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Effects of the biological clock gene *Bmal1* on tumour growth and anti-cancer drug activity

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The *Bmal1* gene plays a key role in controlling circadian rhythms. To better understand how the *Bmal1* gene affects tumour growth and the response to anti-cancer drugs, we examined the effect of knockdown of *Bmal1* by RNAi both *in vitro* and *in vivo*. Down-regulation of *Bmal1* gene expression accelerated cell proliferation *in vitro* and promoted tumour growth in mice. Suppressing *Bmal1* expression in murine colon cancer cells (C26) and fibroblast cells (L929) decreased apoptosis induced by Etoposid, reduced the distribution of cells in the G2/M phases treated by Docetaxel and decreased DNA damage induced by Cisplatin. Loss of *Bmal1* reduced the expression of *per1*, *per2*, *per3*, *wee1* and *p53*. The expression of *p21* and *c-myc* was also altered in certain cell lines. However, *Bmal1* deficiency increased the protein levels of cdc2, cyclin B1, cyclin D1 and cyclin E. Wee1 and cyclin A expression was minimally altered. Thus, the circadian clock gene *Bmal1* plays a role in regulating tumour cell apoptosis, cell-cycle progression and DNA damage response and in homoeostasis regulation. Down-regulation of *Bmal1* accelerates the development of tumours and may influence the response to anti-cancer drugs.

Keywords: anti-cancer drugs/Bmal1/clock gene/circadian rhythm/colon cancer.

Abbreviations: DDP, Cisplatin; DOC, Docetaxel; IEC, intestinal epithelial cells; LMA, low melting agarose; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; NMA, normal melting agarose; OTM, Olive tail moment; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI, propidium iodide; PVDF, polyvinylidene difluoride; RT, room temperature; RT-PCR, reverse transcription polymerase chain reaction; SCGE, single cell gel electrophoresis; SD, standard deviation; SDS, sodium dodecyl sulfate; shRNA, small hairpin RNA; TD, tail DNA percentage; VP-16, Etoposid.

A circadian rhythm is a roughly 24-h cycle in the biochemical, physiological or behavioural processes of living entities, driven by endogenous clocks (*1*, *2*). Research has shown that the biological clock system exists to form and adjust circadian rhythms. It also serves as an important regulatory system apart from the nervous, humoral and immune systems (*3*). Substantial studies have found that problems in biological rhythms can cause many diseases, including immune disorders, heart disease and cancer, among others. Circadian disruptions, exemplified by altering the light/dark cycle in rotating shift work, increase cancer risk in humans. One study followed 78,562 nurses, with no history of breast cancer, for 10 years. Those nurses who worked rotating night shifts for 30 years or longer had a significantly increased risk for developing breast cancer as compared to nurses who never worked night shifts (*4–8*). Similarly, another study of 200 patients with metastatic colorectal cancer showed that survival at 2 years was 5-fold higher in patients with marked activity rhythm as compared to those with rhythm alteration (*9*).

Circadian rhythm is generated by a series of interlocking positive and negative feedback gene transcription and translation loops. Circadian clock genes include *Per* (*Per1*, *Per2*, *Per3*), *Bmal1*, *Clock*, *Cry* (*Cry1*, *Cry2*), *CKIε* and others (*10*). The *Bmal1* gene is the core component of the circadian clocks of mammals, coded as Bmal1 (*11*). Similar to the *Clock* gene, it belongs to the bHLH-PAS structural domain transcription factor family and exerts positive control on the feedback circuit. The combination of BMAL1/CLOCK heterodimers with E-box controls *Per*, *Cry* and *Rev-Erbα* maintains the normal circadian rhythm (*12*).

Per (Per1, Per1 and Per3) is the most studied of the core clock genes. The *Per* genes play key roles in controlling the circadian rhythm and may possess tumour suppressive properties. Alterations in the methylation status in the *Per1* and *Per2* promoters have been found in breast cancers (*13*). Moreover, *Per1* and *Per2* expression is significantly decreased in both sporadic and familial primary tumours as compared with normal breast tissues (*14*). *Per2*-deficient mice show a marked increase in tumour development, and reduced apoptosis in thymocytes, following γ-radiation (*15*). Alternatively, overexpression of *Per2* inhibits tumour proliferation in culture cells (*16*) and in animals (*17*). However, no studies have reported comprehensive data on the effect of the *Bmal1* gene on tumour development and anti-cancer drug activity.

The formation of tumours is an extremely complicated process. Genetic changes that reduce apoptosis are likely to be critical components of tumourigenesis. Tumourigenesis may also be caused by the changes in highly conserved DNA repair and cell-cycle checkpoint pathways. This study examines the effect of the core clock gene *Bmal1* on tumour growth and anti-cancer drug efficacy by identifying changes in apoptosis, the cell cycle and DNA repair. Ultimately, we hope to elucidate the role of the circadian clock gene in homoeostasis regulation and regulating gene expression.

