

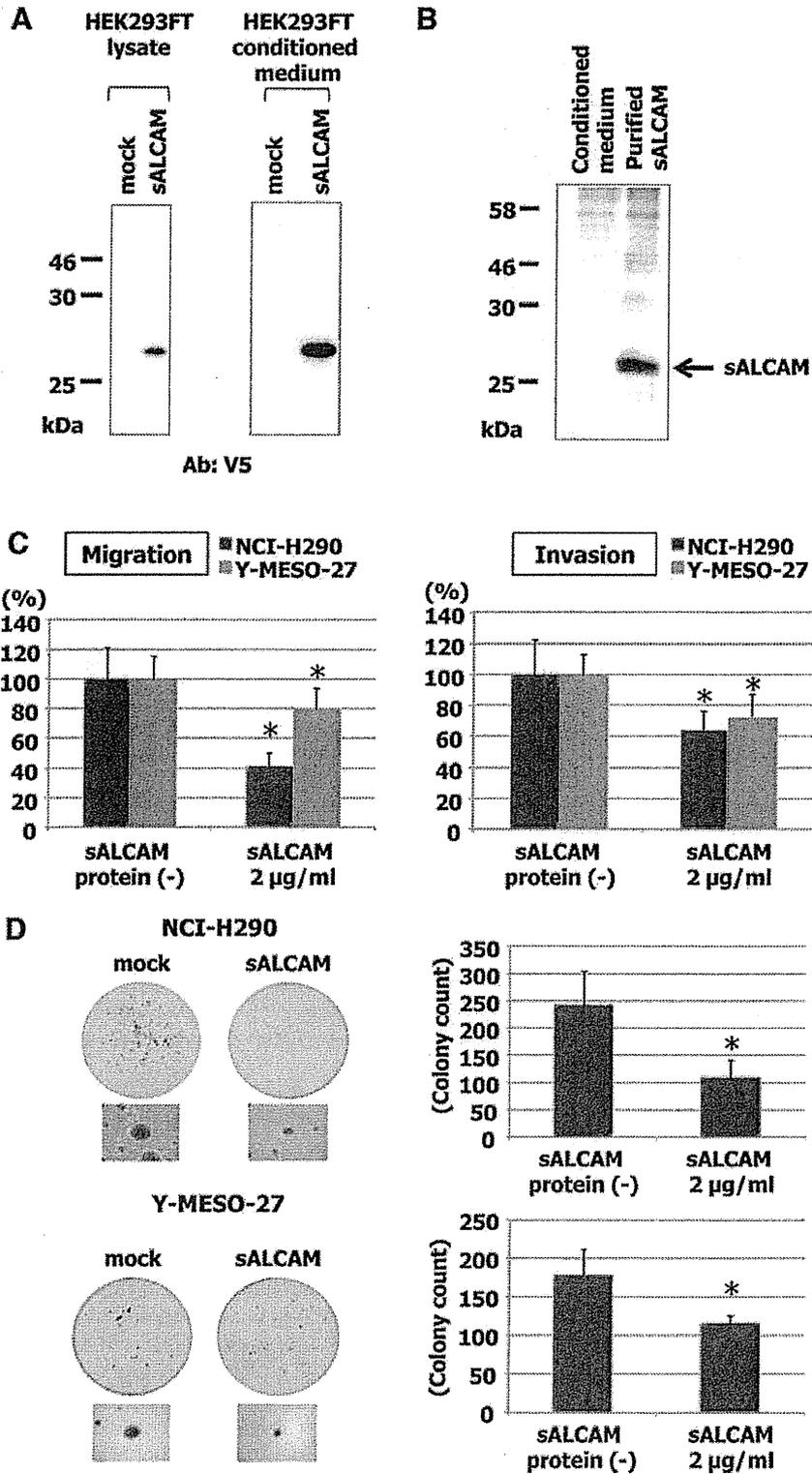
**FIGURE 4.** Enhancement of cell migration and anchorage-independent growth of immortalized mesothelial cells, MeT-5A, with infection of ALCAM expressing virus. (A) Western blot analysis. Whole cell lysates of MeT-5A cells were obtained 96 hours after infection of wild-type ALCAM or green fluorescent protein virus. (B) Immunocytochemical analysis. Positive signals of ALCAM were detected mainly at the cell-cell junction. (C) Cell migration and colony formation analysis of MeT-5A cells. Exogenous ALCAM enhanced migration and anchorage-independent cell growth of the MeT-5A cells. Columns, mean; bars, SD. ALCAM, Activated leukocyte cell-adhesion molecule; MM, malignant mesothelioma. \* $p < 0.05$  versus GFP control.

ALCAM in most MM cell lines and 55% surgical specimens of MMs. ALCAM's oncogenic properties served to promote migration and invasion in MM cells. Moreover, we demonstrated that the soluble isoform, sALCAM, impaired migration and invasion of MM cells in vitro and that sALCAM-expressing virus infection prolonged the survival of MM cell-transplanted mice.

Elevated ALCAM expression was previously reported in melanoma and breast cancer cell lines,<sup>30,42</sup> and immunohistochemical analysis of primary tumors revealed ALCAM expression in 36 to 57% of melanoma, pancreatic cancer, and bladder cancer.<sup>29,34,36</sup> Thus, the frequency of elevated ALCAM expression in MM tumors seemed to be similar to or higher than that of other types of human malignancies. As for the possible mechanisms of ALCAM overexpression, our comparative genomic hybridization analysis did not detect gene amplification of 3q13 locus,<sup>43</sup> which is the locus of the *ALCAM* gene. Meanwhile, a recent report by King et al<sup>42</sup> indicated that nuclear factor kappa B (NF- $\kappa$ B) binds to a consensus-binding motif in the human *ALCAM* promoter and enhances its promoter activity. In this regard, because NF- $\kappa$ B was also shown to be induced in asbestos-exposed mesothelial cells and to play a critical role in the promotion

and progression of MM cells,<sup>44,45</sup> NF- $\kappa$ B might be one of the underlying mechanisms of the ALCAM upregulation in MM cells.

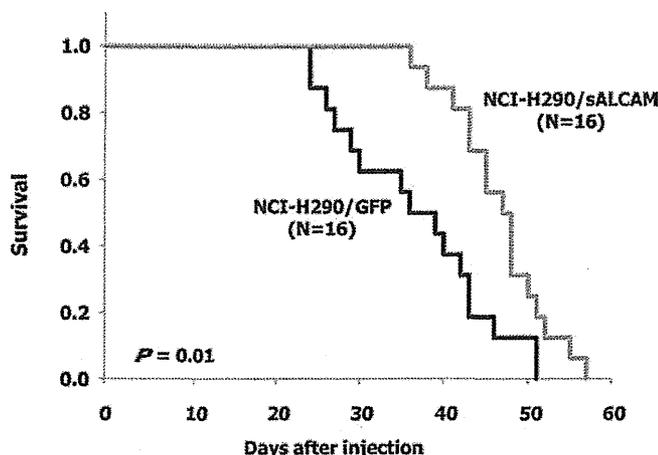
We demonstrated that ALCAM promotes cell migration and invasion, but not cell proliferation, in MM cells. There are several possible mechanisms that the homophilic ALCAM cell-to-cell adhesion confers malignant phenotypes to cells. For instance, ALCAM cell adhesion was shown to contribute to the activation of matrix metalloproteinase-2 (MMP-2), which is involved in tissue invasion, metastases, and angiogenesis.<sup>30,31</sup> It was also indicated that short interfering RNA-mediated ALCAM knockdown inhibited activation of MMP-2 in melanoma and fibrosarcoma cells.<sup>30</sup> Thus, these results suggested that the role of ALCAM might be to initiate a signal to trigger MMP-2 activation. However, we did not confirm a clear MMP-2 inactivation in MM cells infected with the ALCAM-Sh1 expressing virus (data not shown). In addition, although we further performed phospho-kinase and phospho-receptor tyrosin kinase arrays to explore possible cellular mechanisms revealing how ALCAM confers malignant phenotypes to MM cells, our knockdown experiments did not detect significant changes in phosphorylation status of all 88 proteins (data not shown).



