Estrogen suppresses *bcl2* expression through *dnmt1* in Japanese eel ovarian follicle

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Abstract

Japanese eels are commercially valuable species in Asian aquaculture. This study evaluated whether salmon pituitary extract (SPE) or 17 β -estradiol (E₂) treatment can induce cytotoxic activity in eel ovarian follicles. Follicular cells died after exposure SPE for 24-h culture in an *in vitro* culture. Moreover, the E₂ treatment also significantly reduced follicular cell counts. These results reveal that the inhibition of follicular cell numbers by SPE may occur through SPE-induced steroidogenesis. Results of a quantitative PCR analysis indicated that adding E₂ to the culture decreased *bcl2* and increased *dnmt1* mRNA expression in Japanese eel follicular cells after 24 h. The results of a promoter assay revealed that E₂ significantly increase *dnmt1* promoter activity through estrogen receptor-binding site. An *in silico* analysis predicted several putative transcription factors targeting the *bcl2* gene promoter region. Methylation of the *bcl2* promoter accounted for the downregulation of *bcl2* by E₂-mediated *dnmt1*. The DNA methylation level of the *bcl2* gene was significantly higher in E₂-treated follicular cells than that in the control group. Finally, the E₂-induced hypermethylation pattern of the *bcl2* promoter and the reduction in follicular cell numbers were suppressed by adding an MTase inhibitor. Our findings demonstrate that estrogen has a negative effect on the reproductive system of female eels by regulating an epigenetic mechanism during artificial maturation.

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Introduction

Oogenesis is the biological process that generates mature reproductive cells and requires sequential growth processes that are controlled by a wide variety of endocrine factors (Pati & Habibi 2000). Japanese eel (*Anguilla japonica*) is one of the most important commercially cultured species in Taiwan and East Asian countries. Wild immature Japanese eels do not undergo gonadal development under captive conditions, but can be repeatedly induced by the administration of exogenous salmon pituitary extract (SPE) to obtain fertilized eggs and produce viable offspring (Yamamoto & Yamauchi 1974, Ijiri *et al.* 1998, Tanaka *et al.* 2001). Our previous study reported that SPE treatment was an effective and indispensable treatment for artificially hastening vitellogenesis during oocyte maturation (Wang & Lou 2006). In contrast, the SPE or 17 β -estradiol (E₂) treatment significantly reduces total oocyte numbers by increasing the rate of follicular cell apoptosis (Wang & Lou 2007). E₂ is produced by follicle-stimulating hormone (FSH)-induced ovarian steroidogenesis and plays an important role in sexual maturation and differentiation, including vitellogenesis and oogenesis through mechanisms mediated primarily by estrogen receptors (ERs). The role of follicular apoptosis throughout vitellogenesis and

oogenesis has been investigated in teleost reproduction (Janz & Van Der Kraak 1997, Wood & van der Kraak 2002, 2003). Increasing evidences suggests that members of the Bcl2 protein family, including the anti-apoptotic factor Bcl2 and proapoptotic factor Bax, are the critical regulators of mitochondrial cytochrome c release (Williamson *et al.* 1998, Martinou & Youle 2011, Zhang *et al.* 2012). Studies in mammals indicated that follicular cell apoptosis is affected by the change in the Bcl2–Bax expression ratio, which alters oocyte quality in the reproductive process (Liu *et al.* 2000, Yang & Rajamahendran 2002, Hussein 2005).

DNA methylation is an important epigenetic modification of chromosomes that plays a significant role in the regulation of gene transcription and is crucial for development and differentiation (Vanselow *et al.* 2010, Ding *et al.* 2013). DNA methylation is catalyzed by a family of specific enzymes, DNA methyltransferases (MTase), which transfer a methyl group at the N6 position of adenine or N4 or C5 position of cytosine. The C5-cytosine MTase is the most prevalent enzyme in mammals and other vertebrates. According to functional and structural similarities, C5-cytosine MTases have been grouped into several subfamilies (dnmt1–7 and orphans) that share a catalytic domain in eukaryotes (Chen & Li 2004, Ponger & Li 2005). Furthermore, the dnmt1

