ATM expression is attenuated by promoter hypermethylation in human ovarian endometriotic stromal cells

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ABSTRACT

A number of genes involved in the pathogenesis of endometriosis are silenced by the hypermethylation of their promoter regions. We assessed the effect and mechanism of the DNA demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC) (10 μM) on the cell cycle in human endometriotic cyst stromal cells (ECSCs) and normal endometrial stromal cells (NESCs) by flow cytometry. The DNA methylation status of G2/M checkpoint regulators were investigated by methylation-specific polymerase chain reaction (PCR). The expression of ATM and the effect of 5-aza-dC on its expression were also evaluated by quantitative reverse transcription-PCR and western blotting analysis. 5-aza-dC treatment resulted in the cell cycle arrest of ECSCs at the G2/M phase. In contrast, 5-aza-dC did not affect the cell cycle of NESCs. The promoter region of the ataxia telangiectasia mutated *(ATM)* gene was hypermethylated in ECSCs, but not in NESCs. *ATM* mRNA expression was attenuated in ECSCs compared to that in NESCs. Further, 5-aza-dC was found to restore ATM expression of in ECSCs by its promoter demethylation. Our findings indicate that *ATM* promoter hypermethylation occurs in endometriosis, and that *ATM* silencing is involved in tumorigenesis during this disease; moreover, selective DNA demethylating agents and molecular target drugs against ATM silencing are promising for the treatment of endometriosis.

Key words: endometriosis; DNA methylation; ataxia telangiectasia mutated (ATM); DNA demethylating agent; cell cycle arrest

Introduction

Endometriosis is an estrogen-dependent neoplasm that is frequently observed in women of reproductive age (Giudice and Kao, 2004). Histologic features of endometriosis resemble normal endometrium in the proliferative phase (Giudice and Kao, 2004); however, there are many molecular differences at the epigenetic, genetic, mRNA and protein levels (Nasu *et al.*, 2011a, 2011b; Abe *et al.*, 2013). In 2014, endometriosis was classified as a benign tumour (Kurman *et al.*, 2014) and, although rare, this disease is recognised as the origin of secondary malignant ovarian neoplasm.

DNA methylation of CpG islands in gene promoter regions is the best understood epigenetic modification. CpG islands of gene promoters are usually unmethylated and participate in active gene transcription (De Smet *et al.*, 2004). When promoter CpG islands are methylated, expression of the associated gene is typically silenced by the suppression of transcriptional activity (Jones *et al.*, 2002). Aberrrant DNA methylation in promoter regions has been reported in endometriosis, and involves genes such as progesterone receptor (*PR*)-B (Wu *et al.*, 2006), *HOXA10* (Wu *et al.*, 2005), estrogen receptor (*ER*)-β (Xue *et al.*, 2007a), steroidogenic factor-1 (*SF-1*) (Xue *et al.*, 2007b), aromatase (Izawa *et al.*, 2008), *miR-196b* (Abe *et al.*, 2013) and *miR-503* (Hirakawa *et al.*, 2016).

DNA methylation of CpG islands is relatively stable and reversible. The maintenance by DNA methyltransferases (DNMTs) such as DNMT1, DNMT3A, and DNMT3B promote epigenetic inheritance during DNA replication (Nasu *et al.*, 2011b; Wu *et al.*, 2007). DNMT inhibitors are currently tested in clinical trials or already used in clinics, especially for cancer treatment. 5-aza-2'-deoxycytidine (5-aza-dC) is one of the most studied DNMT inhibitors; this compound inhibits DNA methylation and reactivates gene expression, which involves the incorporation of these molecules at cytosine positions during DNA replication (Nasu *et al.*, 2011b; Esteller, 2008).

In the present study, we investigated the effect of 5-aza-dC on the cell proliferation, apoptosis and cell cycle progression of ovarian endometriotic cyst stromal cells (ECSCs) and normal endometrial stroma cells (NESCs). We found that 5-aza-dC treatment resulted in the cell cycle arrest of ECSCs at G2/M phase. Then, we evaluated the promoter methylation status of genes associated with G2 checkpoint control and discussed the epigenetic mechanisms of cell cycle control in endometriosis.

Materials and methods

ECSC and NESC isolation procedure and cell culture conditions

Ovarian endometriotic tissues were obtained from patients during salpingo-oophorectomy or evisceration for ovarian endometriotic cysts (n = 19, aged 26–47 years). NESCs were obtained from premenopausal patients during hysterectomies for subserosal leiomyoma with no evidence of endometriosis (n = 18, aged 38–50 years). Patients who had not received any hormonal treatments for at least two years were chosen for the tissue collection. All specimens were diagnosed as mid- to late-proliferative phase. This study was conducted under the approval by the institutional review board (IRB) of the Faculty of Medicine, Oita University, and with written informed consent of the patients.

ECSCs and NESCs were isolated from ovarian endometriotic cyst and eutopic endometrium, respectively, through enzymatic digestion as previously described (Nishida *et al.*, 2004). Isolated ECSCs and NESCs were cultured in DMEM supplemented with 100 IU/ml of penicillin, 50 mg/ml of streptomycin and 10% heat-inactivated fetal bovine serum (FBS) (all obtained from Gibco-BRL, Gaithersburg, MD, USA), at 37°C with 5% CO_2 in air.

ECSCs and NESCs in monolayer culture after the third passage were >99% pure as determined by immunocytochemical staining with antibodies specific for vimentin, CD10, cytokeratin, factor VIII, and leukocyte common antigen, and were used for the following