**FIGURE 5.** Purification of sALCAM and its inhibitory effects on MM cells. (A) Western blot analysis using anti-V5 antibody. Secreted sALCAM in the conditioned medium as well as in the whole cell lysates was confirmed. (B) Silver staining. Purity of synthesized sALCAM was confirmed with no obvious contaminated proteins. (C) Cell migration and invasion assays. In the presence of sALCAM protein at the indicated final concentration in the media, both migration and invasion abilities of MM cells were significantly inhibited. (D) Anchorage-independent colony formation assay. Anchorage-independent growth of NCI-H290 and Y-MESO-27 cell lines was suppressed with addition of sALCAM. Columns, mean; bars, SD. sALCAM, soluble activated leukocyte cell-adhesion molecule; MM, malignant mesothelioma. \* $p < 0.05$  vs. control.

The extracellular domain of adhesion molecules can be released from cells to yield soluble cell-adhesion molecules, which are generated by proteolytic cleavage or alternative splicing.<sup>46-48</sup> Regulatory roles of several soluble cell adhesion

molecules have been shown to be involved in angiogenesis or tumor growth for intercellular adhesion molecule-1<sup>49,50</sup> and in cell migration for P-selectin.<sup>51</sup> As for the soluble form sALCAM, Kilsdonk et al<sup>31</sup> showed that overexpressed sALCAM



**FIGURE 6.** MM cells with sALCAM expression showed longer survival in vivo. Nude mice were injected with MM cell lines infected with NCI-H290/sALCAM or NCI-H290/GFP ( $1 \times 10^6$ ) virus into the right thoracic cavity. NCI-H290/sALCAM group showed a significantly prolonged survival ( $p = 0.01$  and log-rank test). MM, malignant mesothelioma; sALCAM, soluble activated leukocyte cell-adhesion molecule.

impaired the migration of melanoma cells and diminished lung metastases of melanoma cells in vivo. Thus, their results suggested that sALCAM may also act as a tumor suppressor for the tumor cells with ALCAM expression. As we also showed the inhibitory effect of sALCAM on MM cells, we considered sALCAM to possibly have a therapeutic potential for MM.

ALCAM has also been shown to be a target molecule for immunotherapeutic modality against human cancers. For example, an internalizing single-chain antibody fragment directed to ALCAM, which was conjugated to immunoliposomal cytotoxic agents was shown to effectively target and kill ALCAM-expressing ovarian-cancer and prostate-cancer cells in vitro.<sup>52,53</sup> Wiiger et al<sup>54</sup> also demonstrated the potential role of a human recombinant anti-ALCAM antibody as a therapeutic agent against colon and breast cancers. Thus, these newly developed immunotherapeutic agents might also be beneficial for MM treatment.

In conclusion, our study revealed that overexpression of ALCAM contributes positively to MM progression and that sALCAM effectively inhibits tumor progression. We considered that ALCAM might be a potential therapeutic target of MM. Further investigation about the detailed mechanisms of how cell migration, invasion, and anchorage-independent growth can be enhanced in MM cells by ALCAM is also needed to provide new insights into a more effective strategy to target the ALCAM expressed on MM cells.

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## The circadian clock gene *BMAL1* is a novel therapeutic target for malignant pleural mesothelioma

Momen Elshazley<sup>1</sup>, Mitsuo Sato<sup>1</sup>, Tetsunari Hase<sup>1</sup>, Ryo Yamashita<sup>1</sup>, Kenya Yoshida<sup>1</sup>, Shinya Toyokuni<sup>2</sup>, Futoshi Ishiguro<sup>3,4</sup>, Hirotaka Osada<sup>3</sup>, Yoshitaka Sekido<sup>3</sup>, Kohei Yokoi<sup>4</sup>, Noriyasu Usami<sup>4</sup>, David S. Shames<sup>5</sup>, Masashi Kondo<sup>1</sup>, Adi F. Gazdar<sup>6</sup>, John D. Minna<sup>6</sup>, and Yoshinori Hasegawa<sup>1</sup>

<sup>1</sup>Department of Respiratory Medicine, Nagoya University Graduate School of Medicine, Japan

<sup>2</sup>Department of Pathology and Biological responses, Nagoya University Graduate School of Medicine, Japan

<sup>3</sup>Division of Molecular Oncology, Aichi Cancer Center Research Institute, Japan

<sup>4</sup>Department of Thoracic Surgery, Nagoya University Graduate School of Medicine, Japan

<sup>5</sup>Department of Oncology Diagnostics, Genentech Inc., South San Francisco, CA

<sup>6</sup>Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center at Dallas, 6000 Harry Hines, Dallas, TX

### Abstract

Malignant pleural mesothelioma (MPM) is a highly aggressive neoplasm arising from the mesothelial cells lining the parietal pleura and it exhibits poor prognosis. Although there has been significant progress in MPM treatment, development of more efficient therapeutic approaches is needed. *BMAL1* is a core component of the circadian clock machinery and its constitutive overexpression in MPM has been reported. Here, we demonstrate that *BMAL1* may serve as a molecular target for MPM. The majority of MPM cell lines and a subset of MPM clinical specimens expressed higher levels of *BMAL1* compared to a nontumorigenic mesothelial cell line (MeT-5A) and normal parietal pleural specimens, respectively. A serum shock induced a rhythmical *BMAL1* expression change in MeT-5A but not in ACC-MESO-1, suggesting that the circadian rhythm pathway is deregulated in MPM cells. *BMAL1* knockdown suppressed proliferation and anchorage-dependent and independent clonal growth in two MPM cell lines (ACC-MESO-1 and H290) but not in MeT-5A. Notably, *BMAL1* depletion resulted in cell cycle disruption with a substantial increase in apoptotic and polyploidy cell population in association with downregulation of Wee1, cyclin B and p21<sup>WAF1/CIP1</sup> and upregulation of cyclin E expression. *BMAL1* knockdown induced mitotic catastrophe as denoted by disruption of cell cycle regulators and induction of drastic morphological changes including micronucleation and multiple nuclei in ACC-MESO-1 cells that expressed the highest level of *BMAL1*. Taken together, these findings indicate that *BMAL1* has a critical role in MPM and could serve as an attractive therapeutic target for MPM.

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**Correspondence to:** Mitsuo Sato, Department of Respiratory Medicine, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showaku, Nagoya, Aichi 466-8550, Japan, Tel.: +81-52-744-2167, Fax: +81-52-744-2176, msato@med.nagoya-u.ac.jp.

Additional Supporting Information may be found in the online version of this article.

