMTs, especially DNMT1 (Robert

et al., 2003; Szyf, 2009). All in all, these results further verified that dTERT expression was epigenetically regulated by DNA methylation. Therefore, combining the pieces of evidence above, we postulated that DNMTs, possibly DNMT1, participate in a negative regulation of dTERT transcription through methylation of the CpG island of region S1 in the promoter. However, the potential specific mechanism of methylation in duck TERT regulation requires further study.

In summary, we firstly found that the expression patterns of dTERT varied at different developmental stages and tissues, and then we examined the corresponding relative telomerase activity and relative telomere length. Subsequently, we demonstrated that the varied expression of dTERT was regulated by different methylation status of its promoter. Moreover, we also revealed that the methyltransferase DNMT1 might play an important role in the methylation process. Our findings provide new knowledges to better understand the function and regulation of TERT in poultry species.

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