subfamily includes different classes of MTases: DNMT1 from chordates, MET1 and CMT from plants, DIM2 from *N. crassa* and *M. grisea*, Msc2 from *A. immerses*. The MTase of dnmt3 subfamily includes DNMT3 from chordates and DRM from plants (Ponger & Li 2005). In mammals, the initial methylation pattern is established by the *de novo* MTase DNMT3a/b subfamily in the early embryo, whereas DNMT1 subfamily maintains this pattern during the chromosome replication and repair (Mortusewicz *et al.* 2005, Reese & Bartolomei 2006). The dnmt2 subfamily is the most widespread MTase in eukaryotes, and its cellular function is not clear. Abundant evidence suggests that the expression of certain target gene is susceptible to the effect of DNA methylation during teleost development and that MTase promote tissue-specific growth and development via their activating actions (Mhanni & McGowan 2002, Rai *et al.* 2010, Takayama *et al.* 2014, Dasmahapatra & Khan 2016). Zebrafish *dnmt1* exhibits temporal and spatial patterns of expression in ovarian tissues during early development (Mhanni & McGowan 2002). Some studies indicate that increased levels of sex steroid hormones (e.g. estrogens) trigger inflammation-induced epigenetic gene silencing via aberrant eukaryotic DNA hypermethylation in human cancers (Belinsky *et al.* 2002, Wang *et al.* 2016). Si *et al.* also demonstrated that the anticancer drug paclitaxel augments ER α binding to the DNMT1 and DNMT3B promoters to activate the *DNMT1* and *DNMT3b* genes (Si *et al.* 2016). Furthermore, increased hypermethylation-induced downregulated *BCL2* expression has been demonstrated in breast cancer (Stone *et al.* 2013). This present study examined whether SPE or a sex steroid treatment affects hypermethylation of the *bcl2* gene in Japanese eel ovarian follicles during early gonadal maturation. We also determined whether this effect was mediated by MTase and resulted in decreased follicular cell survival. Here we report differences in the role of methylation in survival gene and concomitant expression levels during artificial maturation of Japanese eel. We demonstrate in an animal model and cell culture that *dnmt1* is robustly induced during the early stages of ovarian folliculogenesis, via estrogen, resulting in a decrease of follicular cells.

Materials and methods

Animal preparation

Female Japanese eel were purchased from a local aquaculture farm in Lukang, Taiwan. Then, the fish were placed in flow-through tank (25 $\pm$ 2°C, 30‰, 14L:10D). All animal research procedures were approved by the National Taiwan University Animal Research Committee (IACUC approval No. NTU104-EL-00105). To examine whether SPE involved in the DNA methylation of follicular cell gene expression via MTase activation, all fish (504 $\pm$ 8.9 g mean weight) received a weekly intraperitoneal injection of SPE at a dose of

20 mg/kg body weight per week (treated) or physiological saline (vehicle control). Extracts were prepared by homogenizing salmon pituitary powder with 0.9% NaCl solution followed by centrifugation at 750 g for 15 min. At the time of collection (after 7 weeks), the ovary of anesthetized fish was removed and weighted to calculate the gonadosomatic index (GSI expressed as a percentage: (gonad weight/total body) $\times$ 100). The ovary fragments were then histologically examined and frozen in liquid nitrogen until assayed.

Histology

Five ovarian fragments per fish group were fixed in Bouin's solution and prepared for histological examination using standard biological procedures. Gonads were embedded in paraffin and sectioned (7 μ m) with a microtome. Each ovary was sectioned, processed for hematoxylin-eosin staining and observed at 200 $\times$ final magnification under a light microscope.

Enzyme-linked immunosorbent assay for estrogen level

Blood of SPE-treated female eel was collected from the caudal vasculature using a 3 mL heparinized syringe and centrifuged. The plasma was then stored at -80°C, and the plasma E₂ level was measured by the ELISA kits (Cusabio Biotech) according to the manufacturer's protocol. The optical density of the solution in each well was then determined within 10 min using a microplate reader set to 450 nm.

Ovarian follicular cell isolation and cell culture

The ovarian follicular cell culture of Japanese eel was prepared according to that for Atlantic croaker (Benninghoff & Thomas 2006) with some modifications. Female eel was killed by deep anesthetization. The ovary was immediately removed and placed in 1 $\times$ Hank's balanced salt solution (HBSS). Ovarian follicles were dissected and washed twice with two volumes of 1 $\times$ HBSS. The follicles were subsequently digested in 0.1% collagenase type 1A for 1.5 h with continual gentle mixing on a rotating shaker. At the end of the incubation period, the follicles were vigorously mixed by repeated pipetting to shear the follicle cells. Disassociated cells were separated from intact oocytes and other large debris by filtering them through a 100 μ m cell strainer (BD Biosciences). The cell suspension was centrifuged at 500 g for 5 min, and the resulting pellet was resuspended in 5 mL Leibovitz's L-15 medium (Thermo Fisher Scientific). The Leibovitz-15 medium consisted of fetal bovine serum (FBS, 10%; Thermo Fisher Scientific), penicillin (100 units/mL) and streptomycin (100 μ g/mL). The cells were incubated at 25°C. Cell density was determined by hemocytometer count, and cell viability, which was generally greater than 90%, was ascertained using the trypan blue exclusion method.

Detection of DNA fragmentation by the TUNEL assay

Follicular cells were seeded at a density of 1.5 $\times$ 10⁵ cells per well on collagen-coated 12-well plates and maintained at

37°C. E₂ (100 nM) was given into totally three 12-well plates and incubated for 24 h. The TUNEL assay was conducted by using the ApoAlert DNA fragmentation assay kit (Clontech). The incorporation of fluorescein-dUTP into the fragmented nuclear DNA generates the green fluorescence detected by a standard fluorescein filter set (520 ± 20 nm). All follicular cells stained with propidium iodide (PI) exhibited strong red cytoplasmic fluorescence when viewed at >620 nm.