## Keywords

apoptosis; *BMAL1*; mesothelioma; targeted therapy; mitotic catastrophe

Malignant pleural mesothelioma (MPM) is a highly aggressive neoplasm arising from the mesothelial or submesothelial cells lining the parietal pleura. MPM has a particularly poor prognosis with a median survival of approximately 12 months from the onset of diagnosis.<sup>1</sup> Asbestos exposure is considered the most important etiologic factor that has been mentioned in relation to MPM.<sup>2</sup> Although a significant progress in MPM treatment has been achieved, there is an urgent need for developing new therapeutic approaches to improve the clinical outcome of patients with MPM.<sup>3</sup>

Many physiological, biological and metabolic processes are controlled by a homeostatic system called the circadian clock. This system is regulated by a circadian pacemaker located in the suprachiasmatic nucleus of the anterior hypothalamus that controls peripheral clocks over the 24 hr.<sup>4</sup> Recent studies have shown that peripheral tissues also have advanced molecular mechanisms that regulate circadian events, many of which have also been observed in established cell lines.<sup>5-7</sup>

Several circadian clock genes have been reported to control circadian rhythms in peripheral tissues, including three period proteins (PER1, PER2 and PER3), two cryptochromes (CRY1 and CRY2), CLOCK, NPAS2 and BMAL proteins. *BMAL1* is an indispensable core component in the circadian clock machinery. It can form heterodimer complexes with *CLOCK* or *NPAS2* genes; this complex drives transcription from E-box elements found in the promoters of circadian-responsive genes.<sup>8</sup> Period and cryptochrome proteins negatively regulate CLOCK/BMAL1 dimer-mediated transcription, thereby forming the feedback loop that regulates the timing of clock gene transcription.<sup>9</sup>

Disruption of the circadian clock has been associated with a wide variety of human disorders including cancer.<sup>10</sup> Previous studies have shown that clock genes are involved in the pathogenesis of human cancers. These genes seem to function primarily as tumor suppressors.<sup>11</sup> Several studies have reported the involvement of *BMAL1* in human cancers. High *BMAL1* expression was associated with poor patients' prognosis and distant metastasis in colorectal and breast cancer.<sup>12,13</sup> In addition, vascular endothelial growth factor is transcriptionally upregulated by *BMAL1*.<sup>14</sup> These reports suggest an oncogenic role for *BMAL1*. By contrast, Taniguchi *et al.*<sup>4</sup> showed that *BMAL1* expression is inactivated by promoter methylation in hematologic malignancies but not in solid cancers and that exogenously overexpressed *BMAL1* suppresses *in vitro* and *in vivo* growth of a lymphoma cell line, indicating a tumor suppressive role of *BMAL1* that may be specific for hematologic malignancies.

Recently, expression microarray analysis of MPM showed overexpression of several circadian rhythm genes compared to normal parietal pleural. Specifically, the *BMAL1* transcript was found to be overexpressed in MPM whereas negative regulators of *BMAL1* were expressed at lower levels. These findings raise the possibility that *BMAL1* could contribute to the aggressive malignant phenotypes of MPM.<sup>15</sup> To the best of our knowledge, no prior studies have analyzed the functional roles of *BMAL1* in MPM and thus we sought to investigate the role of *BMAL1* in the pathogenesis of MPM and its potential utility as a therapeutic target for MPM.

## Material and Methods

### Cell lines and tissue culture

Thirteen MPM cell lines and a nontumorigenic mesothelial cell line (MeT-5A) were used in this study. We purchased H2452, H2052, MSTO-211H, H28 and MeT-5A cell lines from the American Type Culture Collection and confirmed their authenticity by short tandem repeat (STR) analysis. H290 and H2373 were gifts from Dr Adi F. Gazdar (University of Texas Southwestern Medical Center, Dallas, TX). ACC-MESO-1, Y-MESO-12, Y-MESO-9, ACC-MESO-4, Y-MESO-22 (epithelioid) Y-MESO-14 (biphasic), and Y-MESO-8D (sarcomatoid) cell lines are established by ourselves.<sup>16</sup> Cells were grown in monolayer cultures in RPMI 1640 (Sigma-Aldrich Corp., St. Louis, MO, USA) containing 10% fetal bovine serum, 2 mmol/L glutamine and 1 mmol/L sodium pyruvate at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. MeT-5A cells were cultured in Medium 199 with Earle's balanced salt solution, 0.75 mM L-glutamine and 1.25 g/L sodium bicarbonate supplemented with 3.3 nM epidermal growth factor, 400 nM hydrocortisone, 870 nM insulin, 20 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid and 10% fetal bovine serum.

### RNA isolation and quantitative real-time reverse transcriptase-PCR analysis

For mRNA analysis, 5 µg of total RNA isolated using Trizol (Invitrogen, Carlsbad, CA, USA) were reverse transcribed with Super script III First-Strand Synthesis System using Random primer system (Invitrogen, Carlsbad, CA, USA). Quantitative real-time reverse transcriptase-PCR (qRT-PCR) analysis of *BMAL1*, *NPAS2* and *CLOCK* was performed as described previously.<sup>17</sup> GAPDH (Assays-on-Demand; Applied Biosystems, Foster City, CA, USA) was used as an internal control.

### Transfection of short interfering RNA

Cells ( $4.5 \times 10^5$ ) were plated in 10 cm<sup>2</sup> dish plate. Next day, cells were transiently transfected with either 10 nM predesigned short interfering RNA (siRNA) [Stealth Select RNA interference (RNAi)] targeting *BMAL1* or control siRNA purchased from Invitrogen using Lipofectamine RNAiMAX (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's protocol. After 48 hr, the transfected cells were harvested for further analysis or plated for growth assays.

### Western blot analysis

Cells were collected and washed twice in 1× phosphate-buffered saline (PBS), then lysed in ice-cold lysis buffer (0.5 M Tris-HCl with pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10 mM EDTA, 10% NP-40, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 5 mg/mL leupeptin and 10 mg/mL aprotinin) for 5 min. The lysate was centrifuged at 13,000 rpm for 20 min at 4°C, and protein content of the supernatant was measured. Total cell lysates (30 µg/well) were separated by SDS-PAGE and the gels transferred into nitrocellulose membranes (Whatman, Piscataway, NJ). Membranes were blocked with 5% nonfat dry milk in PBS containing Tween-20 (PBST) (1× PBS, 0.1% Tween-20) for 1 hr at room temperature and incubated with primary antibody at 4°C overnight. Membranes were then washed three times with PBST and probed with appropriate horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. The membranes were washed three times in PBST and bands were visualized using Western blot chemiluminescence reagent (BioRad, Hercules, CA 94547, USA). Antibodies were obtained from Santa Cruz (CA, 95060, USA) and Cell Signaling (Danvers, MA 01923, USA) Biotechnologies and used at the following dilutions (*BMAL1*, 1:1,000; Cyclin B, 1:1,000; Cyclin E, 1:1,000; Wee1, 1:1,000; p21<sup>WAF1/CIP1</sup>, 1:1,000; Cleaved caspase 3, 1:1,000; beta-actin, 1:5,000).

### Immunofluorescence staining

Forty-eight-hour post-transfection with *BMAL1*-siRNA or control-siRNA oligos, *in vitro* growing cells were seeded at  $2 \times 10^4$  cells/chamber in two-well Lab-Tek™ Chamber Slide System, incubated overnight at 37°C and 5% CO<sub>2</sub>. Next day, cells were washed with PBS and fixed with 4% paraformaldehyde solution, permeabilized with 0.01% Triton X-100 and blocked in 1% BSA in PBS for 20 min at room temperature. Subsequent antibody incubations were in PBS containing 1% BSA–0.01% Triton X-100. Antibody reagents were mouse anti- $\alpha$ -tubulin (1:100, Sigma-Aldrich St. Louis, MO, USA) and rabbit anti-BMAL1 H-170 (1:200, Santa Cruz Biotechnology, CA. 95060, USA). Incubation with primary antibody was kept overnight at 4°C, followed by three washes with PBS solution. Secondary reagent with Alexa Flour® 488-cojugated goat antimouse IgG, goat antirabbit IgG (1:1,000, Molecular probes, Invitrogen, Eugene, OR 97402, USA) and Alexa Flour® 594-cojugated goat antimouse IgG (1:700, Molecular probes, Invitrogen Eugene, OR 97402, USA). Secondary antibodies were incubated for 1 hr at room temperature. After PBS washing, independent mounting was done with Prolong Gold antifade reagent supplemented with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA, USA). All stained cells were visualized by confocal Eclipse TE 2000-E microscope (Nikon, Tokyo, Japan) with 20 $\times$ , 40 $\times$  and 60 $\times$  objectives.

