Epigenetic silencing of *ARNTL*, a circadian gene and potential tumor suppressor in ovarian cancer

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Received July 24, 2014; Accepted August 13, 2014

DOI: 10.3892/ijo.2014.2627

Abstract. Ovarian cancer is the fifth leading cause of cancer death and the most deadly gynecological malignancy in women. Epigenetic modifications play an important role in regulating gene transcription. Specifically, aberrant promoter hypermethylation has been implicated as a hallmark of cancer. In order to identify genes that are differentially methylated in ovarian cancer, we performed meDIP-chip in various ovarian cancer cell lines using Agilent 244K CpG island microarray. One of the targets, *ARNTL* which is a core component of the circadian clock is methylated in a sub-set of ovarian cancer cell lines. Combined bisulfite restriction analysis (COBRA) confirmed the results of the microarray. Additional analysis using ChIP-PCR revealed that promoter of ARNTL is enriched with the repressive histone mark H3K27me3 in CP70 and MCP2 ovarian cancer cells. Treatment with the EZH2 inhibitor (GSK126) significantly restored *ARNTL* expression in these cells (CP70 and MCP2). Further functional analysis demonstrated that overexpression of *ARNTL* inhibited cell growth and enhanced chemosensitivity of cisplatin in ovarian cancer cells. Finally, overexpression of *ARNTL* restored the rhythmic activity of *c-MYC* in ovarian cancer cells. These results suggested that *ARNTL* may be a tumor suppressor and is epigenetically silenced in ovarian cancer.

Introduction

Circadian rhythms are important mechanisms regulating the daily oscillation of several biological processes (1,2). These mechanisms provide the organisms with survival advantages by organizing their behavior and physiological adaptation to the cyclic changes in the environment (3,4). Disruption of these rhythms may have profound influence on human health (5-7) and has been known as a risk factor in the development of human cancer (7,8). Studies also found that the proliferation of tumor cells followed the autonomous circadian patterns that are out of phase from non-tumor cells (9-12). The evidence suggested that the circadian clock may suppress tumor formation at the systemic, cellular and molecular levels (13-15).

ARNTL (also known as BMAL1), an HLH-containing transcription factor, is an important player in the control of circadian rhythms (15). ARNTL together with CLOCK regulates the transcription of several E-box motif containing genes such as c-myc (16,17). Importantly, expression of ARNTL has been demonstrated to be downregulated in several human cancer including ovarian, head and neck and in blood (18-20). Notably, ARNTL was also found to be epigenetically silenced in leukemia. Re-expression of ARNTL in leukemia cells inhibited tumor growth and restored the expression pattern of circadian genes thus suggesting that it may be a tumor suppressor.

Epigenetic modifications, including DNA methylation play an important role in gene expression and cell fate commitment (21,22). Previous reports including ours have shown that tumor suppressor genes can be epigenetically silenced in ovarian cancer (23-29). In order to identify genes that are suppressed by promoter hypermethylation in ovarian cancer, we performed meDIP-chip using Agilent 244K CpG island microarray in a panel of ovarian cancer cell lines. Our result showed that *ARNTL* is epigenetically silenced by promoter methylation and histone modifications in a sub-set of ovarian cancer cells. Restoration of *ARNTL* suppressed cancer cell growth and restored the expression pattern of *c-MYC* thus suggesting that it may be a tumor suppressor in ovarian cancer.

Materials and methods

Cell culture and epigenetic treatment. Immortalized ovarian surface epithelia cells (IOSE) (30) were maintained in a 1:1

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Key words: ovarian cancer, epigenetic modifications, *ARNTL*, circadian clock, tumor suppressor

Table I. Primer sequences.