Cell viability

To examine the effect of SPE or sex steroid hormone treatment on follicular cell growth, cells were seeded at a density of 1.5×10^5 cells per well on collagen-coated 12-well plates and maintained at 37°C in a 5% CO₂ atmosphere. SPE (the concentration of 0.01 and 1 mg/mL, dissolved in 1× PBS buffer) or steroids (the concentration of 10 and 100 nM, diluted in DMSO) was given into totally three 12-well plates and incubated for 24 h. 17 α -Methyltestosterone (MT) and 17 β -estradiol (E₂) were purchased from Sigma-Aldrich. 5'-aza-2'-deoxycytidine (5'-AZA; Sigma-Aldrich) were dissolved in 1× PBS buffer and used as DNA methylation inhibitor. For Aza treatment, cells were grown in medium supplemented with 0–100 μ M of freshly prepared 5'-AZA for 24 h; four replicates were used at each concentration and the PBS buffer was added in the control group. After the 24-h incubation, 0.5 mg/mL of dimethyl-thiazol-diphenyltetrazoliumbromide (MTT; Sigma-Aldrich) was added into each well. Cell viability was evaluated by the XL800 spectrophotometer (Bio-TEK Instruments, Inc.) at 595 nm.

RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA was isolated from ovarian tissues or follicular cells using TRIzol reagent (Invitrogen). cDNA was synthesized by reverse transcription with 1 μ g total RNA using the SuperScript™ kit (Invitrogen). The 20 μ L reverse transcription products were diluted to 100 μ L and 2–3 μ L was used for real-time PCR. Real-time PCR reactions were performed in a volume of 10 μ L containing specific primers (Supplementary Table 1, see section on [supplementary data](#) given at the end of this article) and 5 μ L 2× Fast SYBR Green Master Mix (Thermo Fisher Scientific) using the following condition: Initial denaturing: 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 40 s and extension at 72°C for 30 s. The threshold cycle number (CT) value for the target gene was normalized against *ef-1 α* (Genbank accession no. AB593812) and calculated as $\Delta CT = CT_{\text{Target gene}} - CT_{\text{ef-1}\alpha}$.

cDNA cloning and sequencing

Total RNA was extracted from fragments of frozen tissues using the TRIzol reagent (Thermo Fisher Scientific). mRNA was isolated using the FastTrack 2.0 RNA isolation kit (Thermo Fisher Scientific). mRNA (1 μ g) was reverse transcribed using Superscript II reverse transcriptase (Thermo Fisher Scientific) and oligo-dT primer. To isolate *dnmt1* and *bcl2* cDNA, the

strategy entailed first generating a partial cDNA by reverse transcription-polymerase chain reaction (RT-PCR) using one set of degenerate primers (primed with DP1/DP2 set for *dnmt1* and BP1/BP2 set for *bcl2*; see Supplementary Table 1). The PCR products were analyzed on agarose gels and cloned into the pGEM-T vector (promega) for DNA sequencing. To construct full-length 5' cDNA, the RACE was performed according to the SMART RACE cDNA Amplification Kit (Clontech). Briefly, 1 μ g of total RNA was utilized to generate 5'-RACE cDNA products with specific primers and reagents provided by the kit in the presence of MMLV reverse transcriptase enzyme (Clontech). 5'-RACE PCR product was carried out using *dnmt1* (DSP1) or *bcl2* (BSP1)-specific primers (Supplementary Table 1) and reagents provided by the kit in the presence of Advantage 2 polymerase mix (Clontech). The diluted primary PCR product was further used to generate the secondary PCR reaction using Nested Universal Primer A (NUP) primer and specific secondary primers (DSP2 for *dnmt1* and BSP2 for *bcl2*; Supplementary Table 1). The amplified products were gel purified using the Gel DNA recovery kit (Zymo Research) and were used as templates for performing a nested PCR using NUP primer and specific nested primers (DSP3 for *dnmt1* and BSP3 for *bcl2*; Supplementary Table 1).

Construction of the Japanese eel *dnmt1* promoter luciferase plasmids

A 0.8 kb fragment of eel *dnmt1* 5'-flanking region was isolated by PCR using the primers listed in Supplementary Table 1. The *dnmt1*-specific primers containing XhoI and HindIII restriction enzyme cleavage sites were designed to amplify the desired promoter fragments of the eel *dnmt1* gene, and the PCR products were cloned into a XhoI- and HindIII-digested pGL4-Basic vector (Promega). To test the activity of the Japanese eel *dnmt1* promoter, several reporter constructs were made. pGL4-dnmt1 0.8 K was used as template, pGL4-dnmt1 0.75 K, pGL4-dnmt1 0.6 K and pGL4-dnmt1 0.2 K were respectively created using PCR.