92 experiments (Nishida *et al.*, 2004). CD10 was used as a marker of Müllerian origin. Each
93 experiment was performed in triplicate and repeated at least three times.
94
95 ***Assessment of the effects of 5-aza-dC on ECSC cell viability***
96 The effects of 5-aza-dC on the cell viability of NESCs and ECSCs were analysed by
97 modified methylthiazoletetrazolium (MTT) assay using the Cell Proliferation Kit I (Roche
98 Diagnostics, Basel, Switzerland), as previously described (Abe *et al.*, 2013; Hirakawa *et al.*,
99 2016). Briefly, 5×10^3 NESCs and ECSCs placed in 96-well flat-bottomed microplates
100 (Corning Inc., Corning, New York, NY, USA) were incubated for 96 h with or without
101 5-aza-dC (10 μM). Thereafter, cell viability was measured according to the manufacturer's
102 instructions. Data were calculated from triplicate samples and are presented as the percent
103 viability relative to those of untreated NESCs.
104
105 ***Assessment of the effects of 5-aza-dC on ECSC apoptosis***
106 The effects of 5-aza-dC on apoptosis in NESCs and ECSCs were analysed by the
107 direct determination of nucleosomal DNA fragmentation using the Cell Death Detection ELISA
108 (Roche Diagnostics), as previously described (Abe *et al.*, 2013; Okamoto *et al.*, 2015;
109 Hirakawa *et al.*, 2016). Briefly, 5×10^3 NESCs and ECSCs placed in 96-well flat-bottomed
110 microplates (Corning Inc.) were incubated for 96 h with or without 5-aza-dC (10 μM).
111 Thereafter, nucleosomal DNA fragmentation was measured according to the manufacturer's
112 instructions. Data were calculated from triplicate samples and are presented as the percent
113 viability relative to those of untreated NESCs.
114 The effects of 5-aza-dC on the the activities of caspase-3 and caspase-7 of NESCs and
115 ECSCs were analysed by the Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA), as
116 described (Abe *et al.*, 2013; Okamoto *et al.*, 2015; Hirakawa *et al.*, 2016). Briefly, 5×10^3

NESCs and ECSCs placed in 96-well flat-bottomed microplates (Promega) were incubated for 96 h with or without 5-aza-dC (10 μM). Thereafter, caspase-3 and caspase-7 activities were measured according to the manufacturer's instructions. Data were calculated from triplicate samples and are presented as the percent viability relative to those of untreated NESCs.

Assessment of the effects of 5-aza-dC on ECSC cell cycle by flow cytometry

The effects of 5-aza-dC on the cell cycle of NESCs and ECSCs were analysed by flow cytometry after 96 h of culture with or without 5-aza-dC (10 μM), as previously described (Abe *et al.*, 2013; Hirakawa *et al.*, 2016). Briefly, ECSCs were cultured at <60% confluence for 4 days with or without 5-aza-dC (10 μM). Flow cytometric analysis of the cell cycle was performed after propidium iodide staining using the CellFIT program (Becton-Dickinson, Sunnyvale, CA, USA), in which the S-phase was calculated using a ModFit model. Data were calculated as the percentage of values obtained for 5-aza-dC-treated cells relative to those of untreated controls.

Methylation-specific PCR (MSP)

Based on a database search using the UCSC Genome Browser on Human, Dec. 2013 (GRCh/hg38) Assembly (http://genome.ucsc.edu/cgi-bin/hgGateway), we detected the presence of dense CpG islands surrounding the genes listed in Figure 1, except for *cdc25A*. Considering the functions of proteins encoded by these genes as the negative regulators of the G2/M checkpoint (Figure 1), methylation status of ataxia telangiectasia mutated (*ATM*), ataxia telangiectasia and Rad3-related (*ATR*), p53, $p21^{Waf1/Cip1}$, checkpoint kinase (*Chk*)*1* and *Chk2* were evaluated by MSP, as described (Abe *et al.*, 2013). Genomic DNA was extracted from cultured NESCs (n = 8) and ECSCs (n = 8) with a QIAamp® DNA Mini kit (Qiagen). Then, genomic DNA (1 μg) was subjected to bisulfate conversion using the EpiTect Bisulfite Kit

142 (Qiagen) and further processed for the PCR amplification of specific CpG island regions of
143 candidate genes. MSP for candidate genes and the predicted size of PCR products are listed in
144 Table 1 and the PCR was performed as previously described (Fruhwald *et al.*, 2001; Brakensiek
145 *et al.*, 2005; Roy *et al.*, 2006; Wang *et al.*, 2010; Mazumder Indra *et al.*, 2011). PCR products
146 were analysed by 2% agarose/ethidium bromide gel electrophoresis.
147
148 ***Quantitative reverse transcription-polymerase chain reaction (RT-PCR)***
149 The expression of ATM mRNA in NESCs and ECSCs was evaluated by quantitative
150 RT-PCR, as described (Abe *et al.*, 2013; Hirakawa *et al.*, 2016). Briefly, total RNA was
151 extracted from NESCs (n = 10) and ECSCs (n = 10), cDNA was synthesised, and quantitative
152 RT-PCR was performed as described (Abe *et al.*, 2013; Hirakawa *et al.*, 2016). Primers specific
153 to *ATM* (Assay ID: Hs01112355, Applied Biosystems) and glyceraldehyde 3-phosphate
154 dehydrogenase (*GAPDH*) (Assay ID: Hs02758991_g1) were used. The candidate mRNA
155 expression levels relative to *GAPDH* mRNA expression were calculated by using calibration
156 curve. The data were calculated from 10 samples and are presented as the percentage of values
157 compared to those of NESCs.
158 The effect of 5-aza-dC on *ATM* mRNA expression in ECSCs was also evaluated by
159 quantitative RT-PCR. Briefly, 96 h after 5-aza-dC (10 μM) treatment, total RNA was extracted
160 from ECSCs and subjected to quantitative RT-PCR, as described. The data were calculated
161 from triplicate samples and are presented as the percentage of the values compared to those of
162 untreated controls.
163
164 ***Protein expression***
165 The expression of ATM protein in NESCs (n = 5) and ECSCs (n = 5) was evaluated by
166 western blotting analysis, as described (Nishida *et al.*, 2004; Abe *et al.*, 2013; Hirakawa *et al.*,

2016). Antibodies against ATM (ab82512, Abcam, Cambridge, England) and GAPDH (mAbcam 9484, Abcam) were used as primary antibodies. The expression of ATM protein relative to that of GAPDH in NESCs was analyded using Image Lab™ software (Bio-Rad Laboratories, Hercules, CA, USA) and the data are presented as the percent values.

The effect of 5-aza-dC on ATM and phosphorylated p53 protein expression in ECSCs was also evaluated by western blotting analysis. An antibody against phosphorylated p53 (#9284, Cell Signaling Technology, Danvers, MA, USA) was used as the primary antibody.

Statistical analysis

All data were obtained from triplicate samples and are presented as percentages relative to the corresponding controls as mean ± SD; these values were appropriately analysed using the Bonferroni test, Student's *t*-test, or Mann-Whitney *U*-test with Statistical Package for Social Science software (IBM SPSS statistics 24; IBM, Armonk, NY). P values < 0.05 were considered statistically significant.