### Tumor specimens of patients with MPM

Sixteen resected MPM tissue specimens were obtained from patients diagnosed with MPM who underwent surgery at the Department of Thoracic Surgery, Nagoya University Hospital between 2005 and 2011. Eleven patients received neoadjuvant chemotherapy with pemetrexed and cisplatin, while one patient received adjuvant chemotherapy and radiotherapy. Fifteen specimens were obtained through extra-pleural pneumonectomy and one was obtained by pleurectomy. Diagnosis of mesothelioma was made based on clinical evaluation, histopathologic examination, and the clinical stage was determined according to the International Mesothelioma Interest Group.<sup>18</sup> Patients' overall survival was defined as the length of time from the date of surgery to that of death. Four noncancerous (normal parietal pleura) tissue specimens were obtained from patients who underwent thoracic surgery for different causes (*i.e.*, not including mesothelioma) and were used as normal controls. The study protocol was approved by the Institutional Review Boards of Nagoya University Graduate School of Medicine. Informed consent was obtained from the patients following institutional guidelines. The expression level of the circadian clock protein, *BMAL1*, was examined in the aforementioned surgically resected specimens. Sections from formalin-fixed paraffin-embedded were treated for immunostaining with commercially available BMAL1 antibody (Sanata Cruz Biotechnology, CA.95060, USA) according to the procedures described elsewhere.<sup>13</sup>

### Cell growth assays

Colorimetric proliferation assay was performed using WST-1 assay kit (Roche, Basel, Switzerland) according to manufacturer's instruction. Liquid and soft agar colony formation assays were done as described previously.<sup>19</sup>

### Cell cycle analysis

Post-transfection with *BMAL1*-siRNA or control oligos, cells were synchronized by serum starvation for 12 hr or by double-thymidine treatment (to block mitosis and induce late G1/early S phase arrest; subconfluent cell cultures were incubated in complete medium containing 2 mM thymidine for 18 hr, and then, the thymidine medium was removed and replaced with complete medium lacking thymidine for 12 hr followed by another 18-hr incubation in the presence of thymidine). Synchronized populations were harvested and

washed in ice-cold PBS. Following centrifugation at 900g for 5 min, cells were suspended in 150  $\mu$ L of cold PBS while vortex gently, and cells were fixed by dropwise addition of 350  $\mu$ L ice-cold ethanol. Fixed cells were stored at  $-20^{\circ}\text{C}$  for at least 30 min. For staining, pelleted cells were washed twice with cold PBS and resuspended in 0.5 mL PBS containing 200  $\mu\text{g}/\text{mL}$  RNase, and stained with propidium iodide 20  $\mu\text{g}$ . Cells were incubated at  $37^{\circ}\text{C}$  for 30 min and maintained at  $4^{\circ}\text{C}$  before analysis, cells were filtered through 40  $\mu\text{m}$  nylon mesh and analyzed by flow cytometry for cell cycle status [FACS Calibur instrument (Becton Dickinson), with BD Cell Quest™ Pro Ver. 5.2.1 (BD) Bioscience, Franklin Lakes, NJ, USA].

### Apoptosis analysis

Apoptosis was quantified by detecting surface exposure of phosphatidylserine in apoptotic cells using a phycoerythrin (PE)—Annexin V Apoptosis Detection Kit I (BD Biosciences). Cells were harvested 5 days after transfection of siRNA oligos, treated according to the manufacturer's instructions and measured with PE 7-amino-actinomycin D (7-AAD) staining using flow cytometry [FACS Calibur instrument (Becton Dickinson), with BD Cell Quest™ Pro Ver. 5.2.1 (BD) Bioscience, Franklin Lakes, NJ, USA].

### Hematoxylin and eosin staining

Post-transfection with *BMAL1*-siRNA or control oligos, ACC-MESO-1 cells were harvested after 48 hr and 10,000 cells were plated/chambered in eight-well Lab-Tek™ Chamber Slide System, and then incubated overnight at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . Next day, cells washed with PBS and fixed with 1% glutaraldehyde for 5 min and subsequently stained with Mayer's Hematoxylin solution (Muto Puro Chemical LTD, Tokyo, Japan) for 2–3 min, rinsed with distilled water and then submerged in Scott's tap water substitute (0.2%  $\text{NaHCO}_3$  and 2%  $\text{MgSO}_4$ ) for bluing, finally cells were washed with distilled water once and examined under the microscope.

### Statistics

SPSS Ver. 18 software was used for all statistics analysis in this study. Mann–Whitney *U*-test was used for analyzing difference between two groups.

## Results

### MPM cell lines express higher levels of *BMAL1* than normal pleural mesothelial cells

Quantitative detection of *BMAL1* and *NPAS2* mRNA in a panel of MPM and MeT-5A cell lines was performed using qRT-PCR. Ten out of 13 (77%) MPM cell lines expressed higher levels of *BMAL1* mRNA than MeT-5A, while *NPAS2* expression profile seems different from *BMAL1* profile in MPM cell lines (Figs. 1a and 1c). Western blot analysis for the same set of cell lines clearly demonstrated variable degrees of BMAL1 protein expression (Fig. 1b). Furthermore, we investigated *BMAL1* expression levels in 16 surgically annotated MPM and four normal parietal pleural specimens (Table 1). Through immunohistochemical analysis, BMAL1 was detected in a subset of MPM specimens with nuclear and/or cytoplasmic localization, while none of the four normal parietal pleural samples showed detectable levels of BMAL1 protein (Fig. 1d), suggesting that BMAL1 may be important in the development of MPM.

### Expression profile of *BMAL1* and *CLOCK* over 24 hr in ACC-MESO-1 and MeT-5A cells

Based on previous data showing that expression of BMAL1 followed a rhythmical pattern in animal models,<sup>20</sup> we decided to examine the expression of *BMAL1* and *CLOCK* by qRT-PCR analysis to evaluate their oscillation over 24 hr in ACC-MESO-1 and MeT-5A cells.

We used the mouse fibroblast cell line (NIH3T3) as a positive control as it shows a rhythmic expression of *BMAL1*.<sup>20</sup> Serum shock was done as described previously.<sup>5</sup> *BMAL1* rhythmic expression was found in serum-shocked normal nontumorigenic mesothelial cells but not in ACC-MESO-1 cells, whereas *CLOCK* showed rhythmic expression in both MPM and normal mesothelial cells (Fig. 2). These findings indicate that *BMAL1* rhythmic expression was intact in normal mesothelial cells but not in MPM cells.