Gene	Primer sequence (5'-3')	Annealing temperature (˚C)	Product size (bp)
RT-PCR			
ARNTL RT-F1	CTGGAGCACGACGTTCTTTCTT	60	123
ARNTL RT-R1	GGATTGTGCAGAAGCTTTTTCG		
COBRA-PCR			
ARNTL BSP F	GGTGTTGTAGGAGTTTTTAAAGGGG	60	342
ARNTL BSP R	ACRAACTTAAACTCCCCAAACCC		
ARNTL cDNA construct			
ARNTL F	AGGGCTAGCGCCCACTAGGAGATGCTATGATTAAT	60	1791
ARNTL R	AAGGGATCCGTAGTGTTTACAGCGGCCATG		
ARNTL ChIP PCR			
ARNTL ChIP F	TGGAAGGAAATGCAATGGAATC	60	130
ARNTL ChIP R	CCCGAGGACTGCAAGTGTTC		

mixture of medium 199 (Sigma, St. Louis, MO, USA) and 105 (Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, Grand Island, NY, USA), 400 ng/ml hydrocortisone (Sigma), 10 ng/ml EGF and 50 U/ml of penicillin/streptomycin (P/S; Gibco). HeyC2 was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FBS and 50 U/ml of P/S, 1X MEM NEAA (Gibco) and 0.01 M of HEPES (Gibco). A2780 and its cisplatin-resistant sublines CP70, MCP2 and MCP3 cells were propagated in RPMI-1640 (Gibco) supplemented with 10% FBS and 50 U/ml of P/S. For demethylation treatment, cells were plated in a 60-mm plate and treated with 0.5 μM 5'-aza-2'-deoxycytidine (5-aza-dC; Sigma) for 3 days. For treatment with the EZH2 inhibitor (GSK126; Cayman Chemical, Ann Arbor, MI, USA), cells were either treated with 10 μM GSK126 alone for 3 days or combined with 5-aza-dC for 3 days. In the control experiment, cells were treated with DMSO. Culture medium and drugs were replaced every 24 h. Cells were then harvested for RNA analysis.

Extraction of RNA and quantitative RT-PCR. RNA extraction was performed using TRizol reagent (Life Technologies) according to the manufacturer's protocol. To remove potential contaminating DNA from the complementary DNA, 1 μg of total RNA was treated with DNase I (Amplification Grade; Invitrogen) prior to reverse transcription. First Strand cDNA Synthesis used MMLV High Performance Reverse Transcriptase (Epicentre, Chicago, IL, USA). The expression of *ARNTL* in ovarian cancer cell line was examined by RT-PCR analysis. Ten-fold diluted cDNA (4 μl) was amplified in a total volume of 20 μl containing 2X SYBR® Green Real-time PCR Master Mix (Toyobo, Osaka, Japan), 0.2 μM of each primer (Table I). Samples were first denaturated at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec, at annealing temperature for 30 sec, and extension at 72˚C for 30 sec, followed by a melting curve. The relative gene expression level was determined by comparing the threshold cycle of the test gene against the Ct of *GAPDH* in a given sample. The qPCR reactions were carried out using ABI StepOne™ Real-Time PCR systems (Applied Biosystems, Foster City, CA, USA).

Protein extraction and western blot analysis. Before protein extraction, $1x10^6$ cells were seeded into 100-mm dish. When the cells reached 90% confluence, the medium was removed and the dish was washed with 1X PBS at 4˚C. The cells were then lysed with 100 μl of PRO-PREP protein extraction solution (iNtRON Biotechnology, Seongnam, Korea) according to the manufacturer's protocol. Samples and pre-stained marker were loaded to a 12.5% polyacrylamide gel for electrophoresis. The proteins were then electrophoretically transferred from the gel onto a PVDF membrane by the Mini Trans-Blot® Electrophoretic Transfer Cell system (Bio-Rad Laboratories, Hercules, CA, USA) at 400 mA for ~90 min. After the transfer, the membrane was incubated with 5% non-fat milk in 1X TBST for 1 h at room temperature. Then the first antibodys, ARNTL antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or actin antibody (Santa Cruz Biotechnology) was diluted with 5% non-fat milk in 1X TBST and was added into the membrane followed by incubation for overnight at 4˚C. The membrane was then washed in 1X TBST at room temperature 3 times. The secondary antibody conjugated with horseradish peroxidase (IgG-HRP antibody) was also diluted with 5% non-fat milk in 1X TBST and incubated with membrane at room temperature for 1 h. The membrane was washed with 1X TBST at room temperature 3 times. The Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA, USA) was prepared by mixing equal volumes of the HRP substrate luminol reagent as well as the HRP substrate peroxide solution and added onto the membrane. Finally, the light-signal was detected by BioSpectrum® 2D Imaging System (UVP, Upland, CA, USA).