Materials and Methods

Cell culture

Mouse colon carcinoma C26 cells and mouse fibroblast L929 cells were cultured in medium containing DMEM supplemented with 10% newborn bovine serum at 37°C and 5% CO_2. Primary cultures of murine intestinal epithelial cells (IECs) were maintained in DMEM supplemented with 5% foetal bovine serum, 2 mmol/l L-glutamine, 2 μg/ml Insulin, 20 ng/ml EGF, 100 U/ml Penicillin and 100 μg/ml Gentamicin at 37°C and 5% CO_2.

siRNA transfection

L929 cells and IEC cells were plated in six-well plates the day before transfection in order to reach 30% confluency at the time of transfection. Ten nano moles of siRNA oligo was transfected using Lipofectmine2000 according to the manufacturer's protocol. The effect of the siRNA was examined 48–72 h after transfection. The sequences of RNA oligos used were as follows (*18*):

Bmal1: 5′-CCACCAACCCAUACACAGAAGCAAA-3′,
Control: 5′-CCACCAAAUACACACGAAGCCCAAA-3′

shRNA construction and transfection

The mouse *Bmal1* small hairpin RNA (shRNA) was constructed in the lentivirus gene transfer vector pGCL-GFP from Genechem Co., Ltd, Shanghai, China (Fig. 1A). The *Bmal1* targeting sequence was the same as the siRNA sequence. The loop sequence was 5′-TTAA-3′. The Control shRNA sequence used was as following: 5′-TTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACA CGTTCGGAGAATTTTTTC-3′.

C26 cells were transfected with lentivirus in the presence of polybrene (6 μg/ml) and sorted by flow cytometry. The C26 cells transfected with lentivirus encoding shRNA against *Bmal1* were named C26_Bmal1 shRNA cells. Cells transfected with the control lentivirus were named C26_Control shRNA cells.

Cell growth assay

C26_Control shRNA cells and C26_Bmal1 shRNA cells were replated in 96-well plates at 48–72 h post-siRNA transfection. Cell growth was measured daily using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to the standard protocol. Each kind of cells was repeated three times.

Animals and tumour tissue collection

Male BALB/c mice ~3–4 weeks old were purchased from Sun Yat-sen University Animal Center (Guangzhou, China). The protocol was approved by the Ethics Committee at the Sun Yat-sen University Cancer Center, where the research was conducted. Animals were synchronized in a 12-h light, 12-h dark cycle for at least 2 weeks before initiating experiments.

C26_Control shRNA cells and C26_Bmal1 shRNA cells were prepared at a concentration of 5×10^6 viable cells/ml. Approximately 200 μl of cells were injected subcutaneously in the axilla of BALB/c mice. Mice bearing C26 tumours were kept under 12-h light and 12-h dark conditions. Tumour growth was monitored weekly in two dimensions. Tumour size was calculated using the formula $V = a^2b/2$, where a and b are the shortest and longest diameters, respectively. When the average of tumour size of each group of mice reached 1000 mm^3, mice were sacrificed at the same time, and the tumour, spleen and thymus weights were determined.

RT–PCR

Total RNA was extracted from C26, L929 and IEC cells using Trizol according to manufacturer's instructions (Invitrogen). Reverse transcription was performed using the Superscript FirstStrand Synthesis System (Invitrogen) with an Oligo $d(T)_{18}$ primer. Details of the primers used in this study for PCR are given in Table I.

Western Blot

Total proteins were extracted from C26 and L929 cells with sodium dodecyl sulphte SDS–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (125 mmol/l Tris–HCl, pH 6.8, 6% SDS, 10% glycerol, 10 mmol/l 2-mercaptoethanol). Proteins were separated by standard SDS–PAGE using the Mini-gel system (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membranes using a transfer apparatus (Bio-Rad). Membranes were incubated in blocking buffer (5% non-fat dry milk in TBST buffer) at room temperature (RT) for 1 h and then incubated with primary antibodies (1 : 200–1 : 2000) overnight at 4°C. After incubation with secondary antibodies, peroxidase activity was detected by enhanced chemiluminescence according to the manufacturer's

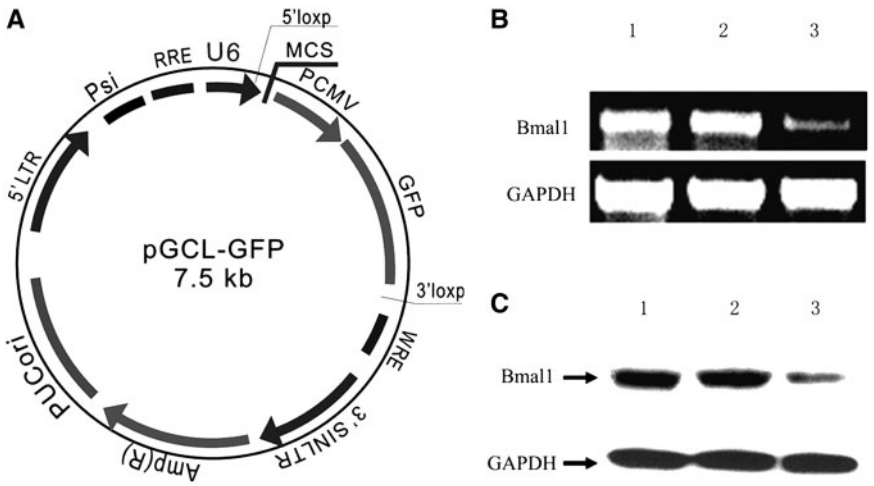

Fig. 1 Stable knockdown of *Bmal1* by shRNA in C26 cells. (A) The lentivirus RNAi vector was derived from pGCL-GFP (Genechem Co., Ltd, Shanghai, China). (B–C) The effect of lentivirus-mediated RNAi on *Bmal1* in C26 cells. (B) The expression of *Bmal1* mRNA was analysed by RT–PCR. (C) The expression of Bmal1 protein was examined by western blotting. Lanes (1–3): C26 cells in the absence of treatment; C26 cells infected with control lentivirus; C26 cells infected with shRNA lentivirus targeting *Bmal1*. GAPDH was used as a loading control.

