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DNA methyltransferase 3a is Correlated with Transgene Expression in Transgenic Quails

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ABSTRACT

DNA methyltransferases (*DNMTs*) are closely associated with the epigenetic change and the gene silencing through the regulation of methylation status in animal genome. But, the role of *DNMTs* in transgene silencing has remained unclear. So, we examined whether the knockdown of DNMT influences the reactivation of transgene expression in the transgenic quails. In this study, we investigated the expression of *DNMT3a*, and *DNMT3b* in blastoderm, quail embryonic fibroblasts (QEFs) and limited embryonic tissues such as gonad, kidney, heart and liver of E6 transgenic quails (TQ2) by RT-PCR. We further analyzed the expression of *DNMT3a* at different stages of whole embryos during early embryonic development by qRT-PCR. *DNMT3a* expression was detected in all test samples; however, it showed the highest expression in E6 whole embryo. Embryonic fibroblasts collected from TQ2 quails were treated with two *DNMT3a*-targeted siRNAs (siDNMT3a-51 and siDNMT3a-88) for RNA interference assay, and changes in expression were then analyzed by qRT-PCR. The siDNMT3a-51 and siDNMT3a-88 reduced 53.34% and 64.64% of *DNMT3a* expression in TQ2 QEFs, respectively. Subsequently the treatment of each siRNA reactivated enhanced green fluorescent protein (EGFP) expression in TQ2 (224% and 114%). Our results might provide a clue for understanding the DNA methylation mechanism responsible for transgenic animal production and stable transgene expression.
(**Key words :** Transgenic quail, *DNMTs*, *DNMT3a*, EGFP, RNA interference)

INTRODUCTION

DNA methylation is the first recognized and most well-characterized epigenetic phenomena. In vertebrates, the addition of a methyl group to the 5-position of the cytosine nucleotide in the CpG sequence to form 5-methylcytosine (m5C) is called DNA methylation. Such DNA methylation mainly occurs in the CpG Island, CG rich region, and promoters (Fatemi et al., 2005; Bernstein et al., 2007). DNA methylation regulates expression of endogenous genes during embryo development including genomic imprinting, X-chromosome inactivation and tissue-specific differentiation and induces silencing of transgene such as retroviral genome and retrotransposable element (Li and Reinberg, 2011). A disruption of DNA methylation often causes tumorigenesis and abnormal embryonic development (Li et al., 1992; Okano et al., 1999; Feinberg and Tycko, 2004; Ballestar and Esteller, 2008).

DNA methyltransferase (*DNMT*) catalyzes the modification at m5C. There are 4 *DNMTs* (*DNMT1, DNMT3a, DNMT3b,* and *DNMT3L*) that have been reported in mammals. During DNA replication, *DNMT1* delivers information ofparental DNA methylation to daughter, thereby recognizing and methylating hemimethylated CpGs. *DNMT3a* and *DNMT3b* are known as *de novo* methyltransferases which are related with silencing of retrotransposon, methylating unmethylated CpGs to initiate methylation (Chen and Li, 2004). Although *DNMT3L* does not have methyltransferase activity, it is closely related with genomic imprinting. Instead of its absence, *DNMT3L* links between *DNMT3a* and *DNMT3b* and reinforces them (Kinney and Pradhan, 2011). *DNMT1*, 3a, and 3b were also expressed in chickens during early embryonic development. Chicken *DNMTs* play similar role as the mammalian *DNMTs* in methylation establishment (Champagne, 2011; Chedin, 2011; Rengaraj et al., 2011).

It has been reported that long terminal repeats (LTRs) of Rous sarcoma viruses (RSVs) have strong promoter activity in various types of cells (Gorman et al., 1982). Production of

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transgenic animals with a high induction of transgene expression has been reported by using RSV promoters (Overbeek et al., 1986; Zhang et al., 1990). RSV promoter has also been utilized for transgene expression in birds. In previous research, transgenic quails and chickens that expressed enhanced green fluorescent protein (*EGFP*) driven by the RSV promoter have been reported (Koo et al., 2004; Shin et al., 2008; Kwon et al., 2010). However, variations in the transcriptional activity of the promoter among tissues and organs in transgenic animals have been a major problem (Overbeek et al., 1986). Likewise, the *EGFP* expression was diverse in the transgenic birds among tissues (Mizuarai et al., 2001). This diversity in transgene expression was controlled by CpG methylation status of RSV promoter in chickens (Park et al., 2010). Moreover, alleviating CpG methylation of the RSV promoter by 5-aza-2'-deoxycytidine (5-azadC, inhibitor of *DNMTs*) reactivated the transgene expression (Jang et al., 2011).