### ***BMAL1* knockdown suppresses proliferation, anchorage-dependent and independent clonal growth of MPM cells**

To investigate the role of *BMAL1* in MPM cell growth, we performed RNAi-mediated gene silencing against *BMAL1*. ACC-MESO-1 and H290 cell lines were selected as the MPM cell models to be used for further investigations (Fig. 1a). MeT-5A was used as a normal control. To reduce the off target effects, we used low dose (10 nM) stealth selected RNAi (Invitrogen Carlsbad, CA, USA) which includes three siRNA oligos with nonoverlapping sequences targeting *BMAL1*. Efficient *BMAL1* knockdown was confirmed by qRT-PCR, western blotting and immunostaining (Fig. 3). Next, to evaluate the effect of *BMAL1* knockdown on cell proliferation in mass culture and clonogenic growth in anchorage-dependent and independent conditions, we performed colorimetric growth, liquid and soft agar colony formation assays. We found that in ACC-MESO-1 and H290 cells, *BMAL1* knockdown significantly suppressed proliferation and dramatically suppressed colony formation in anchorage-dependent (liquid colony formation assay) and anchorage-independent conditions (soft agar assay) (Fig. 4). By contrast, we did not see significant suppression of proliferation in MeT-5A (Supporting Information, Fig. S1a).

### ***BMAL1* depletion induces massive apoptosis in MPM cells, with limited consequences in the normal pleural mesothelial cells**

Next, we investigated whether the antiproliferative effect of *BMAL1* depletion is due to cell death. *BMAL1* knockdown resulted in massive apoptosis and necrosis in MPM cells (Fig. 5a), as evidenced by increases in cleaved caspase-3 activation in ACC-MESO-1 cells (Fig. 5b). By contrast, subtle apoptosis could be found in MeT-5A cells following *BMAL1* knockdown (Fig. 5a). These results indicate that *BMAL1* knockdown-induced growth inhibition occurs in part through apoptotic mechanisms and MPM cells are more dependent on *BMAL1* expression for their survival than normal mesothelial cells.

### ***BMAL1* knockdown leads to cell cycle disruption in ACC-MESO-1 cells with a clear rise of polyploidy**

We investigated whether *BMAL1* knockdown-induced growth inhibition also was caused by cell cycle arrest. Consistent with induction of apoptosis as measured by cleaved caspase-3 expression, there was an increase in subG1 DNA content in ACC-MESO-1 transfected with *BMAL1*-siRNA oligos compared to cells transfected with control oligos (Fig. 5c). The proportion of cells in G1 phase decreased whereas a modest increase in the proportion of cells in G2/M phase. Notably, the profiling also showed an increase in the fraction of cells whose DNA contents exceed 4 *N* that correspond to polyploidy cell population. With much longer times in culture, high percentages of those cells (in G2/M phase and polyploidy region) decreased while subG1 population significantly increased (Fig. 5c), implying that polyploidy cells underwent apoptosis. By contrast, we did not see significant changes in cell cycle profiling of MeT-5A following *BMAL1* knockdown (Supporting Information, Fig. S1b).

### Depletion of *BMAL1* induces drastic morphological alterations indicative of mitotic catastrophe in ACC-MESO-1 cells

We noted that following *BMAL1* knockdown, ACC-MESO-1 cells underwent drastic morphological changes; the cells enlarged and elongated by visual examination under phase contrast microscopy. Next, we performed hematoxylin and eosin (H-E) staining for these cells. As shown in Figure 6a, dramatic morphological alterations were seen in ACC-MESO-1 cells after *BMAL1* knockdown; cells exhibit much enlarged flattened shape, micronucleation, multiple nuclei and vacuolization occasionally were found. Next, we examined *BMAL1* siRNA-treated ACC-MESO-1 cells by detailed morphological analysis of DAPI-stained cell nuclei and  $\alpha$ -tubulin immunostaining to visualize the cytoskeletal alterations associated with changes in nuclear morphology. Multiple morphological defects were identified which are consistent with the findings observed by H-E staining (Figs. 6b and 6c). Importantly, the existence of micronucleation is a highly indicative sign of catastrophic mitosis. To examine the possibility of mitotic catastrophe as a sequel of *BMAL1* knockdown and to explain its role in *BMAL1*-induced cell death during the cell cycle, ACC-MESO-1 cells transfected with *BMAL1*-siRNA or control oligos synchronized using a double thymidine block (to induce pharmacological block of mitosis in these cells). Interestingly, inhibition of mitosis in ACC-MESO-1 transfected with *BMAL1* siRNA resulted in a marked decrease of polyploidy and subG1 population (Fig. 6d), suggesting aberrant mitosis as a cause of cell death after *BMAL1* knockdown. To confirm the occurrence of mitotic catastrophe following *BMAL1* knockdown, we performed time lapse microscopic examination for ACC-MESO-1 cells transfected with *BMAL1*-siRNA or control oligos. Examination showed that cells rounding up as they entered mitosis, then attempting to undergo cytokinesis. ACC-MESO-1 cells transfected with control siRNA successfully completed mitosis, but *BMAL1* siRNA-treated cells failed to divide properly and exhibited large cell volume and micronucleation (Fig. 6e), indicating mitotic catastrophe as a cell fate following *BMAL1* knockdown in those cells.

### RNAi-mediated knockdown of *BMAL1* results in expression alterations of the cell cycle regulators in ACC-MESO-1 cells

Recent data suggest that there is a strong relationship between the circadian clock system and regulation of the cell cycle.<sup>21,22</sup> In particular, *BMAL1* is considered a key regulator of cancer cell proliferation through coordinating the activity of cell cycle proteins including p21<sup>WAF1/CIP1</sup> and cyclin B. Specifically, some data suggest that the circadian clock controls mitotic process through Wee1, known to be clock target gating the G2/M transition.<sup>10,21,23</sup> Therefore, we investigated the link between *BMAL1* knockdown and the status of the cell cycle proteins. Notably, ACC-MESO-1 cells after *BMAL1* knockdown showed profound alterations in cell cycle regulators, including significant decrease of Wee1, p21<sup>WAF1/CIP1</sup>, cyclin B proteins and accumulation of cyclin E protein (Fig. 5b).

### Discussion

In this report, we show that most MPM cell lines and a subset of surgically annotated MPM specimens expressed higher levels of *BMAL1* than MeT-5A and normal parietal pleural specimens, respectively. Silencing of *BMAL1* resulted in suppression of MPM cell growth and induction of apoptosis in these cells but limited effect was observed in MeT-5A. *BMAL1* depletion from ACC-MESO-1 cells, which expressed the highest level of *BMAL1*, led to cell cycle disruption with a substantial increase in apoptotic and polyploidy cell population as well as decreased levels of Wee1, cyclin B and p21<sup>WAF1/CIP1</sup> expression and upregulation of cyclin E. *BMAL1* knockdown in ACC-MESO-1 cells induced mitotic catastrophe denoted by marked disruption of cell cycle regulator proteins and drastic

morphological changes including micronucleation, multiple nuclei and increased cellular volume of ACC-MESO-1 cells.

By immunohistochemical analysis, we demonstrated that *BMAL1* is constitutively expressed in a subset of clinical MPM samples. Two out of three Stage IV-MPM patients were positive for *BMAL1*, whereas three out of 12 Stage III-MPM patients were positive (Table 1). This suggests that *BMAL1* expression may be associated with advanced stage-MPM, but the small patients' number makes it difficult to draw a firm conclusion on this possible association (Supporting Information, Fig. S2). It would be of importance to analyze the association between *BMAL1* expression and clinicopathological features of MPM using a large cohort. In line with our provisional results, high *BMAL1* expression was observed in metastatic breast, colorectal and liver cancers,<sup>12,13</sup> these findings suggest that *BMAL1* has an oncogenic activities in a variety of human cancers.