Transient transfection and luciferase activity assay

Transient transfections were conducted using Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer's instructions. Briefly, hepatocytes were seeded at the density of $2\text{--}4 \times 10^4$ cells/cm² into 12-well plates for 24 h. In each well, the E₂ (10–100 nM)-treated cells were transfected with eel *dnmt1* promoter reporter vectors using Lipofectamine 2000 (Invitrogen). To normalize the transfection efficiency, Renilla Luciferase reporter vectors (Promega) were co-transfected to the eel *dnmt1* promoter reporter vectors. pGL4 Control and pGL4-Basic (Promega) were used as positive and negative controls, respectively. Twenty-four hours post transfection, the cells were harvested in 50 mL 1× passive lysis buffer and luciferase activities were assayed using the Dual-Luciferase Reporter Assay System (Promega). Luminescence intensity was measured by Synergy™ HT Multi-Detection Microplate Reader (BioTek). The fold induction was calculated by reference to an untreated sample.

Primary culture of hepatocyte for reporter vector transfection

Livers were excised from anesthetized eel and isolated hepatocytes were prepared by collagenase digestion (15 mg/50 mL) of a perfused liver at room temperature for 30 min and cultured in Leibovitz-15 medium were kept in a conventional incubator as described previously (Kwon & Mugiya 1994, Yanhong *et al.* 2008).

RNA interference

DNMT1 shRNA in pLKO.1 plasmids (clone ID: TRCN0000021890 (#1), TRCN0000021893 (#2) and TRCN0000021891 (#3)) were obtained from the National RNAi Core Facility in the Academia Sinica (Taipei, Taiwan). Follicular cells were transfected with the empty vector (pLKO.1 puro) or plasmids expressing shRNA (6 µg DNA per 10⁵ cells for 48 h) using Lipofectamine 2000 (Invitrogen) method for 48 h.

Genome DNA isolation and cloning

The preparation of genomic DNA libraries from ovarian fragment of Japanese eel was conducted by using the GenomeWalker kit (Clontech). The libraries were made by cutting the genomic DNA with either *Dra*I, *Eco*RV, *Pvu*II or *Stu*I and ligating each pool of the digested genomic DNA to the GenomeWalker Adaptor. Two PCR amplifications/library were performed. The primary PCR was made with the adaptor primer (AP1) provided in the kit and *dnmt1* or *bcl2* reserve primer (DSPr1 or BSPr1; see Supplementary Table 1 for PCR primer sequences) as the gene-specific primer from the 5'-end of Japanese eel *dnmt1* sequence. The diluted PCR mixture was used as the template for a secondary PCR. This second PCR was carried out using the nested adaptor primer (AP2) and a nested gene-specific primer DSPr2 or BSPr2 (Supplementary Table 1). Using gel electrophoresis, one specific band from the *Stu*I library was purified and cloned into the pGEM-T vector (Promega) for DNA sequencing.

Methylation-specific PCR and sequencing

Genomic DNA was isolated from the Japanese eel follicular cells using the PureLink genomic DNA Kits (Thermo Fisher Scientific) and diluted into 40 ng/µL aliquots. Next, bisulfite conversion was performed using the EZ DNA methylation Gold kit (Zymo research) according to the manufacturer's instruction. In this experiment, the conversion efficiency was tested by means of clone sequencing of a PCR product generated using primers targeting cytosine-free priming (CFP) sites, which was used to quantify the specific conversion of cytosine to uracil as described previously (Holmes *et al.* 2014), that the PCR product generated using primers targeting CFP sites. We designed the primer pair of *bcl2* exon1 fragment to conduct PCR amplification, that the CFP sites of primer pair do not comprise any cytosine and therefore are not altered during the bisulfite conversion. The conversion-unspecified amplification of the primer pair was shown to amplify bisulfite-modified and unmodified DNA. All the ten clones comprising

300 conversion sites were analyzed after sequencing. The results of DNA sequencing showed that the conversion rates (Cmod/(Cmod + Cunmod)) of modified DNA were 97.6 ± 2.1%. Bisulfite sequencing of follicular cell samples was performed as described previously (Wang *et al.* 2012). Bisulfite-modified DNA was amplified by methylation-unbiased primers designed using the MethPrimer program (<http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi>) and used to amplify a 138-bp product of *bcl2* promoter with primer set mentioned at Supplementary Table 1. PCR products were cloned using pGEM-T Vector System. The sequenced promoter region for *bcl2* was confirmed by using the automated sequencer.

Statistical analysis

Results are expressed as mean ± S.E.M. of multiple experiments. Statistical differences were evaluated by Kruskal–Wallis test or Student's *t*-test. *P* < 0.05 was considered statistically significant.