Table I. Primer sequences and predicted sizes of the amplified products for the different genes studied.

Gene	Forward primer	Reverse primer	Product length (bp)
mGAPDH	5′-CATCAACGGGAAGCCCATCA-3′	5′-GGGATGCCTTGCCCACAG-3′	461
mBmal1	5′-TGTCACAGGCAAGTTTTACAGAC-3′	5′-ACAGTGGGATGAGTCCTCTTTG-3′	281
mper1	5′-AGAGGACCAGGGGACAT-3′	5′-CCATGGACATGTCTACT-3′	982
mper2	5′-GTGAAGCAGGTGAAGGCTAATG-3′	5′-AAGCTTGTAAGGGGTGGTGTAG-3′	312
mper3	5′-TCCTGATGGTAAGACATTCCAG-3′	5′-GCGTGAACAATCACACTCACTT-3′	500
mcry1	5′-CGTCTGTTTGTGATTCGGGG-3′	5′-ATTCACGCCACAGGAGTTGC-3′	669
mrev-erbα	5′-TGGCCTCAGGCTTCCACTATG-3′	5′-CCGTTGCTTCTCTCTCTTGGG-3′	233
mwee1	5′-GAAACAAGACCTGCCAAAAGAA-3′	5′-GCATCCATCTAACCTCTTCACAC-3′	140
mc-myc	5′-TGATGTGGTGTCTGTGGAGAAG-3′	5′-CGTAGTTGTGCTGGTGAGTGG-3′	142
mp53	5′-CTCAAAAAACTTACCAGGGC-3′	5′-CACCACGCTGTGGCGAAAAGTCTG-3′	356
mp21	5′-GCAGACCAGCCTGACAGATTT-3′	5′-GAGAGGGCAGGCAGCGTAT-3′	210

instructions (Amersham Biosciences, USA) using Image Quant analysis software (Quantity One).

Flow cytometric analyses of the cell cycle

C26 and L929 cells were stimulated with 50% foetal bovine serum for 2 h for synchronization. Cells were then harvested, washed with PBS and fixed in ice-cold 70% ethanol overnight. The fixed cells were incubated with 100 μg/ml RNaseA and 50 μg/ml propidium iodide (PI). Cellular DNA content was determined using a flow cytometer (Beckman Coulter, Fullerton, CA. USA). At least 10,000 cells per sample were analysed.

Flow cytometric analyses of apoptosis

C26 and L929 cells were harvested, washed with PBS and incubated with 20 μg/ml Annexin V-FITC and 50 μg/ml PI. The rate of apoptosis was analysed by flow cytometry within 1 h.

Single cell gel electrophoresis/comet assay

C26 and L929 cells were measured by single cell gel electrophoresis (SCGE) assay. This procedure was conducted under low, indirect incandescent light at 2–8°C to minimize cellular damage. The assay was performed according to Singh *et al.* (*19*). Results were expressed as the percentage of DNA in the tail (Tail DNA %, TD) and as Olive tail moment (OTM), which represents the integrated value of DNA density multiplied by the migration distance.

Statistical analysis

Statistical analysis was performed by SPSS 13.0 software. Average values were expressed as mean ± SD. Statistical significance between different groups was determined by the Student's *t*-test, and $P<0.05$ were considered significant.

Results

The construction of C26 cells infected with lentivirus RNAi targeting Bmal1

To achieve a stable reduction in *Bmal1* expression, we transfected C26 cells with a lentivirus vector encoding short hairpin RNA (shRNA) targeting the *Bmal1* gene. Lentivirus-mediated RNAi markedly reduced *Bmal1* expression at the mRNA and protein levels (Fig. 1B). Bmal1 expression was inhibited >80%.

Down-regulation of Bmal1 accelerated the growth of cultured cells

The effects of *Bmal1* knockdown on C26, IEC and L929 cell growth were examined. C26_Bmal1 shRNA cell growth was accelerated 51.3% on Day 4 ($P<0.05$) and 28.4% on Day 5 ($P<0.05$) as compared to C26_Control shRNA cells (Fig. 2A). Following 48 h of siRNA treatment, IEC cells were replated and the living cells were assayed each day by MTT assay. The IEC cells growth increased 17.6% on Day 4 ($P<0.05$) and 36.4% on Day 5 ($P<0.05$) (Fig. 2B). With the same treatment as IEC, L929 cells grew 29.9% faster on Day 4 ($P<0.05$) (Fig. 2C).

Down-regulation of Bmal1 accelerated the growth of cells in vivo

The relationship between reduced *Bmal1* gene expression and tumour growth in mice was examined. The weights of C26_Control shRNA and C26_Bmal1 shRNA tumours were 1.10 ± 0.28 and 2.24 ± 0.69 g, respectively. The C26 tumours inhibiting *Bmal1* expression grew faster than the control tumours ($P<0.05$) (Fig. 2D). The thymic index of mice inoculated with C26_Control shRNA and C26_Bmal1 shRNA cells was 13.55 ± 2.08 and 10.3 ± 2.88, respectively. It was significantly decreased when mice inoculated with C26_Bmal1 control cells ($P<0.05$).