Results

Marginal effects of 5-aza-dC on cell viability and apoptosis of ECSCs

The effects of 5-aza-dC on the cell viability of NESCs and ECSCs were determined by modified MTT assay. Although there was no significance, 5-aza-dC tends to attenuate the cell viability of NESCs and ECSCs (Figure 2A).

The effects of 5-aza-dC on apoptosis of NESCs and ECSCs were determined by the Cell Death Detection ELISA. As shown in Figure 2B, 5-aza-dC significantly induced the apoptosis of NESCs. However, 5-aza-dC showed a marginal effect on the apoptosis of ECSCs.

The effects of 5-aza-dC on the caspase 3/7 activities in NESCs and ECSCs were

192 determined by the Caspase-Glo 3/7 Assay. As shown in Figure 2C, 5-aza-dC significantly
193 activated caspase 3/7 in NESCs and ECSCs.
194
195 ***5-aza-dC induces G2/M phase cell cycle arrest in ECSCs***
196 The effects of 5-aza-dC on the cell cycle of NESCs and ECSCs were determined by
197 flow cytometry. As shown in Figure 3, compared to that in control cells, culture of ECSCs for
198 96 h in the presence of 5-aza-dC (10 μM) resulted in an accumulation of cells in the G2/M
199 phase of the cell cycle (12.6 ± 0.4% vs. 21.3 ± 0.7%, respectively; $p < 0.0001$), with a
200 concomitant decrease in the proportion of these cells in the G0/G1 phase (83.7 ± 0.5 vs. 73.5 ±
201 0.6, respectively; $p<0.0001$). In contrast, 5-aza-dC (10 μM) did not affect the cell cycle of
202 NESCs.
203
204 ***Hypermethylation of ATM in ECSCs***
205 The methylation status of genes known as negative regulators of the G2/M checkpoint
206 including *ATM*, *ATR*, *p53*, $p21^{Waf1/Cip1}$, *Chk1*, and *Chk2*, was evaluated by MSP. As shown in
207 Figure 4A, CpG islands in the promoter region of ATM (Table 2) were hypermethylated in
208 ECSCs, but not in NESCs. In contrast, CpG islands in the promoter region of *Chk2* were
209 hypermethylated in both NESCs and ECSCs. CpG islands in the promoter regions of *ATR*, *p53*,
210 $p21^{Waf1/Cip1}$, and *Chk1* were hypomethylated in both NESCs or ECSCs.
211
212 ***ATM mRNA and protein expression in ECSCs***
213 Next, we evaluated *ATM* mRNA levels in NESCs and ECSCs using quantitative
214 RT-PCR. As shown in Figure 4B, ATM mRNA expression was significantly attenuated in
215 ECSCs, compared to that in NESCs (45.9 ± 25.6 vs. 100.0 ± 42.2, respectively; $p < 0.01$).
216 However, ATM protein expression in ECSCs (n=5) was similar to that in NESCs (n=5)

(88.9 ± 45.3 vs. 100.0 ± 47.5, respectively). (Figure 4C).

Demethylation of ATM promoter, restoration of ATM mRNA and protein expression, and phosphorylation of p53 in ECSCs by 5-aza-dC

Finally, we confirmed that 5-aza-dC (10 μM) treatment induced the demethylation of *ATM* promoter (Figure 5A). Simultaneously, 5-aza-dC (10 μM) treatment significantly induced the mRNA expression of ATM ($p < 0.0001$) (Figure 5B). The protein levels of ATM and phosphorylated p53 were also increased by 5-aza-dC (10 μM) treatment (Figure 5C).

Discussion

In the present study, we investigated the effects of a DNA demethylating agent, namely 5-aza-dC, oncell viability, apoptosis and the cell cycle of NESCs and ECSCs. We demonstrated for the first time that 5-aza-dC treatment results in the cell cycle arrest of ECSCs at the G2/M phase. In contrast, 5-aza-dC did not affect the cell cycle of NESCs. However, 5-aza-dC treatment revealed stronger effects on apoptosis in NESCs compared to that of ECSCs. It is suggested that the mechanism of apoptosis is different from that of cell cycle arrest in these cells. Thereafter, we focused on the mechanisms of cell cycle arrest induced by 5-aza-dC. We evaluated the promoter methylation status of genes that negatively regulate the G2/M checkpoint and found that the promoter of *ATM* was hypermethylated in ECSCs. *ATM* mRNA expression was also found to be attenuated in ECSCs compared to that in NESCs. Finally, 5-aza-dC was found to restore the mRNA expression of *ATM*. These findings suggest that promoter hypermethylation of *ATM* occurs in endometriosis. Further, silencing of this gene could be involved in the tumorigenesis of this benign disease by mediating the escape from cell cycle arrest at the G2/M phase. Moreover, with the development of novel DNA demethylating agents selective for hypermethylated DNA, these drugs would become promising options for

the treatment of endometriosis. Alternatively, molecular target drugs against ATM silencing are also promising.

Cell cycle checkpoints including G1/S, intra-S and G2/M are involved in DNA damage response reactions. When DNA is damaged, the G2/M checkpoint functions to prevent damaged DNA from being segregated into daughter cells, and defects in this checkpoint pathway can result in genomic instability, cell death and tumorigenesis (Molinari, 2000; Abraham, 2001). As shown in Figure 1, a G2/M checkpoint is initiated by the activation of ATM and ATR kinases in response to DNA damage (Shiloh, 2003). Activated ATM and ATR mediate subsequent signal transduction cascades that include Chk1, Chk2 and p53/p21$^{Waf1/Cip1}$ (Bartek and Lukas, 2003; Lobrich and Jeggo, 2007). Chk1 and Chk2 inhibit the activity of their shared downstream substrates, cell division cycle 25A (cdc25A), cdc25B and cdc25C. Inhibition of cdc25A/B/C activity results in the induction of G2/M cell cycle arrest through inhibition of cdc2 and its effectors, cyclin B1 and cyclin B2 (Liu *et al.*, 2000; Niida and Nakanishi, 2006; Lobrich and Jeggo, 2007). ATM and ATR also activate tumour suppressors p53 and p21$^{Waf1/Cip1}$ (Bartek and Lukas, 2003). Activation of p21$^{Waf1/Cip1}$ negatively regulates cdc2 and induces G2/M cell cycle arrest (Zhan *et al.*, 1999).