Results

SPE treatment affects Japanese eel ovarian follicular growth and survival

Our previous study showed that ovarian development in Japanese eel is stimulated by SPE and final maturation is induced after long-term repetitive injections. However, our results also show that the apoptosis-mediated atresia of ovarian follicles is sensitive to SPE or estrogen treatment. To further elucidate the regulatory mechanisms of SPE-induced cell death, we performed experiments to evaluate the effect of SPE on follicular cells during the previtellogenic and early vitellogenic stages. The results revealed that SPE artificially induced ovarian growth in Japanese eel at the early vitellogenic stage compared to a control group of eels in the previtellogenic stage, as shown in Fig. 1A. The gonadosomatic index value was significantly higher in the SPE group than that in the control group (Fig. 1B). To test the effect of SPE treatment on cell death of eel ovarian follicles, we measured the abundance of *bcl2* or *bax* mRNA using the quantitative polymerase chain reaction (qPCR). The results showed that seven weekly SPE injections significantly decreased *bcl2* mRNA levels (Fig. 1C). To further identify the effect of SPE treatment on follicular cells, we isolated and prepared a follicular cell culture from eel ovarian follicles. After adding various doses of SPH (10⁻⁴–10⁰ mg/mL), the cell counts and the levels of E₂ secreted by follicular cells were measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and an ELISA, respectively. The cell counts were lower at the high dose of SPE than at the low dose of SPE following a 24-h culture (Fig. 1D). The effects of SPE on ovarian development and cell death were mediated by activating steroidogenesis, as SPE significantly increased the secretion of E₂ in a dose-dependent fashion after a 24-h cell culture (Supplementary Fig. 1). Furthermore,

Figure 1 SPE and sex steroid treatment promoted the cell death of follicular cells. (A) Representative stained sections for previtellogenic (control group) and early vitellogenic stage (SPE group) of ovarian development of Japanese eel after seven weekly injections. Scale bar = 100 μm. (B) Change in GSI distribution. (C) Relative expression of *bcl2* and *bax* genes in ovarian follicle of Japanese eel after seven weekly injections. (D) The *in vitro* study, follicular cells were incubated with SPE for 24 h and the cell number assessed using the MTT assay to measure mitochondrial enzyme activity. (E) Follicular cells treated with MT or E₂ (10 nM or 100 nM; dissolved in 0.1% DMSO) for 24 h, and the cell number was determined using the MTT assay. (F) The TUNEL assay for follicular cells treated with E₂ (100 nM). Scale bar, 100 μm. **P* < 0.05 and ***P* < 0.01 versus the control group. Data are the means ± S.E.M. of three independent experiments in triplicate. VC, vehicle control (1 × PBS buffer for SPE treatment; 0.1% DMSO for steroid treatment).

17α-methyltestosterone (MT) or E₂ was added to follicular cells, and cell counts were determined. After a 24–72 h incubation, the MT treatment has no significant effect on the follicular cell counts at both the low (10 nM) and high doses (100 nM; Supplementary Fig. 2). However, the same dose of E₂ significantly decreased the follicular cell counts (Fig. 1E). Terminal deoxynucleotidyl transferase dUTP nick end labeling assay results revealed that E₂ (100 nM) induced apoptosis in follicular cells after a 24-h treatment (Fig. 1F).

E₂ influences *bcl2* and *dnmt1* expression

This study further analyzed whether the number of E₂-suppressed cells was mediated by activating the cell death program in Japanese eel follicular cells. The results of a qPCR analysis showed that *bcl2* mRNA expression decreased by adding E₂ (10 nM) for 24 h (Fig. 2A). In contrast, the expression of *bax* mRNA was significantly higher in the E₂ group than that in the control group (Fig. 2A). The relative *bcl2* and *bax* mRNA expression levels were measured (expressed as the relative *bcl2/bax* ratio), and the results indicated that the *bcl2/bax* ratio decreased significantly in the E₂ group compared to the control group (Fig. 2B). Therefore, this study attempted to elucidate the regulatory mechanism on SPE-mediated

E₂/ER action in both genes. The *dnmt1* and *dnmt3b* mRNA expression was measured in follicular cells after adding E₂ for 24 h, and the *dnmt1* mRNA levels increased significantly (Fig. 2C). Not surprisingly, SPE-treated ovarian tissues showed increased expression of *dnmt1* mRNA (Supplementary Fig. 3).

E₂ decreases *dnmt1* promoter activity through E₂/ER action

Our findings show that the E₂ treatment affected follicular cell growth and the *dnmt1* transcript expression. To further determine whether the E₂/ER complex binds to the *dnmt1* promoter region, the Japanese eel *dnmt1* promoter sequence was cloned to study its possible role in E₂/ER upregulation of the *dnmt1* mRNA transcript. A Japanese eel genomic library was screened, and one specific band from the library was cloned for DNA sequencing. The complete sequence analysis obtained 804 bp of the 5-flanking region, and the TATA box was located 30 bp upstream from the transcription starter site. Figure 3A illustrates the complete sequence and a map of the Japanese eel *dnmt1* promoter landmarks. PROMO software (ver 3.0.2) predicted several putative transcription factors (CREB, ER, SP1, AP1 and NF-κB) targeting this core region. To determine whether *dnmt1*