The influence of RNAi targeting Bmal1 on the activity of anti-cancer drugs

The anti-proliferative effects of Docetaxel (DOC) and Etoposid (VP-16) on C26_Control shRNA and C26_Bmal1 shRNA cells were measured by MTT. The IC50 (inhibitor concentration yielding 50% inhibition) of DOC in C26_Control shRNA and C26_Bmal1 shRNA cells was 5.31 ± 0.48 and 13.77 ± 3.09 μM, respectively. The IC50 of DOC in C26_Bmal1 shRNA cells was increased by 159.3% ($P<0.05$) as compared to the C26_Control shRNA cells. The IC50 of VP-16 in C26_Control shRNA and C26_Bmal1 shRNA cells was 29.53 ± 7.59 and 49.98 ± 0.49 μM, respectively. The IC50 of VP-16 was increased by 69.3% ($P<0.05$) in C26_Bmal1 shRNA cells.

The effect of RNAi targeting Bmal1 on apoptosis of cells

The rates of apoptosis in C26_Control shRNA and C26_Bmal1 shRNA cells were 4.9 ± 0.3 and 3.1 ± 0.2%, respectively. Following treatment with 75 μM and 150 μM VP-16 for 24 h, the percentages of apoptotic cells were 24.6 ± 7.5 and 29.4 ± 7.1%, respectively, for the C26_Control shRNA cells and 9.0 ± 3.5 and 11.2 ± 5.3%, respectively, for the C26_Bmal1 shRNA cells. Therefore, inhibiting *Bmal1*