Of these negative regulators of the G2/M checkpoint, we found that only *ATM* expression was attenuated by methylation in ECSCs. Disruption of the tumour suppressor function of this protein can allow the cell to bypass the G2/M checkpoint. Accordingly, loss of functional ATM is associated with both decreased genomic integrity and increased cancer risk. Repression of ATM expression by DNA hypermethylation has been reported in head and neck carcinomas (Ai *et al.*, 2004), oral squamous cell carcinoma (Rigi-Ladiz *et al.*, 2011), colorectal cancers (Bai *et al.*, 2004), non-small cell lung cancer (Safar *et al.*, 2007), breast cancers (Vo *et al.*, 2004) and malignant lymphomas (Huang *et al.*, 2007). It has also been suggested that epigenetically repressed *ATM* might be responsible for resistance to DNA damage signals and

the acquisition of the proliferative characteristics of endometriosis. ATM is a pleiotropic nuclear protein that is activated by endogenous DNA breaks or DNA-damaging agents (Kastan and Lim, 2000; Khanna and Jackson, 2001; Shiloh, 2003; Lavin *et al.*, 2005; Shiloh and Ziv, 2013). ATM promotes cell cycle arrest to prevent the processing of damaged DNA, activate DNA-repair pathways, and induce apoptosis after severe DNAdamage (Kastan and Lim, 2000; Lavin *et al.*, 2005). Regarding the mechanism associated with the G2/M checkpoint, ATM further activates negative regulators of the cell-cycle checkpoint including p53, Chk1, and Chk2 after DNA damage, as summarised in Figure 1.

We demonstrated that 5-aza-dC treatment results in G2/M cell cycle arrest by restoring ATM expression in ECSCs. It has been reported that DNMT1, DNMT3A and DNMT3B, the enzymes responsible for DNA methylation, are overexpressed in endometriosis (Wu *et al.*, 2007), suggesting the presence of rampant hypermethylation in corresponding genes associated with endometriosis pathogenesis (Nasu *et al.*, 2011b). The mechanism of action of the DNA demethylating agent 5-aza-dC is associated with its incorporation into DNA by inhibiting DNA methyltransferase activity (Haaf, 1995). Consequently, genes silenced by hypermethylation are demethylated and re-expressed. 5-aza-dC is one of the most studied nucleoside analogs of cytosine and the mechanism through which it inhibits DNA methylation involves its incorporation at cytosine positions during DNA replication (Nasu *et al.*, 2011b; Esteller, 2008). It is widely recognised that nucleoside analogs of cytosine, such as 5-aza-dC, exert their anti-tumour effects by reactivating aberrantly hypermethylated growth regulatory genes. Further studies on DNA demethylating agents in endometriosis and the selective reactivation of genes repressed by DNA hypermethylation might lead to promising epigenetic treatment strategies for endometriosis. Otherwise, molecular target drugs against ATM silencing are also hopeful for the treatment of endometriosis.

Hypermethylation of gene promoters and silencing of the corresponding genes have

been reported in endometriosis, for as *PR*-B (Wu *et al.*, 2006), *HOXA10* (Wu *et al.*, 2005), *ER*-β (Xue *et al.*, 2007a), aromatase (Izawa *et al.*, 2008), *STRA6* (Yamagata *et al.*, 2014), *HSD17β2* (Yamagata *et al.*, 2014), *miR-196b* (Abe *et al.*, 2013) and *miR-503* (Hirakawa *et al.*, 2016). However, the promoter regions of *GATA6*, *SF-1* and *STAR* genes are hypomethylated in endometriosis (Xue *et al.*, 2007b; Dyson *et al.*, 2014; Yamagata *et al.*, 2014; Izawa *et al.*, 2018). It is suggested that the alteration of these gene expressions are favourable for the progression of endometriosis. Recently, DNA methylation microarray techniques have demonstrated the aberrant methylation status in endometriosis (Dyson *et al.*, 2014; Yamagata *et al.*, 2014; Yotova *et al.*, 2017; Izawa *et al.*, 2018). Yamagata *et al.* (2014) compared the methylation status of eutopic endometrial stromal cells from women with endometriosis and to those from women without endometriosis and found that the methylation status of some genes were different between these cell types. It is considered that the changes of methylation status might be associated with pathogenesis of endometriosis. The methylation status of *ATM* was not described in these reports. However raw data of DNA methylation microarrays were available in public databases in two of the reports (Yamagata *et al.,* 2014; Yotova *et al.*, 2017), and these showed a tendency toward hypermethylation of *ATM*. Interestingly, *ATM* promoter methylation status had marginal effect on the ATM protein expression. It is considered that the post-transcriptional regulatory mechanisms of ATM is complicated.

The limitation of the present study is that we only used in-vitro culture model of stromal cells to evaluate the mechanism associated with 5-aza-dC. Since endometriotic tissues consist of a variety of cell types, further studies with endometriotic epithelial cells as well as the whole tissues are necessary to fully understand the epigenetic changes in this disease. In addition, similar experiments with eutopic endometrial stromal cells from women with endometriosis are necessary to strengthen the findings of the present study. Moreover, considering the malignant transformation that can accompany endometriosis, the methylation

317 status of *ATM* and its related genes in secondary malignant neoplasms should be examined.

318 In conclusion, we observed that *ATM* expression was repressed in ECSCs via the
319 hypermethylation of its promoter. Further, 5-aza-dC induced the cell cycle arrest of ECSCs at
320 the G2/M phase by restoring *ATM* expression. These findings provide important evidence that
321 endometriosis is ultimately an epigenetic and neoplastic disease. These results and further
322 studies on the methylation status of other genes associated with the development of
323 endometriosis might lead to novel treatment strategies for this disease.

324

325

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Authors' roles

K.N. and H.N. participated in the study design, data analysis and manuscript drafting. T.H., Y.A., K.T. and R.Z. executed the study.

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Conflict of interest

None declared.

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468

Figure Legends

Figure 1. Regulatory mechanism of G2/M checkpoint. Representative regulators of G2/M checkpoint and their signal pathways are shown. Except for cdc25A, all of these molecules posess dense CpG islands in the promoter region of their genes.