Bisulfite conversion and combined bisulfite restriction analysis (COBRA). DNA was bisulfite modified using EZ DNA methylation kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. For COBRA analysis, 4 μl of bisulfite

converted DNA was first amplified using specific primers (Table I) targeting promoter region of *ARNTL* followed by digested with 20 U of *BstUI* (CGCG) (New England Biolabs, Ipswich, MA, USA). *In vitro* methylated DNA (IVD) (Merck-Millipore) was used as a positive control for methylation and water was used as negative control. For the un-digested control, water instead of *BstUI* was added. Digested products were separated on 1.5% agarose gel for visualization. The PCR reactions were carried out using the Veriti® 96-Well Thermal Cycler (Applied Biosystems).

Plasmid constructs and transfection. The CDS of *ARNTL* was amplified by PCR using specific primers (Table I) from cDNA of IOSE cells which expressed *ARNTL*. The PCR products were ligated into pIRES2-EGFP vector (Promega, Madison, WI, USA) for sequencing confirmation. *ARNTL*-pIRES was then digested with *Nhe*I (New England Biolabs) and *Bam*HI (New England Biolabs), and inserted into multiple cloning site of pIRES2-EGFP expression vector, which was predigested with *Nhe*I and *Bam*HI. *ARNTL* expression vector or empty vector were transfected into CP70 and MCP2 cells using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. The PCR reactions were carried out using the Veriti® 96-Well Thermal Cycler (Applied Biosystems).

Stable cell lines. Following transient transfection, the transfected cells were cultivated with fresh culture medium containing 400 μg/ml geneticin (G418; Sigma) and replaced every 3 days. Each single colony that formed was selected for further culture.

Colony forming assay. CP70 and MCP2 cells were plated at $1x10^6$ cells/plate in a 100-mm culture dish one day before transfect *ARNTL*. Transfection was performed as previously described. On the second day after the transfection, cells were plated into 3 plates with fresh culture medium containing 400 μg/ml G418 (Sigma) and replaced every 3 days. Surviving colonies were stained with 0.4% crystal violet (Sigma) in 50% methanol and visible colonies were counted. The colony numbers were counted using Image-Pro 3D Suite software version 5.1.1.38 for windows (Media Cybernetics, Inc., Bethesda, MD, USA).

Cell proliferation assay. Cell growth was assessed by MTS assay, as previously described. In brief, for MTS assays, $1x10^3$ cells were seeded in 96-well plates for 4 days with or without various concentrations of cisplatin (Sigma). Cell growth was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega), according to the manufacture's protocol. Relative cell numbers were assessed using a 96-well ELISA plate reader (Multiskan® FC Microplate Photometer; Thermo Fisher Scientific Inc., Waltham, MA) with an absorbance set at 492 nm.

Soft agar assay for colony formation. Base layer of agar (2.5 ml) (0.5% agar in culture medium) was allowed to solidify in a 6-well flat bottomed plate before the addition of 2 ml of cell suspensions containing $1x10^4$ cells in 0.3% agar in culture medium. The cell-containing layer was then solidified at room temperature for 20 min. Colonies were allowed to grow for 14-21 days at 37˚C with 5% CO_2 before imaging. Plates were stained with 1 mg/ml iodonitrotetrazolium chloride (Sigma) overnight at 37˚C. The colonies which contained at least 50 cells were counted. The colony numbers were counted using Image-Pro 3D Suite software version 5.1.1.38 for windows (Media Cybernetics).

Analysis of apoptosis by propidium iodide staining and flow cytometry. Cells ($1x10^5$) were seeded in 60-mm plates for 2 days with or without 1 mg/ml of cisplatin (Sigma). The supernatant was carefully collected, and then wash in PBS. Followed by centrifugation at 200 x g for 5 min at room temperature. The supernatant was aspirated, the pellet resuspended in ~5 ml cold 1X PBS, and then centrifuged for 5 min at 200 x g. Cells were fixed by adding 4.5 ml of 70% (v/v) cold ethanol to the cell suspension keeping the tubes on ice. The cells were stored in ethanol solution at -20˚C at least for 2 days. To remove the ethanol solution, the supernatant was centrifuged at 400 x g for 5 min, the cells were washed in 5 ml of PBS and centrifuged at 400 x g for 5 min. The supernatant was removed and the cells were resuspended in 500 μl of propidium iodide (1 mg/ml), then incubated for at least 30 min at room temperature in the dark. Cells were analyzed by flow cytometry.