A prior study reported that clock genes oscillation was found in different tissues including muscle, liver and adipose tissues.<sup>24</sup> *BMAL1* rhythmic expression was observed also in NIH3T3 cell following synchronization by serum shock.<sup>25</sup> Here, we found that the rhythmic expression of *BMAL1* over the 24 hr period was intact and clearly observed in NIH3T3 cells and MeT-5A as well. Intriguingly, ACC-MESO-1 cells showed constant levels of *BMAL1* mRNA. This result is consistent with a recent report<sup>20</sup> showing that *BMAL1* is rhythmically expressed in mouse prostate while serum-shocked synchronized prostate cancer cells showed disrupted circadian rhythmicity of *BMAL1* gene. In line with Roe *et al.*'s<sup>15</sup> results who reported that *BMAL1* was found to be overexpressed concomitantly with downregulation of its negative counterpart in MPM compared to the normal parietal pleural tissue, our findings support the notion that the circadian rhythm pathway could be deregulated in MPM.

We demonstrated that inhibition of *BMAL1* expression using RNAi technique significantly suppressed proliferation, anchorage-dependent and independent clonal growth in MPM cells. To explore how *BMAL1* knockdown induced growth inhibition, we performed apoptosis assays and found that depletion of *BMAL1* expression by siRNA resulted in a significant increase in the fraction of apoptotic and necrotic cells in the MPM cell lines (ACC-MESO-1 and H290) with limited effect in MeT-5A. We also examined the regulatory role of *BMAL1* in MPM cell cycle and its importance in sustained cell proliferation. Interestingly, we found that RNAi-mediated knockdown of *BMAL1* resulted in cell cycle disruption of ACC-MESO-1 cells but not in MeT-5A cells.

Following *BMAL1* transient knockdown, we observed multiple morphological abnormalities consistent with aberrant mitotic process. There was an increase of binucleated cells, which may correspond to the modest increase in the G2/M phase cell population. Furthermore, some other cells (about 10%) showed micronucleation. Bergman *et al.*<sup>26</sup> reported that the occurrence of cells with double nucleus suggests that these cells undergo cell division without segregating their DNA and could be explained by the increase in cells with 4 NDNA content seen with flow cytometry. Importantly, micronucleation is highly indicative of mitotic catastrophe and could be resulted from chromosomal mis-segregation caused by DNA breaks. It is quite possible that ACC-MESO-1 cells underwent mitotic catastrophe due to impairment of cell cycle regulator proteins, such as, cyclin B, p21<sup>WAF1/CIP1</sup> and Wee1 (Fig. 6f). It is demonstrated that decreasing cyclin B below a critical level results in mitosis skipping and enhances polyploidy formation, which is considered as one of the characteristics of mitotic catastrophe.<sup>27</sup> Polyploidy cells pass an extra round of cell cycle and finally undergo apoptosis.<sup>28</sup> In addition, Wee1 downregulation could contribute to eventual escape from G2/M arrest, which in turns participates in impairment of mitotic integrity. In this study, we observed downregulation of p21<sup>WAF1/CIP1</sup>

and cyclin E upregulation following *BMAL1* knockdown, and the latter could also lead to increased number of polyploidy cells. Other investigators noted that *BMAL1* modulate the transcriptional activity of p53 toward its target p21 and clearly showed that *BMAL1* knockdown caused a decrease in p21 level.<sup>22</sup> Moreover, a previous study reported that overexpression of cyclin E led to impairment of mitosis and polyploidy formation.<sup>29</sup> We believe that accumulation of polyploidy, large multinucleated cells and micronucleation in ACC-MESO-1 after *BMAL1* knockdown were consistent with cell death mechanism involving mitotic catastrophe. It has been shown that mitotic catastrophe could be considered as an important safeguard to prevent the proliferation of polyploidy cells<sup>30</sup> and apoptosis frequently follows mitotic catastrophe.<sup>31</sup> Taken together, these findings show that *BMAL1* plays a critical role in the mitotic process of cancer cells. To our knowledge, this study is the first to shed light on the involvement of *BMAL1* in polyploidy formation, impairment of mitotic events, and also to highlight the role of circadian clock genes in MPM. In conclusion, we provide evidence that *BMAL1* plays an important role and it may be a promising therapeutic target for MPM, but careful consideration is needed to avoid the counter effect on tissues dependant on *BMAL1* such as muscles and liver.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Abbreviations

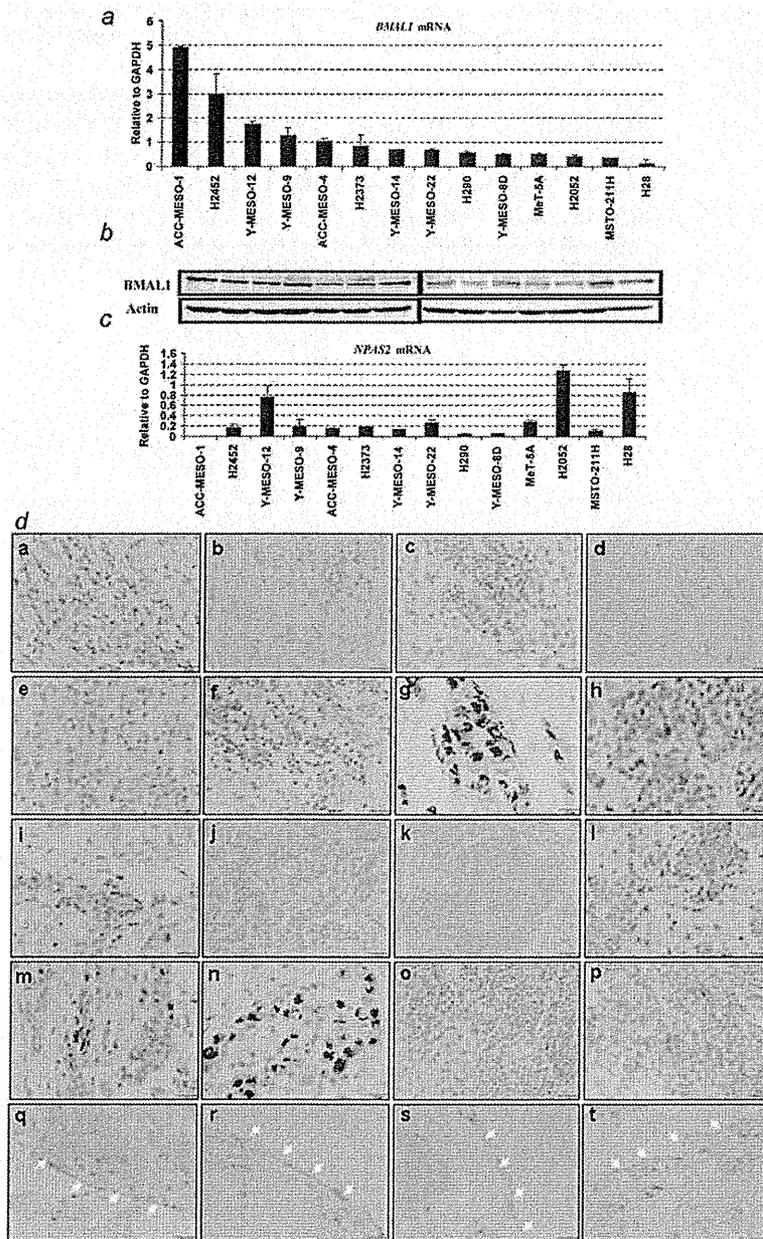
<b>BMAL1</b>	brain and muscle ARNT-like protein-1
<b>STR</b>	short tandem repeat
<b>GAPDH</b>	glyceraldehyde 3-phosphate dehydrogenase
<b>BSS</b>	balanced salt solution
<b>H-E</b>	Hematoxlin and Eosin
<b>HEPES</b>	4-2-hydroxyethyl-1-piperazineetha-nesulfonic acid
<b>MPM</b>	malignant pleural mesothelioma
<b>NPAS2</b>	neuronal PAS domain protein 2
<b>qRT-PCR</b>	quantitative real-time reverse transcriptase-PCR
<b>siRNA</b>	short interfering RNA
<b>DAPI</b>	4,6-diamidino-2-phenylindole