Figure 2 qPCR analysis of the relative apoptotic factor and DNMTs mRNA expression in follicular cells. (A) The mRNA expression levels of *bcl2* and *bax* were quantified by RT-qPCR and (B) the *bcl2/bax* ratio was determined. (C) The transcript levels of *dnmt1* and *dnmt3b* were quantified. The mRNA expression was normalized to that of *ef-1α*. Data are the means $\pm$ S.E.M. of three independent experiments in triplicate. * $P < 0.05$ versus the control group.

transcriptional expression was mediated by E₂, an experiment was performed to confirm whether E₂/ER would bind directly to the promoter of the *dnmt1* gene and regulate its transcriptional activation. Genomic regions containing 0.8K bp upstream of the *dnmt1* promoter region were cloned into the pGL4-luciferase reporter vector using follicular cells. The results showed a significant increase in *dnmt1* promoter activity after the E₂ treatment (Fig. 3B). To explore the core region responsible for E₂-induced *dnmt1* promoter activity, various fragments were cloned, covering different regions of the promoter but excluding the predicted estrogen response element (ERE) sites. As shown in Fig. 3C, deleting the core region between -749bp and -597bp yielded the most significant change in luciferase activity. These results indicate that the ER-binding site -702/-690bp upstream of the *dnmt1* promoter is responsible for E₂-induced upregulation of *dnmt1* expression.

Effect of *dnmt1* shRNA on E₂-treated *bcl2* expression

To confirm whether the downregulation of *dnmt1* attenuates the E₂-treated *bcl2* mRNA expression, three clones (#1, #2 and #3) of *dnmt1* shRNA plasmids were transfected into follicular cells. Using RT-qPCR,

it was found that *dnmt1* shRNA (#2) could reduce *dnmt1* expression (Fig. 4A). It was also found that the knockdowns of *Dnmt1* by shRNA attenuated E₂-decreased *bcl2* mRNA expression (Fig. 4B).

The CpG island on the *bcl2* promoter region of Japanese eel

As mentioned earlier, activating *dnmt1* and silencing *bcl2* expression may mediate the inhibitory effect of E₂ on follicular cell numbers. Experiments were performed to explore whether the *bcl2* gene promoter region contains a CpG island, and whether *dnmt1*-mediated methylation affects promoter activity to epigenetically alter Japanese eel follicular cells. The promoter region of the Japanese eel *bcl2* gene was isolated from a genomic library using PCR cloning. The complete sequence was used to predict the putative transcriptional factors using PROMO software (ver 3.0.2) and screened for the presence of CpG islands using the MethPrimer program. Supplementary Figure 4A illustrates the sequence and a map of the *bcl2* promoter landmarks. A CpG island prediction analysis detected two putative CpG islands at -1529/-1192bp (35 CG sites) and -877/-705bp (11 CG sites) upstream from the transcription start site (Supplementary Fig. 4B). The transcription factor analysis predicted several putative transcription factors (as illustrated in Supplementary Fig. 4C) targeting the *bcl2* gene promoter region, and only the CpG island 2 region contained a binding site for two transcription factor (AR and STAT3)-binding site.

Bcl2 methylation in E₂-treated follicular cells

To determine whether the *bcl2* locus undergoes promoter hypermethylation in E₂-treated follicular cells, the methylation status of CpG island 2 located in the *bcl2* gene was checked using the bisulfite-specific PCR and sequencing method. We first measured the methylation degree of eight CpG sites in the promoter (nucleotides -890 to -752). The percentage of methylation of the *bcl2* gene was higher in E₂-treated follicular cells compared to those in the control group (Fig. 5A). However, E₂-induced hypermethylation of the *bcl2* gene was suppressed by adding a MTase inhibitor (5'-aza-2-deoxycytidine (5'-AZA): 100μM). Further experiments were performed to determine whether *dnmt1*-mediated methylation affects follicular cell growth and development via E₂/ER action, and 5'-AZA was utilized to suppress the inhibitory effect of E₂ on follicular cell counts and *bcl2* mRNA expression. The MTT assay results showed that adding 5'-AZA alone did not affect the follicular cell counts (Fig. 5B). However, 5'-AZA significantly recovered the E₂-induced decrease of follicular cell counts in a dose-dependent manner (25–100μM). Furthermore, the qPCR results showed that the decreased levels of *bcl2* mRNA

Figure 3 Validation of the ER-binding site in the Japanese eel *dnmt1* promoter. Structure of the Japanese eel *dnmt1* promoter. A 1-kb genomic clone of the Japanese eel *dnmt1* gene was sequenced, mapped and predicted by PROMO programs analyzed for consensus sequences to known transcription factors. (A) The nucleotide sequence for the promoter region, where underlined sequences correspond to conservative homologous matches to the noted transcription factor or promoter element. (B) A luciferase reporter vector containing full length of the eel *dnmt1* promoter region was constructed and transfected into Japanese eel hepatocytes, and estrogen exerted a dose-dependent stimulation on *dnmt1* promoter activity after 24-h incubation. (C) A map of the eel *dnmt1* promoter showing the relative locations of the putative transcription factor response elements. Luciferase reporter vectors containing various lengths of the eel *dnmt1* promoter region. The left schematic diagram indicates truncated *dnmt1* promoter sequences linked to luciferase, where the bars on the right indicate luciferase activity relative to the pGL4-Basic control vector. Data are the means $\pm$ S.E.M. of three independent experiments in triplicate. * $P < 0.05$ and ** $P < 0.01$ vs the vehicle control group. VC, vehicle control (0.1% DMSO).