Serum shock for detecting circadian rhythmic activity. Cells were grown to confluence in 100-mm culture dishes in RPMI-1640 media (Invitrogen) supplemented with 10% FBS, followed by culture for 2 days with starvation medium (0.5% serum). At time t=0, the medium was exchanged for 50% serum in RPMI-1640; after 2 h, the medium was replaced with serum-free RPMI-1640. At the indicated times (0, 4, 8, 12, 16 and 20 h) the dishes were prepared for immediate RNA extraction.

Quantitative ChIP-PCR. Cells ($1x10^6$) were cross-linked with 1% fresh formaldehyde and then washed with cold 1X PBS in the presence of the protease inhibitor. Cell were homogenized and the chromatin was subjected to ChiP pull down using magnetic Dynabeads (Invitrogen) and antibodies (against H3K4me3; Active Motif, Carlsbad, CA, USA; against H3K27me3; Merck-Millipore; EZH2; Cell Signaling Technology, Danvers, MA, USA; and IgG; Cell Signaling Technology). The quantity of ChIP DNA was measured by quantitative-PCR analysis using 200 pg of pull-down DNA amplified by *ARNTL* promoter specific primer (Table I), and then quantified as a percentage of the input signal. The PCR reactions were carried out using the Veriti® 96-Well Thermal Cycler (Applied Biosystems).

Statistical analysis. Comparison between groups were assessed by the unpaired t-test. All statistical calculations were done using the statistical package SPSS version 13.0 for windows (SPSS, Inc., Chicago, IL, USA). A P-value <0.05 was considered statistically significant.

Results

ARNTL is frequently downregulated in ovarian cancer cell lines and epigenetic treatments restore ARNTL expression.

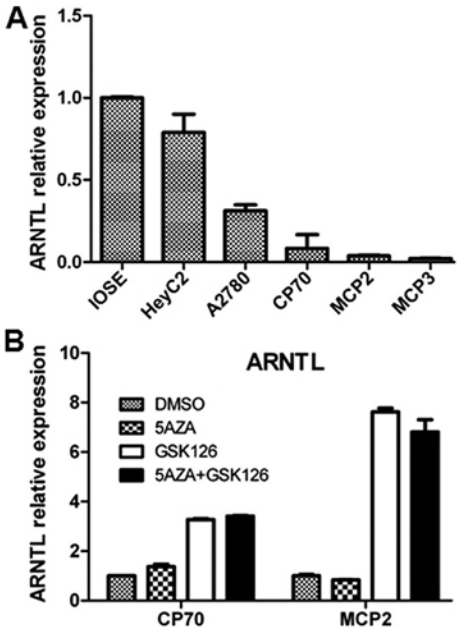

Figure 1. Downregulation of *ARNTL* in ovarian cancer cell lines. (A) Expression of *ARNTL* in immortalized ovarian surface epithelium cells (IOSE) and a panel of ovarian cancer cell lines were determined by quantitative RT-PCR relative to *GAPDH* as an internal control. As compared to IOSE cells, *ARNTL* was downregulated in A2780, CP70, MCP2 and MCP3 ovarian cancer cells. (B) CP70 and MCP2 ovarian cancers were treated with 5-aza-2'-deoxycytidine (5AZA) and/or EZH2 inhibitor (GSK126). Following the designated treatment schemes, mRNAs were harvested, and expression levels of *ARNTL* in treated cells were measured by quantitative RT-PCR. Expression of *ARNTL* was restored after epigenetic drug treatment.

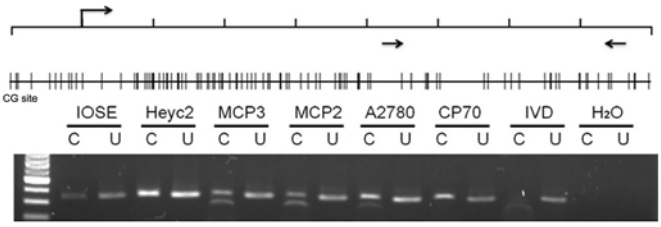

Figure 2. Promoter methylation of *ARNTL* in ovarian cancer cell lines. Methylation analysis of *ARNTL* promoter in ovarian cancer patients was performed by COBRA-assay. U, uncut; C, restriction enzyme *BstU1* cut; IVD, *in vitro* methylated DNA, which was completely digested by *BstU1* and served as positive control for COBRA reaction.