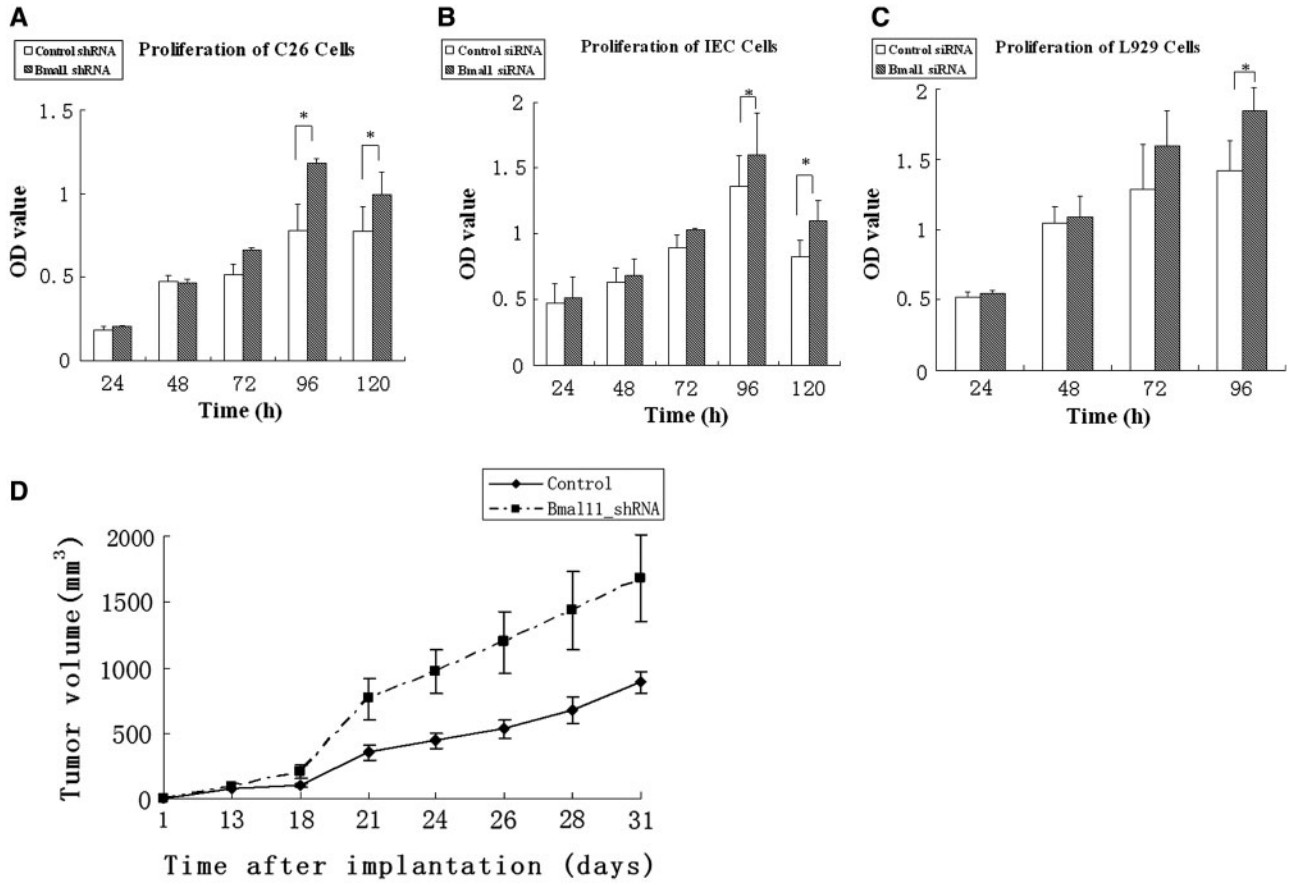

Fig. 2 ***Bmal1*** **inhibition increased cell proliferation.** (A) The C26 cells were infected with control shRNA vectors or shRNA vectors targeting *Bmal1*; (B) The primary culture of IECs were treated with siRNA Control or *Bmal1*; (C) The L929 cells were treated with siRNA Control or *Bmal1*. Data are shown as mean ± SD from three independent experiments. *$P<0.05$, Student's *t*-test; (D) Down-regulation of *Bmal1* increased the growth of C26 cells in BALB/c mice. The results are reported as the mean ± SD.

caused a significant decrease in VP-16-induced apoptosis ($P<0.05$) (Fig. 3A).

The rates of apoptosis in L929 control and Bmal1-knockdown cells were 15.9 ± 1.8 and 7.1 ± 0.7%, respectively. Following treatment with 75 μM and 150 μM VP-16, apoptosis occurred in 34.8 ± 8.6 and 39.9 ± 7.2% of L929_Control siRNA cells, respectively, and in 11.3 ± 3.3 and 18.5 ± 4.6% of L929_Bmal1 siRNA cells, respectively. Thus, inhibition of *Bmal1* caused a significant reduction in the percentage of VP-16-induced apoptotic cells ($P<0.05$) (Fig. 3B).

The effect of RNAi targeting Bmal1 on the cell cycle

The distribution of C26 cells in the G2/M phase of the cell cycle decreased by 28.2% following knockdown of *Bmal1*. After treatment with 1 μM and 5 μM DOC, the percentage of C26_Bmal1 shRNA cells in the G2/M phase reduced significantly by 25.1 and 11.7%, respectively, as compared to the C26_Control shRNA cells (Table II).

Bmal1 inhibition in L929 cells resulted in a 19.8% decrease in the number of cells in G2/M. The G2/M distribution of Bmal1-knockdown cells treated with 2.5 μM and 5 μM DOC was significantly decreased by 7.6 and 8.7%, respectively, as compared to the L929 control cells (Table III).

The effect of RNAi targeting Bmal1 on DNA damage

C26 and L929 cells with suppressed *Bmal1* were treated with cisplatin (DDP) for 24 h and then detected by SCGE. DNA damage was quantitated by Tail DNA percentage (TD) and OTM. The TD and OTM were significantly decreased in *Bmal1*-suppressed C26 and L929 cells (Table IV).

The effects of Bmal1 inhibition on circadian clock genes, cell-cycle control genes and apoptosis related genes

Knockdown of *Bmal1* in C26 cells resulted in down-regulation of *per1*, *per2* and *per3* and up-regulation of *rev-erbα*. The expression of *wee1*, *p53* and *p21* was reduced, and that of *c-myc* was barely altered.

Down-regulation of *Bmal1* in IEC cells resulted in decreased expression of *per1*, *per2* and *per3* and increased expression of *rev-erbα*. The expression of *wee1* and *p53* was down-regulated, and *p21* and *c-myc* expression levels were up-regulated.

Suppression of *Bmal1* in L929 cells caused reduced expression of *per1*, *per2* and *per3* but little change in *rev-erbα* expression. The expression of *wee1*, *p53* and *p21* was down-regulated, but there was little change in *c-myc* expression (Fig. 4).