Figure 1

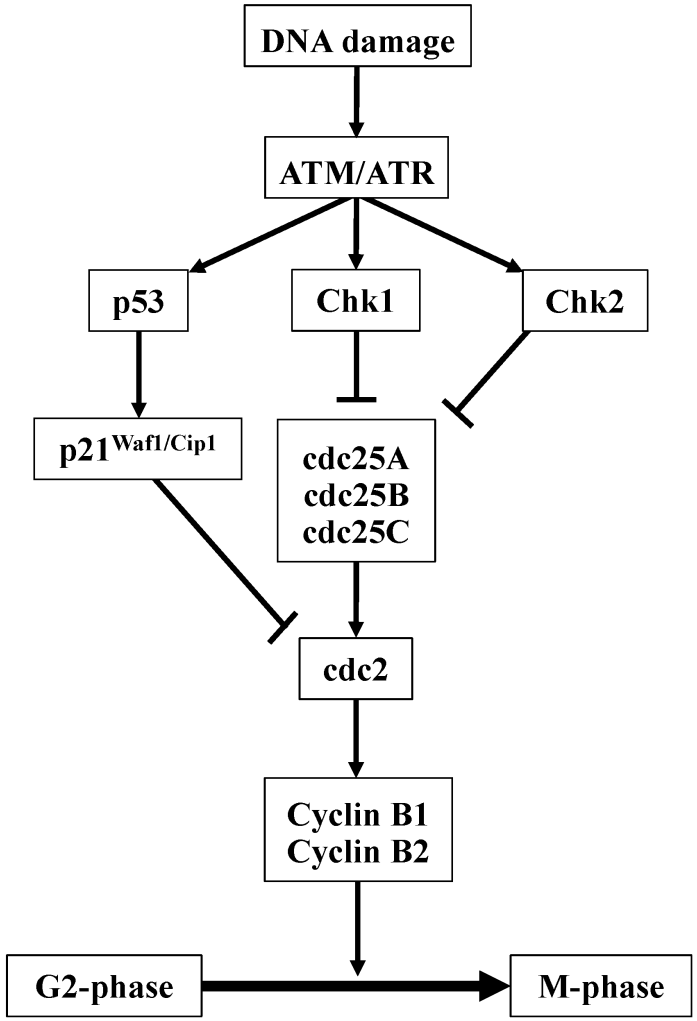

Figure 2. Effects of 5-aza-dC on cell viability and apoptosis of NESCs and ECSCs. (A) Modified MTT assay. 5-aza-dC treatment had marginal effects on the cell viability of NESCs and ECSCs. (B) Cell death detection ELISA. 5-aza-dC significantly induced the apoptosis of NESCs, but had a marginal effect on the apoptosis of ECSCs. (C) Caspase-Glo 3/7 assay. 5-aza-dC significantly activated caspase 3/7 in NESCs and ECSCs. Data were presented as mean ± SD. *$p<0.01$, **$p<0.005$ vs. controls (Bonferroni test).

Figure 2

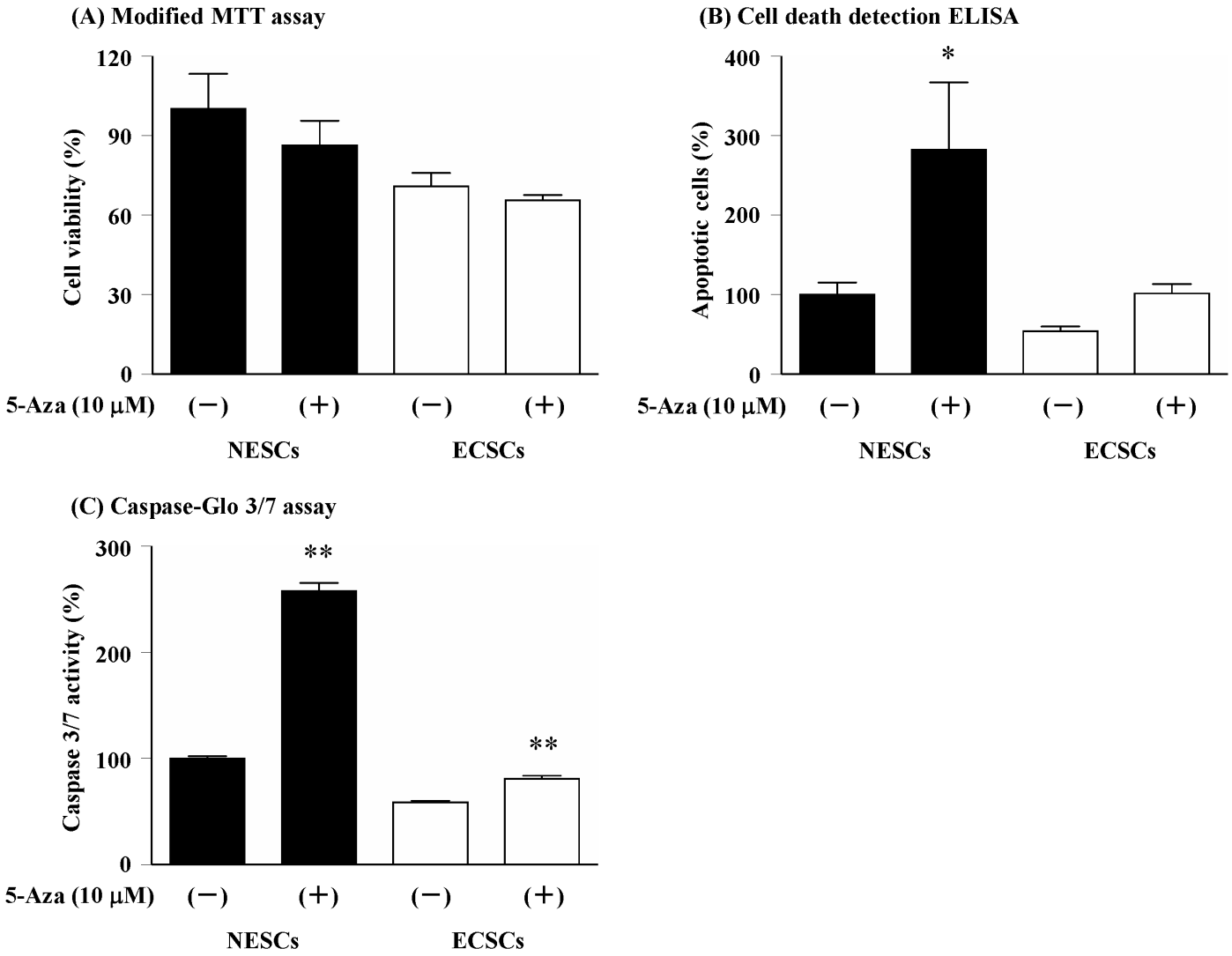

Figure 3. Effects of 5-aza-dC on the cell cycle of NESCs (A, C, E) and ECSCs (B, D, F). Treatment with 5-aza-dC (10 μM) for 96 h did not affect the cell cycle of NESCs (n=3), whereas, 5-aza-dC treatment induced the cell cycle arrest of ECSCs (n=3) at G2/M phase. Error bars show the mean ± SD. *$p<0.05$, **$p<0.0001$ vs. controls (Bonferroni test).