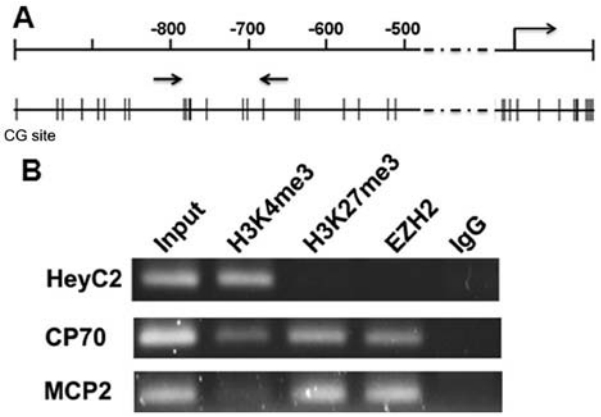

Figure 3. Histone modifications of *ARNTL* promoter in ovarian cancer cell lines. ChIP-PCR assays were performed with antibodies directed against trimethyl-H3-K4, trimethyl-H3-K27 and EZH2 in *ARNTL* promoter in HeyC2, CP70 and MCP2 cells. (A) Schematic diagram showing the corresponding CpG site and location for ChIP-PCR primer in the promoter region of *ARNTL*. (B) The relative binding of each antibody to the corresponding region was measured by PCR.

To investigate genes that are hypermethylated in ovarian cancer cell lines, we performed meDIP-Chip using Agilent 244K CpG island microarray in IOSE and a panel of ovarian cancer cell lines. One of the targets that showed promoter hypermethylation in a sub-set of ovarian cancer cell is *ARNTL* (also known as *BMAL1*). To confirm our result, we performed quantitative real-time RT-PCR to examine the mRNA level of *ARNTL* in these cancer cell lines. Expression of *ARNTL* was downregulated in A2780, CP70, MCP2 and MCP3 ovarian cancer cell lines as compared with IOSE (Fig. 1A). To examine if epigenetic modifications contribute to this downregulation, we treated the cells with DNA methylation inhibitor, 5-aza and/or the EZH2 inhibitor, GSK126 (31) in CP70, MCP2 ovarian cancer cell lines. The resulted indicated that treatment of 5-aza alone resulted in a partial re-expression of *ARNTL* in CP70 cells, while treatment with GSK126 resulted in a robust re-expression of *ARNTL* in these cells, thus, suggesting that *ARNTL* silencing may occur through epigenetic events (Fig. 1B).

The transcription of ARNTL is associated with promoter methylation. To further confirm if epigenetic modifications contribute to the silencing of *ARNTL*, we examined DNA methylation in the promoter region of *ARNTL* in the ovarian cancer cell lines. Combined bisulphite restriction analysis (COBRA) was conducted to determine the methylation status of 342 bp CpG island spanning (+421 bp to +763 bp) region around the transcription start site in the promoter region of *ARNTL*. Promoter methylation was obvious in MCP2, MCP3 and A2780 cells (Fig. 2). However, promoter methylation was not observed in IOSE and HeyC2 cells which expressed *ARNTL*. Notably, methylation was not observed in CP70 cells which did not express *ARNTL*.

Histone modification of ARNTL promoter in ovarian cancer cell lines. Beside DNA methylation, histone modifications also control gene expression through their effects on chromatin structure. We then analyzed the histone modifications for the active and repressive chromatin marks in the *ARNTL* promoter. Chromatin immunoprecipitation coupled with PCR (ChIP-PCR) was performed using antibodies against trimethylated lysine 4 of histone H3 (H3K4me3), trimethylated lysine 27 of histone H3 (H3K27me3) and EZH2. As expected, active histone mark trimethyl-H3K4 but not repressive mark trimethyl-H3K27 was enriched in ARNTL-expressing HeyC2 cells. On the contrary, trimethyl-H3K27 and EZH2 but not trimethyl-H3K4 were enriched at the promoter region of CP70 and MCP2 cells (Fig. 3B). Hence, histone modifications were also involved in the gene silencing mechanism of *ARNTL* in ovarian cancer cell lines.