In this study, we hypothesize a specific *DNMT* modulates transgene expression at specific stages or tissues. To prove it, we first investigated the expression of *DNMTs* in quail cells and embryos. Next, we designed the sequence for the RNA interference of *DNMT3a* in quail. Finally, we analyzed the knockdown effect of *DNMT3a* on the silencing of transgene. Through these studies, we described here the elevated *EGFP* expression in embryonic fibroblast of transgenic quails by quail *DNMT3a* knockdown.

MATERIALS AND METHODS

1. Animal care and general experimental procedures

The care and experimental use of Japanese quails (*Coturnix japonica*) was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). Japanese quails were maintained in a standard management program at the University Animal Farm, Seoul National University, Korea. Procedures involved animal management, reproduction, and embryo manipulation adhered to the standard operating protocols of our laboratory. Eggs were brought to the laboratory within 1 to 3 h of oviposition for stage X embryos. Developing embryos under a relative humidity of 60~70% at 37.8°C were staged according to the Hamburger and Hamilton (HH) classification. The production of transgenic quails has been described in previous reports, and homozygous transgenic quail line (TQ2) was used throughout this study.

2. Culture of quail embryonic fibroblasts

We retrieved quail embryonic fibroblasts (QEFs) from embryonic day 6 (E6) TQ2 quail embryos using our standard procedure. Embryos were freed from the yolk by rinsing with calcium- and magnesium-free phosphate-buffered saline (PBS), and the embryonic bodies were retrieved after removal of embryo heads, arms, legs, tails, and all internal organs with sharp tweezers under a stereomicroscope. The embryonic bodies were collected from a total of five embryos. The bodies were dissociated by gentle pipetting in 0.05% (v/v) trypsin solution supplemented with 0.53 mM *EDTA*. The QEFs were then cultured in Dulbecco's modified Eagle's medium (*DMEM*; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific, Inc.), 2 mM l-glutamine (Invitrogen, Carlsbad, CA, USA), 0.1 mM MEM nonessential amino acids (Invitrogen), and 1% antibiotic-antimycotic (Invitrogen) in a 5% CO_2 atmosphere at 37°C. The expression of enhanced green fluorescent protein (*EGFP*) was visualized under a fluorescent microscope (Carl Zeiss, Oberkochen, Germany).

3. 5-Aza-2'-deoxycytidine treatment

Primary cultured QEFs were seeded at a density of 5.2×10^4 cells/cm^2. The cells were treated with 50 μM of 5-azadC (Sigma-Aldrich, St. Louis, MO, USA) for 48 h, and then, 5-azadC was withdrawn and the QEFs were continuously cultured under normal culture condition.

4. Knockdown assay

The quail *DNMT3a* knockdown probes were synthesized by Bioneer Inc. (Daejeon, Korea) (Table 1) and no complementary sequence in the chicken genome was used as a control for the knockdown experiments. For transfection, liposome mediated gene transfer was performed according to manufacturer's procedure (Invitrogen). Briefly, after culture media removal, TQ2 QEFs were resuspended at 2×10^5 cells per 250 μl Opti-MEM. Mixture of knockdown probes and lipofectamin 2000 solution that was incubated for 20 min was added into resuspended TQ2 QEFs, and the treated QEFs were cultured as mentioned above.