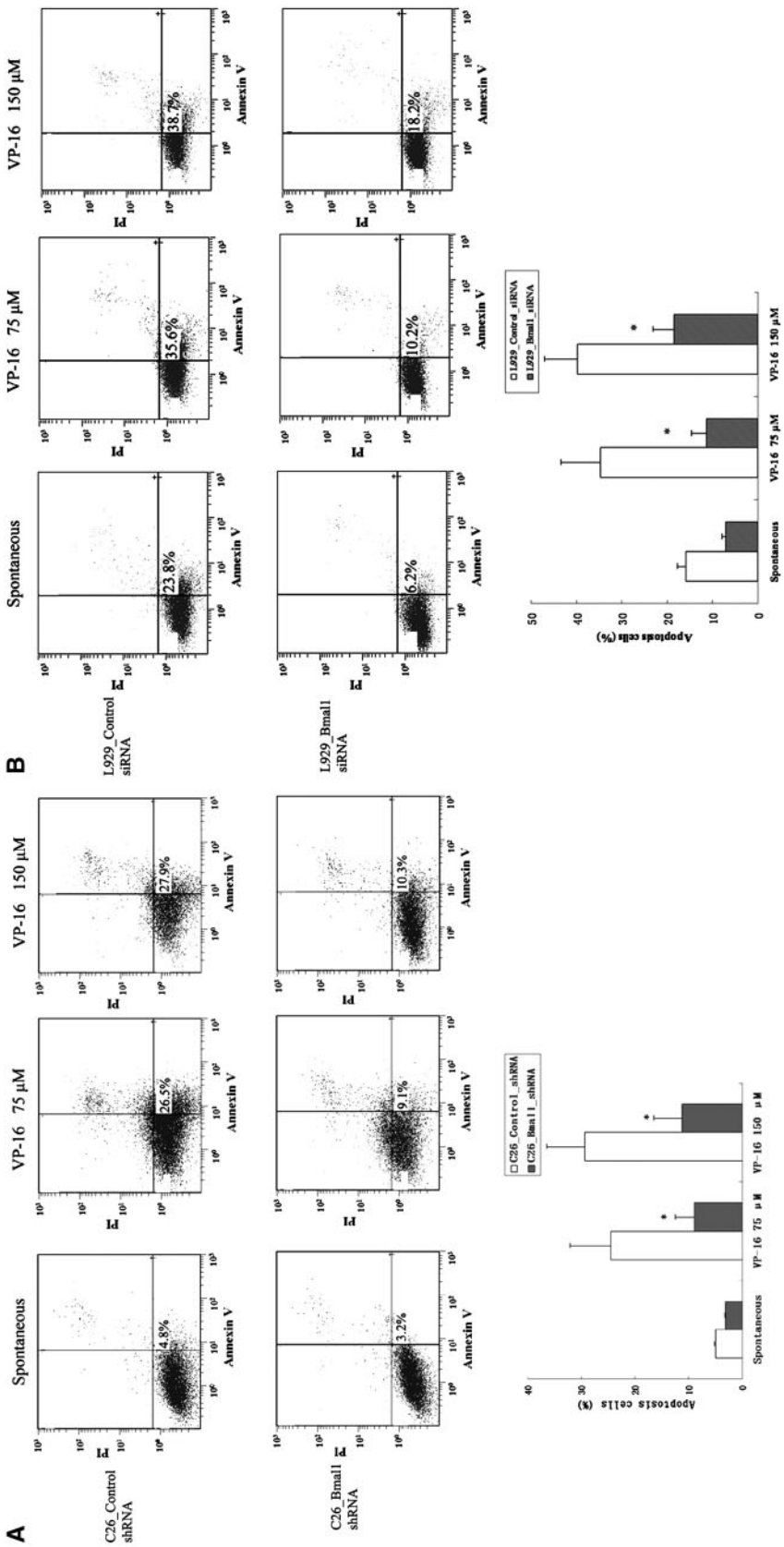

Fig. 3 Apoptosis induced by RNAi-mediated inhibition of *Bmal1* expression. Cells with or without a 24 h VP-16 treatment were collected, and apoptotic cells were measured by flow cytometry. The mean percentage ± SD from three experiments are shown. * $P < 0.05$, Student's *t*-test. (A) In C26 cells; (B) in L929 cells.

Table II. Cell-cycle distribution for C26 cells after RNAi-mediated inhibition of *Bmal1* expression.

C26	G1 (%)	G2/M (%)	S (%)
Control shRNA	36.8 ± 4.8	21.9 ± 1.6	41.3 ± 2.3
Control shRNA			
DOC 1 μM	10.6 ± 1.9	69.8 ± 3.8	19.6 ± 3.2
DOC 5 μM	4.4 ± 3.2	75.4 ± 5.4	20.6 ± 2.5
Bmal1 shRNA	40.5 ± 2.4	13.3 ± 3.9*	46.2 ± 5.3
Bmal1 shRNA			
DOC 1 μM	23.4 ± 2.9*	52.3 ± 4.7*	24.3 ± 3.1
DOC 5 μM	11.0 ± 3.7*	66.6 ± 4.2*	22.4 ± 3.5

*$P < 0.05$, Student's *t*-test.

Table III. Cell-cycle distribution for siRNA-mediated *Bmal1* knockdown in L929 cells after treatment with DOC.

L929	G1 (%)	G2/M (%)	S (%)
Control siRNA	59.0 ± 6.5	9.1 ± 4.3	31.9 ± 5.3
Control siRNA			
DOC 2.5 μM	15.0 ± 3.8	63.2 ± 4.2	21.7 ± 4.9
DOC 5 μM	15.0 ± 4.7	80.7 ± 7.8	4.3 ± 3.0
Bmal1 siRNA	69.5 ± 8.9	7.3 ± 4.0	23.1 ± 6.2
Bmal1 siRNA			
DOC 2.5 μM	13.3 ± 1.8	58.4 ± 6.1*	28.2 ± 7.2*
DOC 5 μM	15.9 ± 3.2	73.7 ± 9.9*	10.4 ± 4.5*

*$P < 0.05$, Student's *t*-test.

Table IV. DNA damage resulting from *Bmal1* inhibition, as detected by SCAG after 24-h treatment with DDP.

	DDP	Tail DNA %	Tail Moment
C26			
Control siRNA	5 μM	5.43 ± 2.49	2.70 ± 1.40
Bmal1 siRNA	5 μM	1.89 ± 3.00*	0.91 ± 0.53*
Control siRNA	20 μM	30.66 ± 6.22	9.32 ± 5.94
Bmal1 siRNA	20 μM	9.92 ± 6.82*	2.17 ± 1.85*
L929			
Control siRNA	500 μM	44.03 ± 15.40	21.21 ± 9.62
Bmal1 siRNA	500 μM	30.01 ± 11.96*	13.35 ± 8.02*

*$P < 0.05$, Student's *t*-test.