ARNTL inhibits cell proliferation and colony formation. Having demonstrated that *ARNTL* is epigenetically silenced

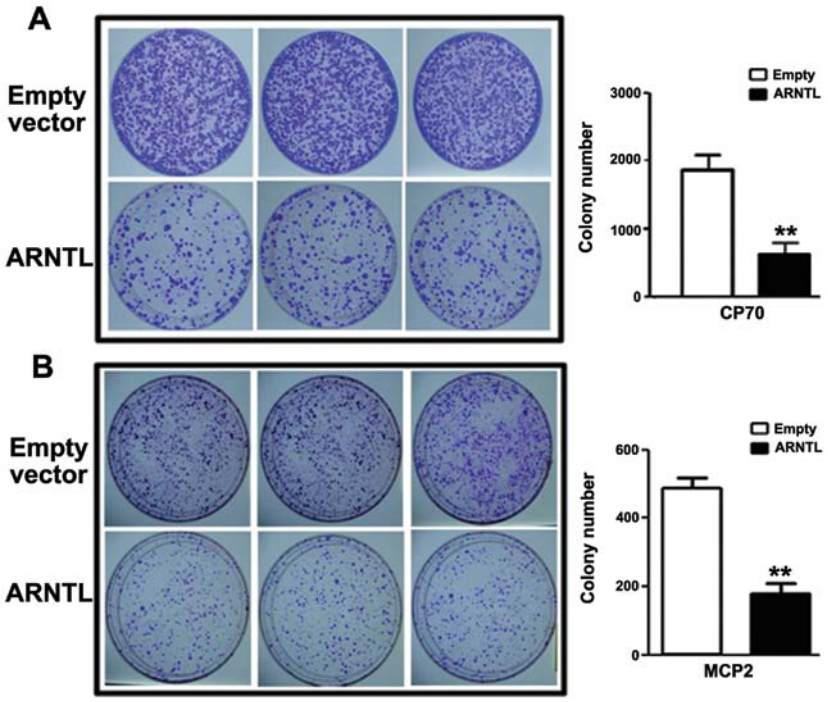

Figure 4. Overexpression of *ARNTL* suppressed the growth of CP70 and MCP2 by colony formation assay. Expression of *ARNTL* inhibited the tumor growth of (A) CP70 and (B) MCP2 ovarian cancer cells as demonstrated in the colony forming assay. Cells transfected with *ARNTL* expressing vector showed a significant reduction in the number of colonies as compared with control (left panel). Right panel shows the quantitative analysis of the colony forming assay. Each error bar represents standard error calculated from triplicate experiments. $^{**}P<0.01$.

by DNA methylation and histone modifications, we then investigated the role of *ARNTL* in ovarian cancer cell lines. By colony formation assay, CP70 or MCP2 cells transfected with *ARNTL* showed a significant decrease in colony numbers as compared to control cells (Fig. 4).

ARNTL inhibits anchorage-dependent growth and restores chemosensitivity. To investigate the effect of *ARNTL* to anchorage-dependent growth and drug resistance, we selected cells that stably overexpressed *ARNTL* in MCP2 ovarian cancer cells. The expression of *ARNTL* of the stable clones was confirmed by quantitative RT-PCR and western blot analysis (Fig. 5A and B). By soft-agar assay, *ARNTL*-ovexpressed cells demonstrated a dramatic decrease in colony numbers as compared to control cells (Fig. 5C). Finally, we examined if *ARNTL* can affect chemosensitivity in these drug-resistant MCP2 cells. Overexpression of *ARNTL* restored the chemosensitivity to cisplatin in these cells (Fig. 5D). Taken together, these results demonstrated that *ARNTL* may function as a potential tumor suppressor in ovarian cancer.

Enhancement of apoptosis by cisplatin in ARNTL overexpressing cells. To determine the mechanism of *ARNTL* in restoring chemosensitivity in MCP2 cells, we investigated the effect of *ARNTL* on apoptosis. Although *ARNTL* did not increase the number of sub-G1 population under normal condition, overexpression of *ARNTL* showed a marked increase in the number of cells in the sub-G1 population under cisplatin treatment ($P<0.01$; Fig. 5E). These results indicated that *ARNTL* may restore the DNA damage-induced apoptosis in MCP2 ovarian cancer cells.