Table 1. List of quail *DNMT3a*-specific siRNA sequence for RNA interference

Candidate siRNA	Target sequence	Strand	Designed siRNA sequence (5'-3')	Location[a]
siDNMT3a-51	AAAGAAGTTTACACAGAGATG	Sense	AGAAGUUUACACAGAGAUGtt	1299
		Antisense	CAUCUCUGUGUAAACUUCUtt	
siDNMT3a-88	AAATGTCACCCAGAAACACAT	Sense	AUGUCACCCAGAAACACAUtt	2066
		Antisense	AUGUGUUUCUGGGUGACAUtt	

[a] Location refers to the first nucleotide of the target sequence in the cloned quail *DNMT3a* sequence.

5. Reverse transcription PCR and Quantitative Real-Time PCR

RNA extraction, cDNA synthesis, reverse transcription PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR) were performed according to our previous report (Lee et al., 2010; Seo et al., 2010). To estimate time-dependent expression and the effect of gene silencing, total RNA samples were extracted from cultured cells and whole TQ2 embryos during early embryonic development (StageX, E5, E6, E7, E8, and E9). Next, 1 mg of total RNA from the samples was used to create single-stranded cDNA using the Superscript III First-Strand Synthesis System (Invitrogen). Sequence-specific primers based on chicken *DNMTs* and *GAPDH* (Table 2) were designed using the Primer3 program (http://frodo.wi.mit.edu/). Real-time PCR was performed using the iCycler iQ real-time PCR detection system (Bio-Rad) and EvaGreen (Biotium, Hayward, CA, USA). Non-template wells without cDNA were included as negative controls. Each test sample was run in triplicate. The PCR conditions were 94°C for 3 min, followed by 40 cycles at 94°C for 30 sec, 59~61°C for 30 sec, and 72°C for 30 sec, using a melting curve program (increase in temperature from 55 to 95°C at a rate of 0.5°C per 10 sec) and continuous fluorescence measurement. The results are reported as the relative expression after normalization of the transcript to *GAPDH* (endogenous control) and the nonspecific control as a calibrator using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

RESULTS

1. *DNMTs* expression in quail embryo

In our previous study, we observed that *EGFP* expression was increased in TQ2 QEFs when 5-azadC decreased methylation of RSV promoter. Thus, we hypothesized that *DNMTs* acts as a silencing factor of the transgene in TQ2 transgenic quails. To prove the hypothesis, we tested *DNMTs* expression in blastoderm, limited embryonic tissues (gonad, kidney, heart and liver) at E6 and QEFs derived from E6 TQ2 transgenic quail embryos. *DNMT3a* was strongly expressed in all the test samples (blastoderm, gonad, kidney, heart, liver, and QEFs). *DNMT3b* expression was detected in all the test samples except blastoderm (Fig. 1).

Further, we investigated the quantitative expression of *DNMT3a* mRNA in several samples from whole TQ2 embryos during early embryonic development (StageX, E5, E6, E7, E8, and E9). Quail *DNMT3a* showed highest expression level particularly at E6 embryos by qRT-PCR (Fig. 2). These results suggest that *DNMT3a* might act as a

Table 2. List of PCR primers used to examine the expression of *DNMTs, EGFP* and *GAPDH*

Primer	Direction	Sequences	Size (bp)
DNMT1 (NW_001475597)	Forward	CTGAGATGCCCTCCCCCAAG	454
	Reverse	GTCCTCCCGTCGTCCTCCAC	
DNMT3a (NC_006090)	Forward	GCAAGCAGCAGAGCAGGGAA	577
	Reverse	CCACCAACAGGTCCACGCA	
DNMT3b (NC_006107)	Forward	GAACCCAGCCACCTTCCACC	547
	Reverse	AGTGATGTTGCCCTCGTGCC	
EGFP (GQ404376)	Forward	CTTGTACAGCTCGTCCATGC	410
	Reverse	ACGACGGCAACTACAAGACC	
GAPDH (NC_006088)	Forward	GGTGGTGCTAAGCGTGTTAT	453
	Reverse	ACCTCTGTCATCTCTCCACA	