Figure 4 Expression of *bcl2* after knocking down *dnmt1*. E₂-treated follicular cells were transfected with shRNAs (6 µg DNA per 10⁵ cells) specifically targeting *dnmt1*. The data were obtained at 48 h post transfection of shRNAs. (A) Cells were transiently transfected with three clones (#1, #2 and #3) of Dnmt1 shRNA plasmids by the Lipofectamine2000 and the expression of *dnmt1* mRNA was detected by RT-qPCR. (B) Cells were transiently transfected with clone 2 (#2) of Dnmt1 shRNA plasmids and cells were treated with E₂ then incubated for 48 h. The mRNA levels of *bcl2* was examined with by RT-qPCR. The relative expression data were presented after normalization against to *ef-1α*. The results are shown as the mean of three independent experiments. **P* < 0.05 and ***P* < 0.01 versus the empty vector (EV; pLKO.1 puro) group.

were rescued by adding 5'-AZA (50 or 100 µM) to E₂-treated follicular cells.

Discussion

This study demonstrated that methylation of the anti-apoptotic factor *bcl2* in Japanese eel follicular cells was affected by exogenous sexual hormones (SPH or estrogen) during artificial maturation, which resulted in a decrease in follicular cell counts. We previously performed an animal study to indicate that exogenous E₂ increases vitellogenin expression in the liver and decreases the numbers of ovarian follicles by increasing apoptosis of follicular cells (Wang & Lou 2006, 2007). The present investigation confirmed this E₂ effect in a cellular model under an epigenetic pathway. The experimental results demonstrate that E₂ bound to the ERE site of the *dnmt1* promoter region, which induced *dnmt1* gene expression. Additionally, the present study suggests an epigenetic pathway in which E₂ modified DNA hypermethylation in the promoter of the *bcl2* gene. The pathway for the E₂-mediated apoptotic effect was first illuminated during artificial maturation induced by exogenous SPE treatment. Importantly, the *in vitro* treatment of ovarian follicular cells with the DNA methylation inhibitor 5'-AZA reversed the numbers of E₂-suppressed cells.

A previous study suggested that European eel could be used to test chronic exposure to contaminants that may affect the DNA methylation status of ovarian follicles (Pierron *et al.* 2014). Their findings also indicate that among animals from the wild clean region, treatment with carp pituitary extracts increased the DNA hypermethylation level of several certain genes associated with ovarian follicular growth, but did not significantly change their mRNA levels. These findings and our data suggest that exogenous FSH-induced E₂ production may promote hypermethylation of ovarian follicle genes. Several studies have suggested that carp or SPEs, which consist of a variety of pituitary hormones (including FSH and luteinizing hormone), can be applied to promote European or Japanese eel ovarian follicular development and growth (during vitellogenesis and final maturation). Our previous study found that SPE and E₂ induction failed to promote ovarian follicle growth and significantly decreased total oocyte numbers (Wang & Lou 2007). In mammal and teleost, ovarian follicular apoptosis is controlled by hormonal/endocrine factors (i.e., gonadotropins, sex steroids, and several growth factors). FSH plays an essential role in the reproductive system by regulating steroidogenesis and gamete differentiation. Several lines of evidence indicate that FSH induced stronger secretion of E₂ in teleost than testosterone (Montserrat *et al.* 2004, Kamei *et al.* 2006, Luckenbach *et al.* 2011). Our cellular model further

Figure 5 Estrogen-induced cell death was reversed using MTase inhibitor. Follicular cells treated with estrogen w/w/o 5'-AZA (25–100 μM) for 24-h incubation. (A) Schematic diagram of the distribution of *bcl2* promoter CpG 2 islands in follicular cells are shown. White arrows indicated the PCR primer pair of bisulfite sequencing PCR (BSP). PCR primers specific to unmethylated and methylated bisulfite-modified DNA were used to amplify promoter region of the *bcl2* gene in E₂-induced follicular cells and 5'-AZA treatment. Amplified PCR products were cloned, and ten individual clones were sequenced to determine the methylation state of the eight CpGs within the amplified region. Open and filled circles indicate that the CpG site is unmethylated or methylated, respectively. Each row represents one clone. (B) The cell number was determined by using the MTT assay. (C) The mRNA expression levels of *bcl2* were quantified by RT-qPCR. VC, vehicle control (0.1% DMSO for 17β-estradiol or 1× PBS buffer for 5'-AZA treatment). Data are the means ± S.E.M. of three independent experiments in triplicate. **P* < 0.05 and ***P* < 0.01 versus the vehicle control group.

showed that cell counts decreased and *bcl2* mRNA expression was downregulated in response to high concentrations of SPE or E₂, but not to MT. These results might account for estrogen-induced follicular cell apoptosis through SPE-induced steroidogenesis.