Rhythmic activity of c-MYC in ARNTL-overexpressing cells. Finally, we investigated the functional impact of restoring *ARNTL* expression in ovarian cancer cells. This is particularly interesting because the expression of several mammalian cell cycle genes, such as *c-myc* (16,32,33), is regulated in a circadian manner. Therefore, we synchronized the ARNTL stable transfectants by serum starvation. RNA was then extracted after serum restoration at the indicated time for gene expression analysis. Notably, expression of *c-myc* displayed a circadian rhythm in both of the *ARNTL*-expressing MCP2 clones (Fig. 5F), whereas a relatively constant level of expression without any rhythmicity was observed in the control cells.

Discussion

The relationship between circadian rhythm and cancer has been previously discussed (13,33). For example, mice deficient in the *Per2* gene showed reduced apoptosis in response to DNA damage and increased tumor formation (32). Depletion of another core circadian gene, *NPAS2* failed to exhibit cell cycle arrest in response to a mutagen in MCF7 breast cancer cells (34). Furthermore, these circadian regulators were found to be epigenetically silenced in several human cancer (35-37). These results suggested that genes involved in circadian clock may be involved also in tumor suppression and can be epigenetically silenced in human cancer.

In the present study, we found that another core component of the circadian rhythm, *ARNTL*, is epigenetically silenced by promoter methylation and histone modifications in ovarian cancer cell lines. Overexpression of *ARNTL* suppressed tumor growth, restored chemosensitivity and resumed the