Figure 3

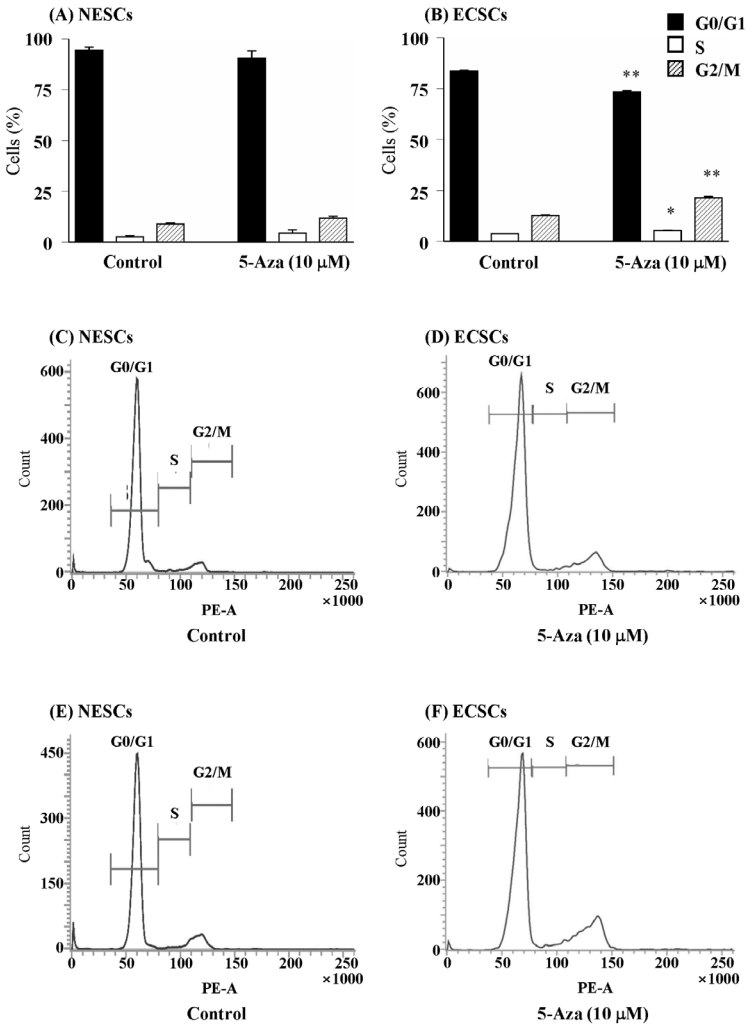

Figure 4. Results of MSP, RT-PCR, and western blotting analysis. (A) Methylation status of the gene promoters of the negative regulators of G2/M checkpoint in NESCs (n=8) and ECSCs (n=8). CpG islands in the promoter region of *ATM* were hypermethylated in ECSCs, but not in NESCs. In contrast, CpG islands in the promoter region of *Chk2* were hypermethylated in both NESCs and ECSCs. CpG islands in the promoter regions of *ATR*, *p53*, *p21* and *Chk1* were hypomethylated in both NESCs or ECSCs. U, unmethylated alleles; M, methylated alleles. (B) *ATM* mRNA expression in ECSCs (n=10) and NESCs (n=10). *ATM* mRNA expression is significantly attenuated in ECSCs compared to that in NESCs. Error bars show the mean ± SD. *$p<0.01$ vs. NESCs (Mann-Whitney *U*-test). (C) ATM protein expression in ECSCs (n=5) and NESCs (n=5). ATM protein expression in ECSCs was similar to that in NESCs (88.9 ± 45.3 vs. 100.0 ± 47.5, respectively).