Accumulating evidence suggests that estrogen plays multiple physiological roles in cellular biology, including apoptosis (Ying 2000, Jaita *et al.* 2005, Zarate *et al.* 2009, Magri *et al.* 2016). Jaita *et al.* demonstrated that E₂ induces apoptosis of anterior pituitary lactotrope by upregulating the Fas/FasL system (Jaita *et al.* 2005). The current study further indicates that E₂ enhanced endometrial glandular cells apoptosis by suppressing the NF-κB system (Chen *et al.* 2014). However, the mechanism of estrogen-induced apoptosis in ovarian follicular cells of teleost fish remains unknown. The present cell culture study showed that SPE or E₂ alone decreased the number of follicular cells by promoting *bcl2* methylation, which, in turn, silenced *bcl2* expression. A sequencing analysis indicated that the Japanese eel *bcl2* promoter region had many contiguous CpG sites within two CpG islands (CpG island 1 and 2), and several putative transcription factors target sites were present (Fig. 4C). Taken together, these results indicate two putative factor-binding sites (AR and STAT3) on the CpG island 2. STAT3 belongs to the signal transducers and activation of transcription (STAT) family. Phosphorylated STATs translocate to the STAT-inducible elements region of the target gene and induce transcription by activating non-genomic ER action (Bjornstrom & Sjoberg 2002). Several reports have demonstrated that E₂ mediates the *bcl2* neuroprotective gene by activating STAT3 (Linnik *et al.* 1995, Jia *et al.* 1996, Dziennis *et al.* 2007). Therefore, the methylation of the STAT3-binding site on the *bcl2* gene most likely accounts for downregulation of *bcl2* by E₂-mediated *dnmt1*.

Several lines of evidence indicate that DNA methylation is catalyzed by *Dnmt1* and *Dnmt3a/b*. *Dnmt3a/b* are responsible for establishing *de novo* methylation (Okano *et al.* 1999). In contrast, *DNMT1* is the primary DNA methyltransferase responsible for copying methylation patterns of hemi-methylated DNA after DNA replication in cells (Chuang *et al.* 1997). *Dnmt1* modifies DNA methylation according to the *Dnmt3*-initiated *de novo* methylation of CpG dinucleotides, although some researchers have speculated that *Dnmt1* shows considerable *de novo* methylation activity in intracisternal A-type particle in mouse embryo (Gaudet *et al.* 2004) and *Dnmt3a*^{-/-}/*3b*^{-/-} double-knockout embryonic stem cells (Lorincz *et al.* 2002). In the present study, the *dnmt3b* mRNA level did not change in follicular cells w/o E₂ treatment, whereas the *bcl2* methylation pattern in follicular cells depended on the *dnmt1* expression level. Our results are consistent with the concept that establishment of the methylation pattern depends on coordination between *Dnmt1* and

Dnmt3 (Fatemi *et al.* 2002, Kim *et al.* 2002). An ERE site was found on the promoter region of Japanese eel *dnmt1*, explaining how E₂ regulates *dnmt1* expression through an analysis of the transcription factor prediction program. The regulation of gene expression by steroids is a multifactorial process, involving both genomic and non-genomic actions that converge at certain response elements located in the promoter of the target gene (Bjornstrom & Sjoberg 2005, Levin 2005). In the present study, a transcription factor prediction program detected several putative transcription factors (CREB, SP1, AP1 and NF-κB), in addition to the ERE, which target sites at this core region. Previous studies have suggested that these transcription factors play pivotal roles, and control a number of cellular processes including cell differentiation, proliferation and apoptosis (Gaub *et al.* 1990, Galien & Garcia 1997, O'Lone *et al.* 2004). Results from the promoter assay indicate that E₂ binds to the ERE-binding site of the Japanese eel *dnmt1* promoter and involves E₂-increased *dnmt1* gene expression through direct genomic activity. A similar pattern of *Dnmt1* transcriptional activity has also been reported in the ER-positive MCF-7 cell lines, which consists of two ERE site on the *Dnmt1* promoter region (Shi *et al.* 2012). These observations suggest that the DNA methylation mechanism is involved in sex steroid-sensitive cells.

Conclusion

This study demonstrated that FSH-mediated estrogen action indirectly affects the growth of Japanese eel ovarian follicular cells by epigenetic modification. E₂ interacted with its ER to enhance *dnmt1* expression, which, in turn downregulated anti-apoptotic factor *bcl2* expression. This study provides insight into the understanding of the reproductive physiology of teleost fish and offers an improved method for Japanese eel.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0484>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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