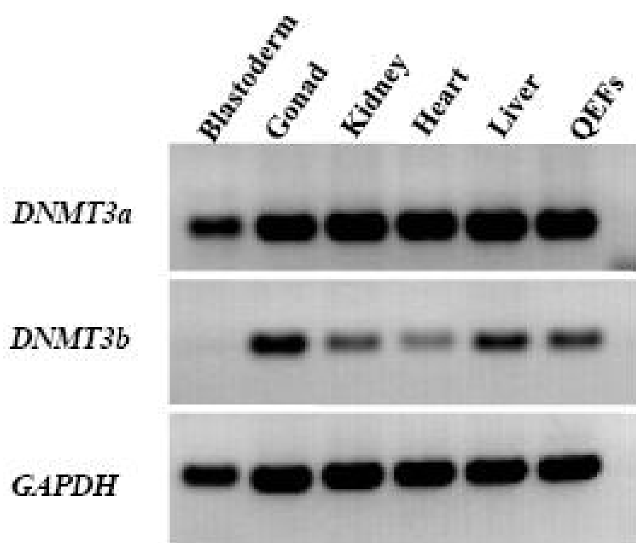

Fig. 1. Expression analysis of *DNMT* family members (*DNMT3a* and *DNMT3b*) in TQ2 transgenic quail blastoderms, limited series of tissues (gonad, kidney, heart and liver) at E6 and QEFs by RT-PCR.

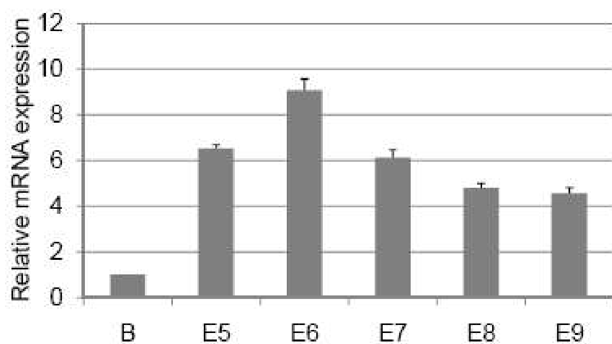

Fig. 2. Expression of *DNMT3a* in early embryos of TQ2 transgenic quails by real-time PCR (mean ± SEM; n=3). The relative mRNA expression of *DNMT3a* was normalized with the expression of *GAPDH*. B: quail blastoderm, E: embryonic day.

major *DNMT* among *DNMTs* in TQ2 QEFs derived from E6 embryos.

2. *DNMT3a* knockdown and *EGFP* expression in TQ2 QEFs

To investigate the correlation between *DNMT3a* and *EGFP* expression, we constructed two *DNMT3a*-targeted siRNAs (siDNMT3a-51 and siDNMT3a-88) for *DNMT3a* gene knockdown (Table 1). When the siRNAs were treated with TQ2 QEFs, siDNMT3a-51 and siDNMT3a-88 showed 53% and 64% knockdown efficiency by qRT-PCR, respectively (Fig. 3A). And then the transcriptional level of EGFP was increased up to 224% and 114% in siDNMT3a-51 and siDNMT3a-88, respectively when treated with TQ2 QEFs (Fig. 3B). The increased *EGFP* expression in siDNMT3a-51 and siDNMT3a-88 treated TQ2 QEFs were observed under fluorescence microscope (Fig. 4). When TQ2 QEFs were treated with 50 μM 5-azadC, 5-azadC treatment showed similar *EGFP* expression compared with *DNMT3a* knockdown in TQ2 QEFs (Fig. 4). These data propose that, among *DNMTs*, *DNMT3a* can suppress *EGFP* expression in TQ2 QEFs.

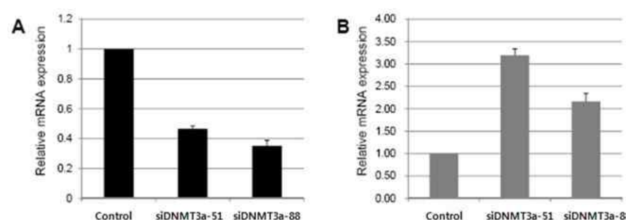

Fig. 3. Increase of *EGFP* expression in TQ2 QEFs by *DNMT3a* knockdown. (A) Knockdown efficiency of *DNMT3a* in TQ2 QEFs by lipofection. (B) *EGFP* expression in TQ2 QEFs after *DNMT3a*-knockdown. Relative expression of *DNMT3a* and *EGFP* were analyzed by qRT-PCR (mean ± SEM; n=3). The relative mRNA expression of *DNMT3a* and *EGFP* were normalized with the expression of *GAPDH*. Control: siRNA which have no complementary sequence in chickens.