Figure 4A

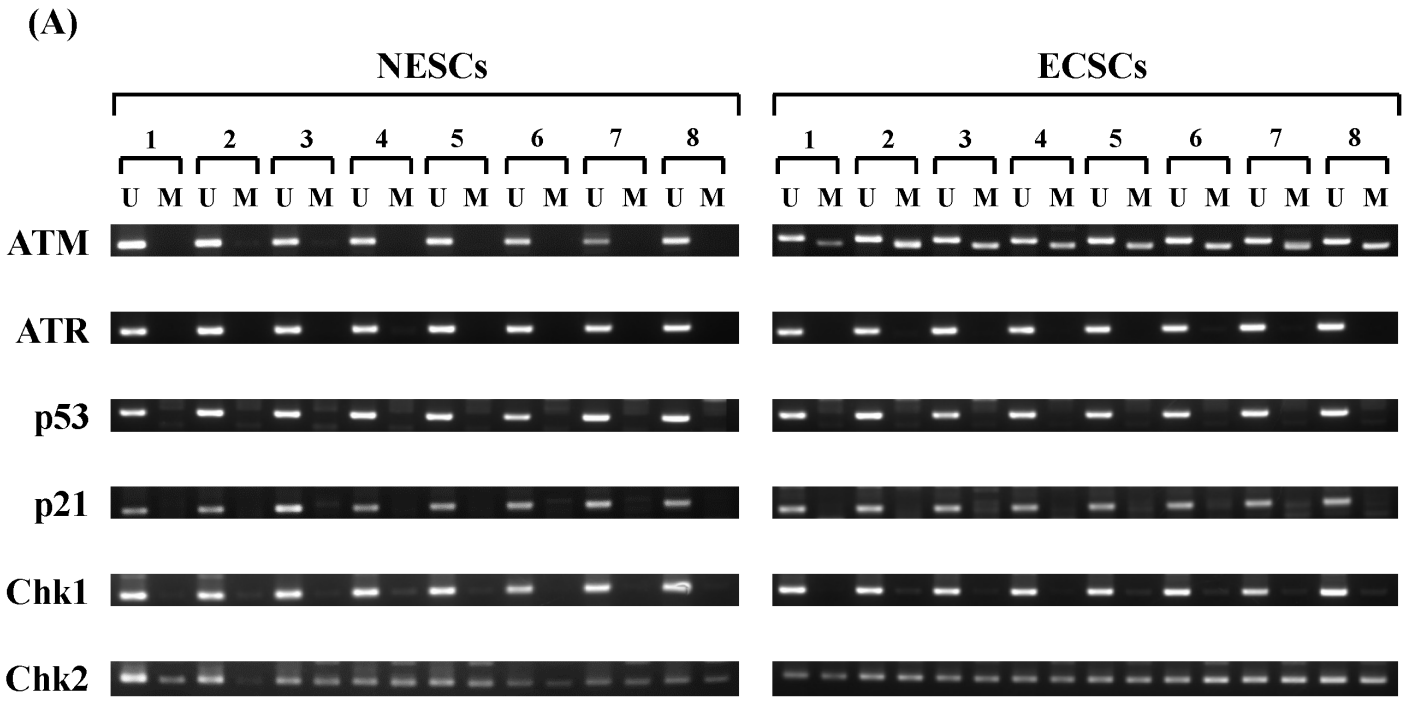

Figure 4B,C

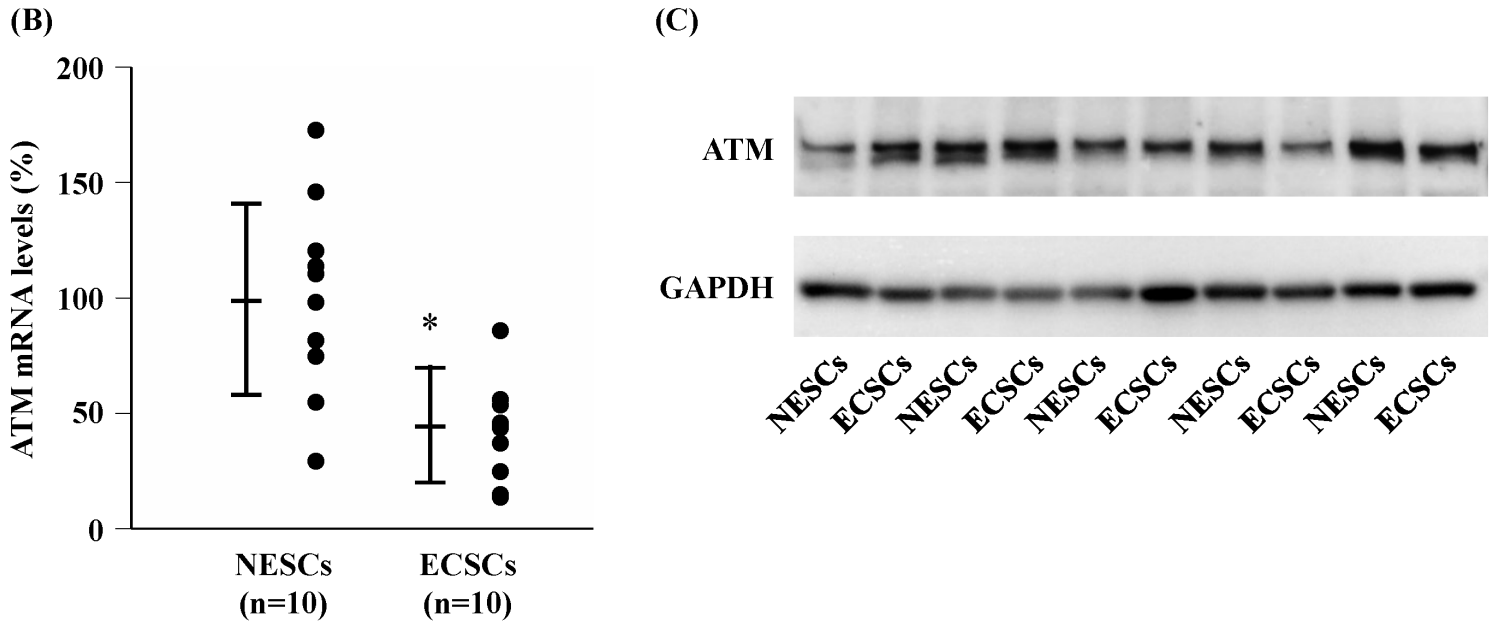

Figure 5. Effects of 5-aza-dC on the methylation status of *ATM* promoter, *ATM* expression, and phosphorylation of p53. (A) Treatment with 5-aza-dC (10 μM) for 96 h induced demethylation of *ATM* promoter in ECSCs (n=8). (B) Treatment with 5-aza-dC (10 μM) for 96 h induced *ATM* mRNA expression in ECSCs (n=3). Error bars show the mean ± SD. *p<0.0001 vs. controls (Student's *t*-test). (C) Treatment with 5-aza-dC (10 μM) for 96 h upregulated the ATM protein levels and induced the phosphorylation of p53 in ECSCs. Representative results of three repeated experiments are shown.