The influence of Bmal1 inhibition on cell-cycle control proteins

Knockdown of *Bmal1* in C26 and L929 cells increased the protein expression of cdc2, cyclin B1, cyclin D and cyclin E. However, there was minimal change in the protein levels of wee1 and cyclin A (Fig. 5).

Discussion

In this study, deregulation of *Bmal1* increased the growth rate of IEC, C26 and L929 cells *in vitro* and C26 tumour growth *in vivo*. The mechanism of action involves a decrease in apoptosis, a reduction of cells in the G2/M phase of the cell cycle and a decrease in DNA damage.

We observed a lower rate of apoptosis in *Bmal1*-suppressed L929 and C26 cells following treatment with VP-16. C26 and L929 cells containing reduced Bmal1 protein showed decreases in *p53* and *p21*, which may have contributed to their enhanced proliferation. Alternatively, according to the most well characterized positive feedback loop, the transcription of *Per* is directly activated by BMAL1/CLOCK heterodimers. Our study showed

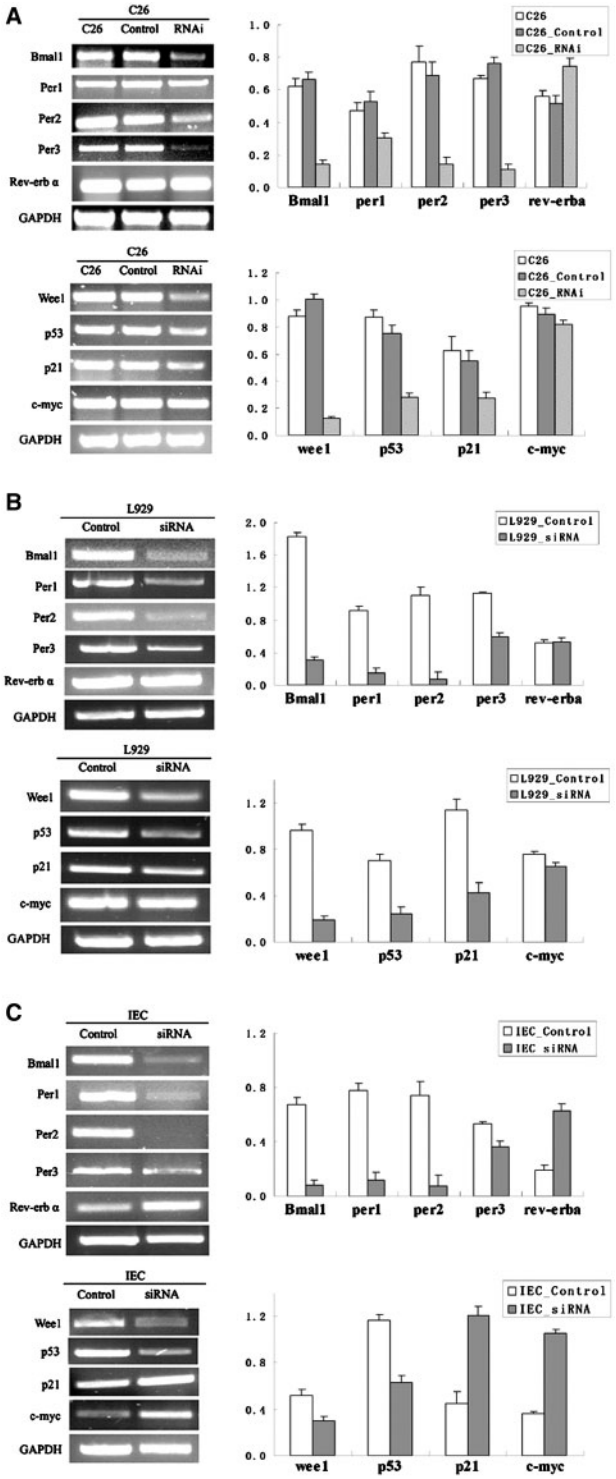

Fig. 4 mRNA levels in control cells and RNAi cells. (A) Untreated C26 cells (C26), C26_control shRNA cells (Control), C26_Bmal1 shRNA cells (RNAi); (B) siRNA-control L929 cells (Control), siRNA-Bmal1 L929 cells (siRNA); (C) siRNA-control IEC cells (Control), siRNA-Bmal1 IEC cells (siRNA). The expression of mRNA was analysed by RT–PCR.