DISCUSSION

Since transgenesis methods introduced retroviral and lentiviral system, they have continuously evolved and contributed tremendously to implementing gene therapy and transgenic animal production. HIV-1-based lentiviral vectors were once acknowledged to be not easily silenced, but it has become apparent that genes delivered by lentiviral vectors can be silenced (Hotta and Ellis, 2008; Pearson et al., 2008; Escors and Breckpot, 2010). After genomic integration of transgenes, epigenetic modifications cause silencing of the transgenes. DNA methylation of the epigenetic modifications is linked to transcriptional silencing, and is important for gene regulation, development, and tumorigenesis (Li et al., 1992; Feinberg and Tycko, 2004; Li and Reinberg, 2011).

In our previous research, we also established transgenic

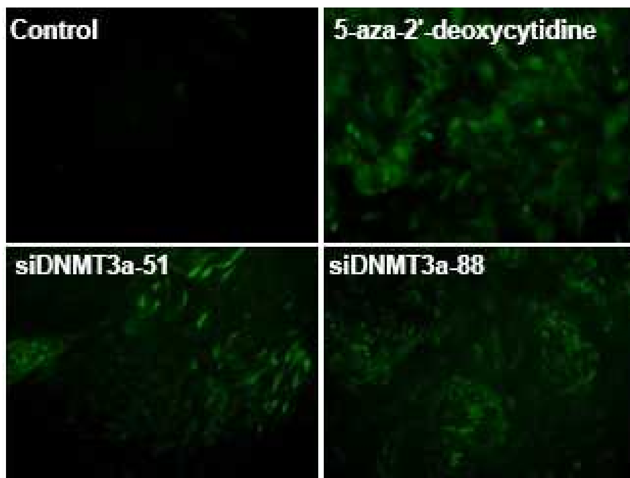

Fig. 4. *EGFP* expression in TQ2 QEFs after *DNMT3a* knockdown and treated with 5-aza-2'-deoxycytidine. *EGFP* expressionin TQ2 was detected under fluorescent microscopy. Control: siRNA which have no complementary sequence in chickens.

quails and chickens that expressed *EGFP* driven by the RSV promoter. However, the *EGFP* expression was varied in the transgenic birds among tissues, and even among the same types of cells in the same tissues (Shin et al., 2008; Kim et al., 2010; Park et al., 2010). We also revealed that this variation in transgene expression was modulated by CpG methylation status of RSV promoter in chickens (Park et al., 2010). In addition, we showed that alleviating CpG methylation of the RSV promoter reactivated the transgene expression (Jang et al., 2011).

In this study, QEFs expressed *DNMT3a* and 3b (Fig. 1). Especially, *DNMT3a* showed stronger and more ubiquitous expression than *DNMT3b* in the analyzed tissues. And *DNMT3a* from whole embryo was also strongly expressed during early embryonic stages (Fig. 2). Actually, *DNMT3a* is one of the *de novo* methyltransferases correlated to silencing of retrotransposon, methylating unmethylated CpGs to initiate methylation to protect host genome against transgene (Okano et al., 1999; Chedin, 2011; Kinney and Pradhan, 2011). In the present investigation, we demonstrated knockdown of *DNMT3a* increased EGFP expression in TQ2 QEFs. Moreover, TQ2 QEFs showed similar EGFP expression between knockdown of *DNMT3a* and 5-azadC treatment which inhibits all types of *DNMTs* (*DNMT1*, 3a, and 3b) (Fig. 4). These results suggest that *DNMT3a* might mainly establish DNA methylation during these stages in quail embryos. Thus, we recommend that *DNMT3a* is a strong candidate to control transgene silencing in quails or its ortholog species.

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