Figure 5

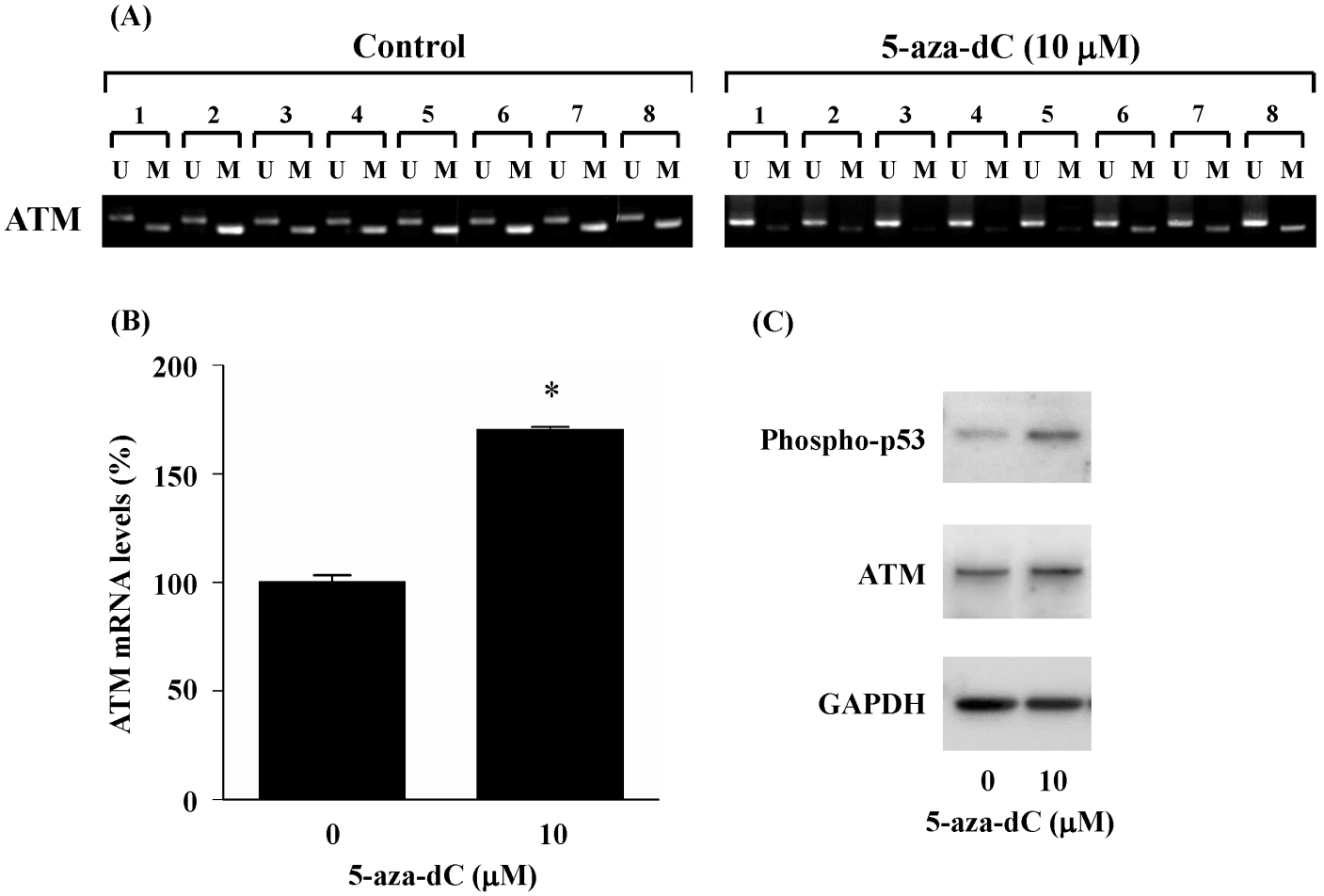

Table 1. Primers for MSP and size of their PCR products

Gene name	Methylation status	Primer sequence	PCR product	References
ATM	Methylated	Forward: GGAGTTCGAGTCGAAGGGC Reverse: CTACCTACTCCCGCTTCCGA	239bp	Roy *et al.*, 2006
	Unmethylated	Forward: GTTTTGGAGTTTGAGTTGAAGGGT Reverse: AACTACCTACTCCCACTTCCAA	246bp	
$p21^{Waf1/Cip1}$	Methylated	Forward: TACGCGAGGTTTCGGGATC Reverse: CCCTAATATACAACCGCCCCG	171bp	Brakensiek *et al.*, 2005
	Unmethylated	Forward: GGATTGGTTGGTTTGTTGGAATTT Reverse: ACAACCCTAATATACAACCACCCCA	161bp	
p53	Methylated	Forward: TTCGGTAGGCGGATTATTTG Reverse: AAATATCCCCGAAACCCAAC	139bp	Fruhwald *et al.*, 2001
	Unmethylated	Forward: TTGGTAGGTGGATTATTTGTTT Reverse: CCAATCCAAAAAAACATATCAC	247bp	
Chk1	Methylated	Forward: GGGGGTAGGAGGGATTAATTC Reverse: AAAAACGATATAAAACAAAAAACGC	194bp	Mazumder Indra *et al.*, 2011
	Unmethylated	Forward: GGGGTAGGAGGGATTAATTT Reverse: AAAAAACAATATAAAACAAAAAACC	195bp	
Chk2	Methylated	Forward: TTACGTTTGTTTTTTAGATTTTCGT Reverse: AAATTCTTCTACCCACAATACCG	213bp	Wang et al., 2010
	Unmethylated	Forward: TTATGTTTGTTTTTTAGATTTTTGT Reverse: CAAATTCTTCTACCCACAATACCA	213bp	

Table 2. A map of the CpG islands in the *ATM* promoter.

ATAGCCGGGTCCAATAACCCTCCATCCCGCGTCCGCGCTTACCCAATACAAGCCGGGCTACGTCCGAGGGTAACAACATGATC
AAAACCACAGCAGGAACCACAATAAGGAACAAGACTCAGGTTAAAGCAAACACAGCGACAGCTCCTGCGCCGCATCTCCTGG
TTCCAGTGGCGGCACTGAACTCGCGGCAATTTGTCCCGCCTCTTTCGCTTCACGGCAGCCAATCGCTTCCGCCAGAGAAAGAAA
GGCGCCGAAATGAAACCCGCCTCCGTTCGCCTTCGGAACTGTCGTCACTTCCGTCCTCAGACTTGGAGGGGCGGGGATGAGGA
GGGCGGGGAGGACGACGAGGGCGAAGAGGGTGGGTGAGA GCCCCGGAGCCCGAGCCGAAGGGC GAGCCGCAAACGCTAA
GTCGCTGGCCATTGGTGGACATGGCGCAGGCGCGTTTGCTCCGACGGGCCGAATGTTTTGGGGCAGTGTTTTGAGCGCGGAGA
CCGCGTGATACTGGATGCGCATGGGCATACCGTGCTCTGCGGCTGCTTGGCGTTGCTTCTTCCTCCAGAAGTGGGCGCTGGGCA
GTCACGCAGGGTTTGAA CCGGAAGCGGGAGTAGGTAGCT GCGTGGCTAACGGAGAAAAGAAGCCGTGGCCGCGGGAGGA
GGCGAGAGGAGTCGGGATCTGCGCTGCAGCCACCGCCGCGGTTGATACTACTTTGACCTTCCGAGTGCAGTGGTAGGGGCGCG
GAGGCAACGCAGCGGCTTCTGCGCTGGGAAATTCAGTCGTGTGCGACCCAGTCTGTCCTCTCCCCAGACCGCCAATCTCATGC
ACCCCTCCAGAG

Underline indicates the CpG islands. Primer binding sites for unmethylated DNA are indicated by a box. Primer binding sites for methylated DNA are indicated by halftone meshing.