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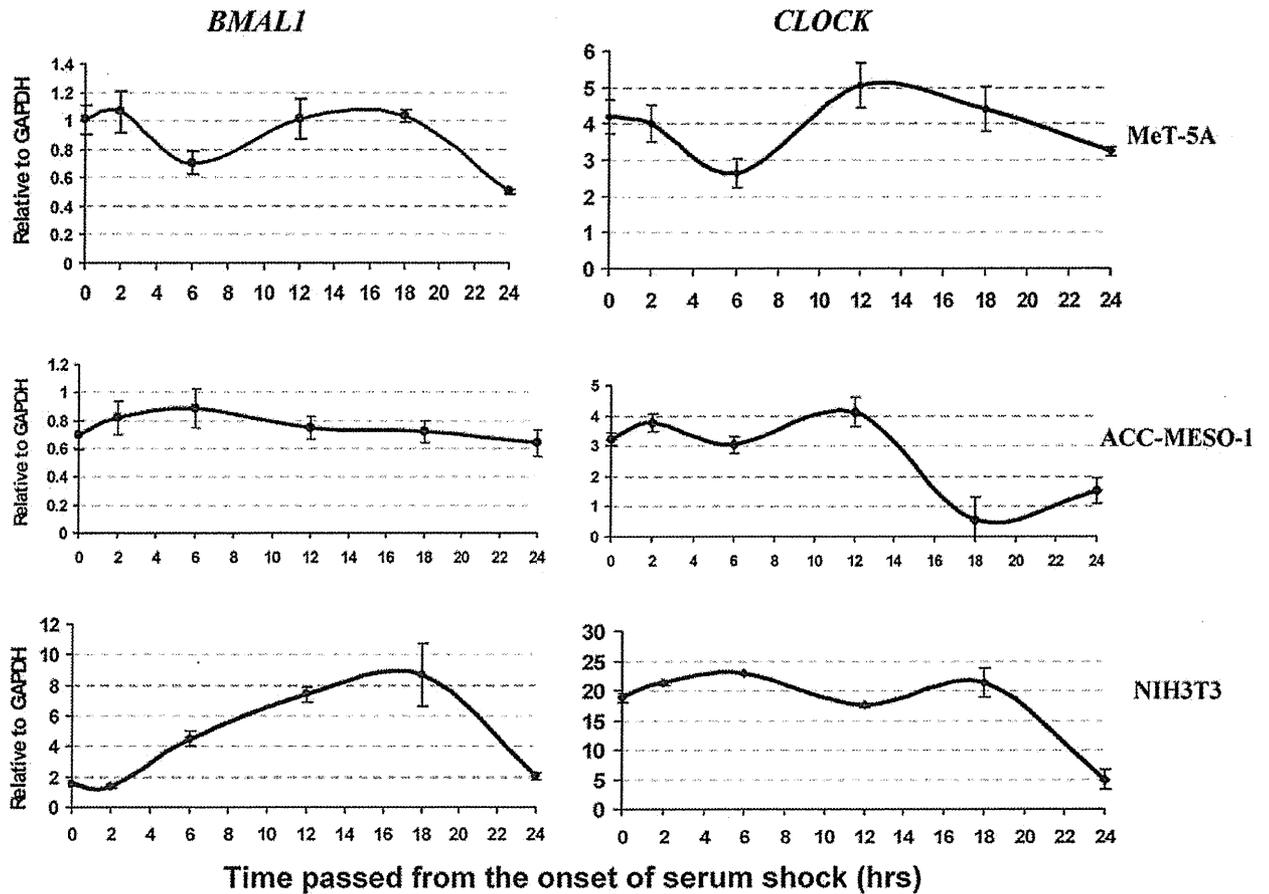
**Figure 1.** *BMAL1* expression levels in MPM and normal parietal pleura. (a) qRT-PCR analysis of *BMAL1* in 13 MPM cell lines and an immortalized pleural mesothelial cell line MeT-5A (control). The cell lines are aligned by expression levels of *BMAL1* mRNA from high (left) to low (right). The result is a representative of two independent qRT-PCR experiments done in duplicated reactions. (b) Western blots of *BMAL1* in MPM cell lines. Actin was used as a loading control. (c) qRT-PCR analysis of *NPAS2* in 13 MPM cell lines and MeT-5A. (d) Immunohistochemical staining of *BMAL1* in surgically annotated MPM and normal parietal pleural specimens. Immunohistochemical analysis showed an overexpression of *BMAL1* in a subset of MPM specimen (a–p), whereas normal pleural mesothelial cells showed negative

BMAL1 immunoreactivity (white arrows, q–t). Details of MPM and normal parietal pleural specimens were mentioned in “Material and Methods” Section. ×400 magnification and 25 μm scale bars were used for all images. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