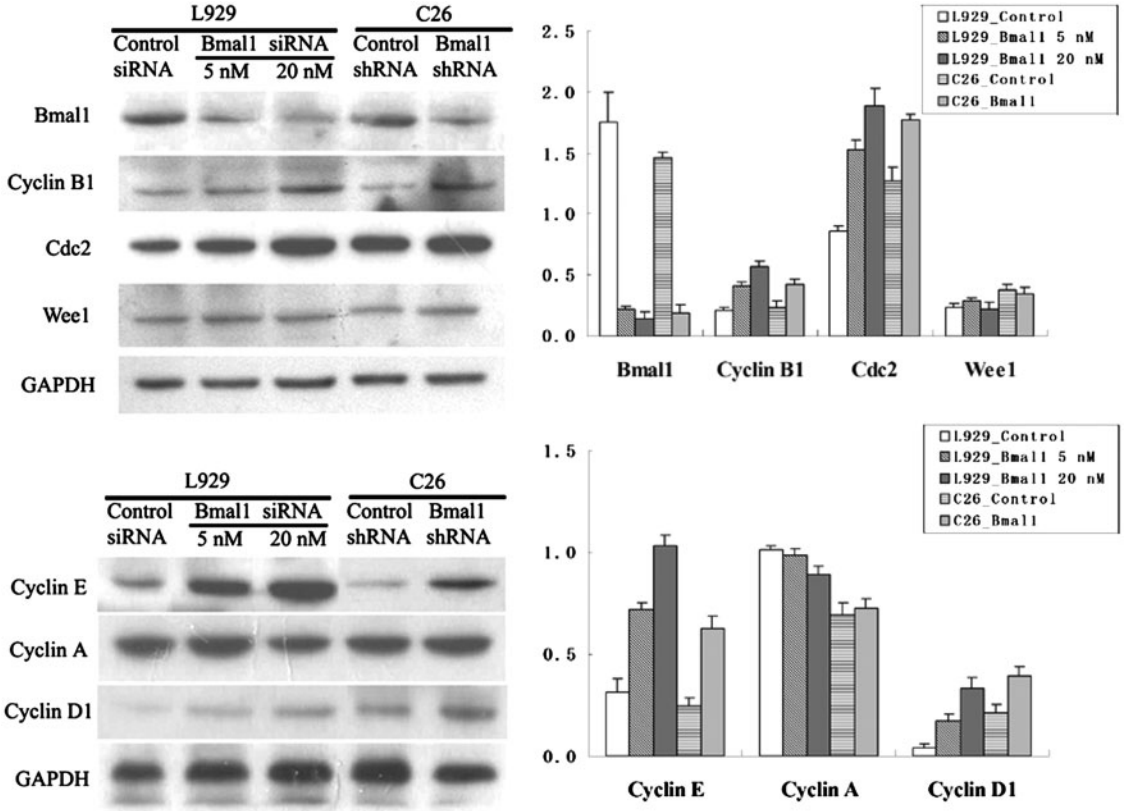

Fig. 5 Protein levels in L929 and C26 cells treated with RNAi. The C26 cells were infected with control shRNA vectors or shRNA vectors targeting Bmal1; The L929 cells were treated with siRNA Control or Bmal1. Protein expression was analysed by western blot.

that all of the *Per* genes, including *Per1*, *Per2* and *Per3*, were expressed at lower levels in the *Bmal1*-suppressed C26, L929 and IEC cells. This may be one reason for reduced apoptosis in the injured cells.

It has reported that human fibroblast cells with suppressed *Bmal1* contain lower *p21* levels and fail to arrest upon *p53* activation (*20*). This finding is associated with an inability to activate the *p53* target gene *p21*. However, a different study showed that circadian expression of p21 was dramatically increased, and no longer rhythmic, in clock-deficient Bmal1$^{-/-}$ mice (*21*). In our study, *p21* expression was increased in the *Bmal1*-suppressed IEC cells and decreased in *Bmal1*-suppressed C26 and L929 cells. The expression of *p53* was decreased in these cells. We speculated that there was a *p53*-independent *p21* pathway in IEC cells. *c-Myc* mRNA was elevated in *Bmal1*-suppressed IEC cells. In L929 and C26 cells, knockdown of *Bmal1* did not change the mRNA expression of *c-Myc*. Thus, the relationship between *Bmal1* and *c-Myc* appears to be cell-type specific. It is unknown whether the *c-Myc* pathway was altered in this cancer cell line.

The reduction of cells in the G2/M phase of the cell cycle is another reason that growth was accelerated in *Bmal1* knockdown cells. In our experiments, the cells expressing low levels of Bmal1 protein had increased levels of cyclin B1 and CDC2 proteins, which likely contributed to the lower distribution of cells in G2/M. Meanwhile, the expression of cyclin D1 and cyclin E proteins were increased in the *Bmal1* knockdown cells. This contributed to the accelerated cell proliferation. The *wee1* gene may act as a negative regulator of mitotic entry (G2 to M transition) by protecting the nucleus from cytoplasmically activated cyclin B1-complexed CDC2 before the onset of mitosis. Wee1 activity increases during the S and G2 phases and decreases at M phase upon hyperphosphorylation. A correlated decrease in protein occurs at the M/G1 phase, likely due to its degradation. Specifically phosphorylated and inactivated cyclin B1-complexed CDC2 reaches a maximum during G2 phase and a minimum as cells enter M phase. Phosphorylation of cyclin B1-CDC2 occurs exclusively on 'Tyr-15', and monomeric CDC2 cannot be phosphorylated (*22*). We observed that the *wee1* gene was down-regulated at the mRNA level, but there were no obvious changes at the protein level. Thus, we deduced that there was post-transcriptional control of *wee1* expression.

The reduction in DNA damage also contributes to the increased growth of *Bmal1*-suppressed cells. DNA damage in the L929 and C26 *Bmal1* knockdown cells was reduced following a 24-h treatment with DDP. These data suggest that *Bmal1* participates in the cell damage and repair pathways.

We found that the circadian clock gene *Bmal1* participates in the regulation of tumour cell apoptosis, cell-cycle progression and DNA damage response. It also plays an important role in homoeostasis

regulation. Upon *Bmal1* suppression, tumour formation is accelerated, and the response to anti-cancer drugs is altered.

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Conflict of interest
None declared.

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