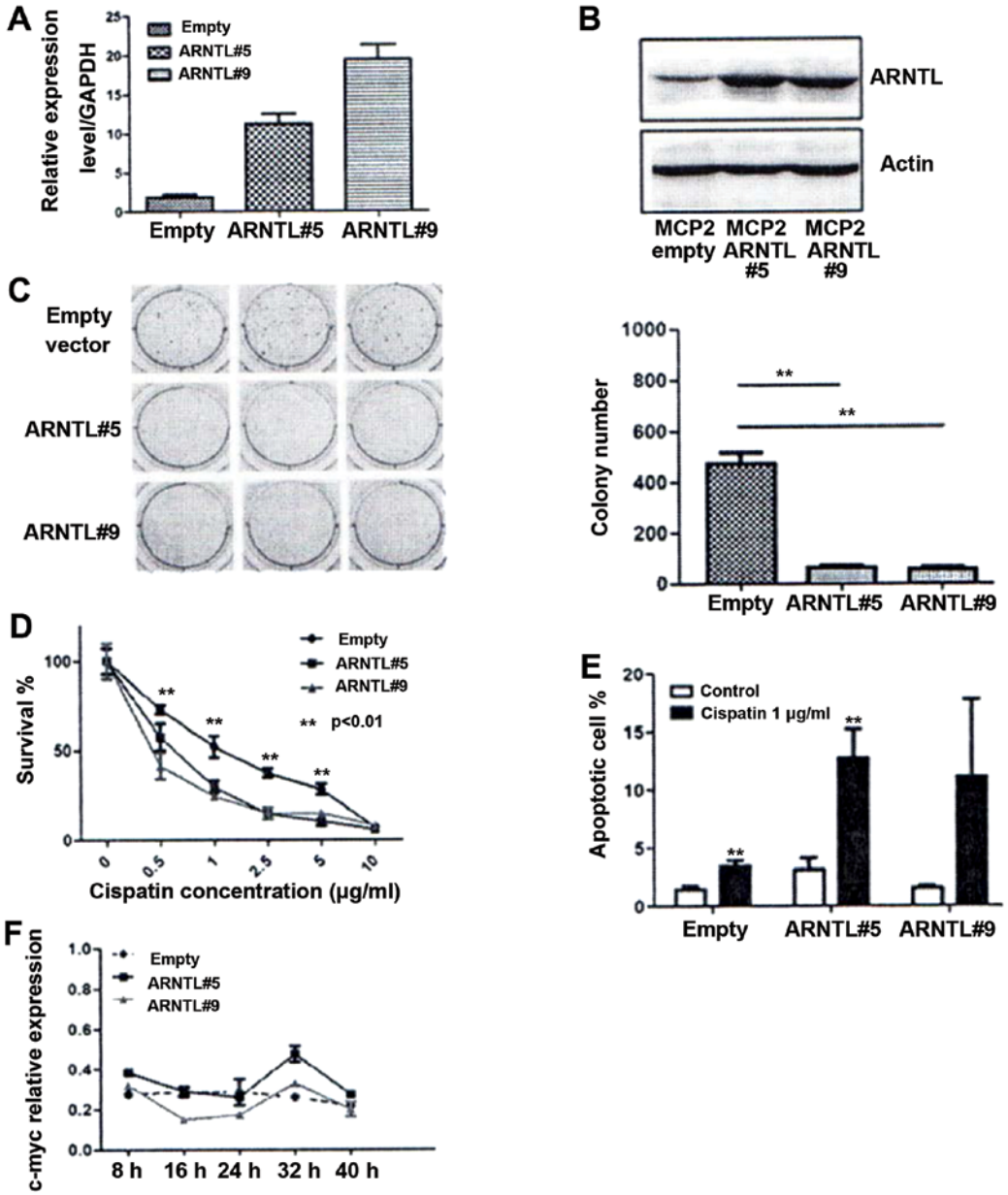

Figure 5. *ARNTL* functions as a tumor suppressor in ovarian cancer. *ARNTL* stable transfectants were selected and the expression level was confirmed by (A) quantitative RT-PCR and (B) western blotting. (C) Overexpression of ARNTL inhibited anchorage-independent growth as demonstrated in soft agar assay (left panel). Right panel shows the quantitative analysis of the soft agar assay. (D) Overexpression of *ARNTL* restored chemosensitivity to cisplatin in MCP2 cells. (E) Overexpression of *ARNTL* induced robust apoptosis under cisplatin treatment. (F) Quantitative RT-PCR analysis of the expression of *c-MYC* in synchronized *ARNT*L-expressing cells. Circadian rhythm of *c-myc* was restored in *ARNT*L-expressing cells, whereas a constant level of expression without any rhythmicity was observed in the control cells. **P<0.01.

rhythmic activity of *c-myc* in ovarian cancer cells. Our results were consistent with a previous report that *ARNTL* exhibited promoter hypermethylation and might act as a tumor suppressor gene in hematologic malignancies (36). Although the role of ARNTL in cancer is not fully understood, studies suggested that ARNTL may be a regulator of the p53 tumor suppressor pathway (38). More recently, it was found that ARNTL can suppress cancer invasion by inhibiting the AKT signaling pathway (39). The molecular mechanism of ARNTL in ovarian cancer warrants further investigation.

Epigenetics involving complex silencing mechanisms require cross-talk and coordination of multiple regulatory events. Promoter DNA methylation plays a crucial role in gene inactivation and its role in tumorigenesis has been established for decades (21). Over the past few years, increased attention has been given to histone methylation, in particular the repressive H3K27me3-mediated gene silencing in cancer (40,41). Previous studies showed that EZH2 may directly control DNA methylation for gene silencing (42). DNA methyltransferases can be recruited to target loci for *de novo* methylation through polycomb-mediated H3K27me3 within DNA methylation. The present study showed that treatment of 5-aza resulted in a moderate re-expression of *ARNTL* in CP70 cell, while treatment with GSK126 resulted in a robust re-expression of ARNTL in CP70 and MCP2 cells thus suggesting that *ARNTL* silencing

may occur through a repressive chromatin complex involving a polycomb repressor and DNA methylation. It is suggested that H3K27me3-mediated gene silencing may constitute a key epigenetic event repressing developmental genes in early stage of tumorigenesis. The therapeutic effect of using inhibitors against DNMT and EZH2 in the treatment of ovarian cancer deserves further investigation.

In conclusion, the circadian gene *ARNTL* may be a potential tumor suppressor in ovarian cancer. Epigenetic silencing of *ARNTL* may be required for proliferation, anchorage-independent ability and drug resistance in ovarian cancer.

Acknowledgements

The present study was supported by the research grants from the National Science Council, Taiwan (NSC102-2320-B-194-006) and the NCCU Interdisciplinary Science grant (103-03), National Chung Cheng University, Taiwan.

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