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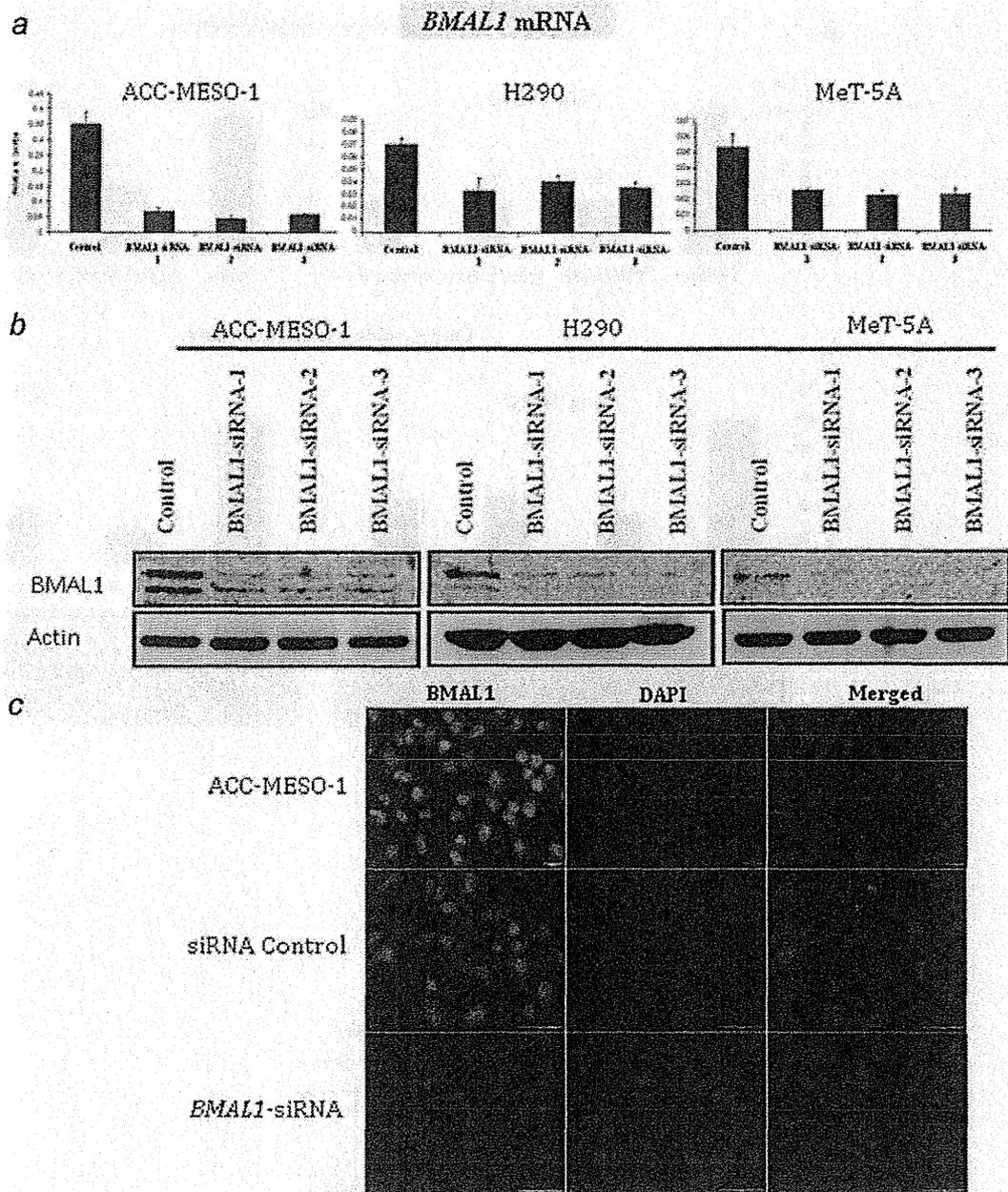
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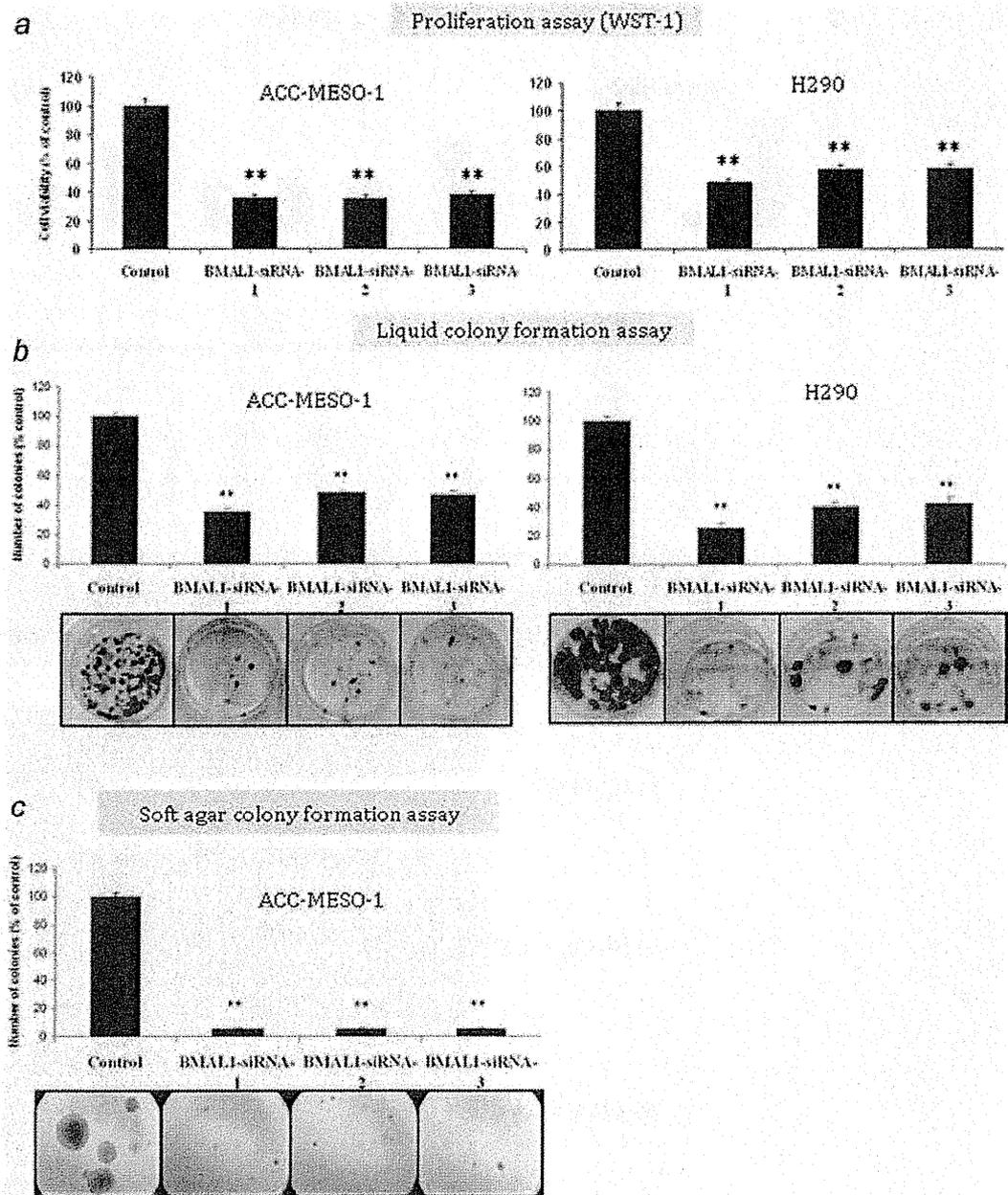


**Figure 2.**

Expression profiles of *BMAL1* and *CLOCK* genes over 24 hr in ACC-MESO-1 and MeT-5A cells. qRT-PCR analysis of *BMAL1* and *CLOCK* mRNA in ACC-MESO-1, MeT-5A at time indicated above. NIH3T3 cells were used as a positive control. Serum shock was performed as mentioned in the "Materials and Methods" Section. We found that serum shock induces rhythmical expression changes of *BMAL1* and *CLOCK* mRNA in NIH3T3 as well as MeT-5A cells. Serum shocked ACC-MESO-1 cells showed rhythmical changes of *CLOCK* mRNA, but not *BMAL1* (GAPDH was used as an internal control). The results are averages of two independent experiments done in duplicate.



**Figure 3.** *BMAL1* knockdown in MPM and MeT-5A cells. Confirmation of *BMAL1* knockdown by (a) qRT-PCR, (b) Western blot analysis and (c) immunofluorescence (IF) assay. IF was used to examine *BMAL1* levels in the parental, siRNA control or siRNA-*BMAL1*-treated ACC-MESO-1 cells. All data are averages of three independent experiments done in duplicates. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]



**Figure 4.** *BMAL1* knockdown inhibits proliferation and suppresses clonal growth of MPM cells in anchorage-dependent and -independent conditions. (a) WST-1 proliferation. (b) Liquid colony formation assays for ACC-MESO-1 and H290 cells transfected with *BMAL1*-siRNA or control oligos. (c) Soft agar colony formation assay for ACC-MESO-1 cells transfected with *BMAL1*-siRNA or control oligos. Results are from three independent experiments and shown as mean  $\pm$  SD. In liquid colony and soft agar assay colony, numbers of cells transfected with control oligos are set as 100%. \*\* indicate  $p < 0.01$  (Mann-Whitney *U* test). All data are averages of three independent experiments done in duplicates. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]