의학박사 학위논문

Study on the effects of acidic tumor microenvironment on the expression of circadian gene *BMAL1* in breast cancer

유방암에서 산성 종양 미세환경이 일주기 유전자 *BMAL1*발현에 미치는 영향에 관한 연구

2022년 8월

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A thesis of the Degree of Doctor of Philosophy

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August 2022

The Department of Biomedical Sciences,
Seoul National University
College of Medicine
(Major in Biomedical Sciences)

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지도 교수 예 상 규

이 논문을 의학박사 학위논문으로 제출함
2022년 4월

서울대학교 대학원
의과학과 의과학전공
권 용 진

권용진의 의학박사 학위논문을 인준함
2022년 7월

위 원 장 _____ (인)

부위원장 _____ (인)

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Study on the effects of acidic tumor microenvironment on the expression of circadian gene *BMAL1* in breast cancer

by

Yong-Jin Kwon

A thesis submitted to the Department of Biomedical Sciences in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biomedical Sciences at Seoul National University College of Medicine

April 2022

Approved by Thesis Committee:

Chair _____(Seal)

Vice Chair _____(Seal)

Examiner _____(Seal)

Examiner _____(Seal)

Examiner _____(Seal)

ABSTRACT

Circadian oscillation is an essential process that influences many physiological and biological mechanisms. Disrupted circadian rhythms and decreased circadian genes are associated with many diseases such as cancer. Despite many efforts to identify the detailed mechanisms for decreasing circadian genes and recovering reduced circadian genes in cancer, it is still largely unknown.

I found that expression of BMAL1 is reduced in tumor hypoxia-induced acidosis and restored by selectively targeting the acidic pH in breast cancer cell lines. The expression of BMAL1 was reduced by both decrease of protein stability and inhibition of transcription in the acidic tumor microenvironment. Melatonin significantly prevented acidosis-mediated decrease of BMAL1 expression by inhibiting lactate dehydrogenase-A (LDH-A) under hypoxia. The acidosis-mediated metastasis was significantly alleviated by BMAL1 overexpression in breast cancer cells.

I therefore suggest that tumor hypoxia-induced acidosis promotes metastatic potency by decreasing the expression of BMAL1, and the acidic tumor microenvironment could be a target for preventing breast cancer metastasis by sustaining BMAL1.

Keyword : circadian clock; BMAL1; hypoxia; acidic tumor microenvironment; breast cancer; metastasis; lactate dehydrogenase-A (LDH-A), melatonin

Student Number : 2016-21972

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LIST OF ABBREVIATIONS

AKT (Protein kinase B)

ARNTL (Aryl hydrocarbon receptor nuclear translocator-like protein 1)

ATCC (American type culture collection)

ATP (Adenosine triphosphate)

BMAL1 (Brain and muscle arnt-like protein-1)

CHX (Cycloheximide)

CLOCK (Circadian locomotor output cycles kaput)

CRY (Cryptochrome)

DMEM (Dulbecco's modified eagle medium)

DMEM/F-12 (Dulbecco's modified eagle medium/nutrient mixture F-12)

ECL (Enhanced luminol-based chemiluminescent)

EDTA (Ethylenediaminetetraacetic acid)

EMT (Epithelial-Mesenchymal Transition)

FBS (Fetal bovine serum)

G418 (G418 disulfate salts)

GEO dataset (Gene expression omnibus dataset)

GFP (Green fluorescent protein)

GSEA (Gene set enrichment analysis)

HCM (Hypoxic conditioned media)

HIF-1α (Hypoxia-inducible factor-1α)

HRP (Horseradish peroxidase)

IHC (Immunohistochemistry)

JAK (Janus kinase)

LAMP2 (Lysosomal associated membrane protein-2)

LDH (Lactate dehydrogenase)

LLC (Lewis lung carcinoma)

MCT (Monocarboxylate transporter)

MEM (Minimum essential medium)

MMP (Matrix metalloproteinase)

mTOR (Mammalian target of rapamycin)

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

NCBI (National center for biotechnology information)

NCM (Normoxic conditioned media)

NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells)

OS (Overall survival)

PBS (Phosphate buffered saline)

PER (Period)

RFS (Relapse free survival)

RCF (Relative centrifugal force)

ROS (Reactive oxygen species)

RPM (Revolutions per minute)

qRT-PCR (Quantitative reverse transcription polymerase chain reaction)

SCN (Suprachiasmatic nucleus)

SD (Standard deviation)

SDS (Sodium dodecyl sulfate)

STAT3 (Signal transducer and activator of transcription 3)

VEGF (Vascular endothelial growth factor)

WT (Wild-type)

ZO-1 (Zonula occludens-1)

INTRODUCTION

Breast cancer is the most common cancer in females worldwide, and the second most cancer-related death reported annually [1,2]. Cancer metastasis represents progression of the primary cancer [3,4], with over 80% of breast cancer-related deaths diagnosed with metastasis [5]. Despite extensive studies of breast cancer, the incidence and metastasis rates have not been reduced [6]. A better understanding of cancer and new treatment strategies are therefore needed to prevent metastasis of breast cancer.

The circadian clock maintains daily oscillation rhythms with a 24 h periodicity in all living organisms. The suprachiasmatic nucleus (SCN) in the hypothalamus is an internal factor regulating the circadian rhythm and it responds to stimuli from external environments such as light, which affect pathological and physiological functions [7]. Several genes are associated with the circadian clock, including brain and muscle Arnt-like protein-1(*BMAL1*; encoded by *ARNTL* gene), circadian locomotor output cycles kaput (*CLOCK*), period (PERs; *Per1*, *Per2*, and *Per3*), and cryptochromes (CRYs; *Cry1* and *Cry2*), which form a complex network of transcription–translation feedback loops, post-translational modifications, and degradation [8,9].

Many people disrupt their daily circadian rhythm due to irregular patterns of life. Disrupted circadian rhythms are associated with a number

of diseases, including cancer [10,11]. Because disrupted circadian rhythms and patient prognosis are known to be directly related, it can be very important to analyze tumor progression according to disrupted circadian rhythms in tumors. In previous epidemiological studies, it was suggested that night shift workers with irregular circadian rhythms are closely associated with increased risk of breast, colon, lung and prostate cancer [12–14]. The cancer tissue has been shown to have lower expression of circadian genes than surrounding normal tissues, and more advanced states of cancer exhibit lower expression of circadian genes [15,16]. In addition, pattern of circadian genes expression was disrupted in MCF-7, MDA-MB-231, T47D, and Hs578T (breast cancer cell line) compared to MCF-10A (normal breast cell line) and HME1 (mammary epithelial cell line) [17,18]. *BMAL1*, which is one of the most important circadian clock genes, regulates overall circadian oscillations in humans, and previous reports have suggested that reduced BMAL1 is closely associated with tumor metastasis, proliferation, colonization and chemo-resistance in cancer cells [19–21]. In particular, Loss of *BMAL1* increased tumor initiation by regulating intestinal stem cell signaling such as Hippo and Wnt signaling pathway, and knocked-out BMAL1 MDA-MB-231 breast cancer cells promoted tumor metastasis [22,23]. Although BMAL1 is known to inhibit tumor progression, the mechanism for decreasing BMAL1 in cancer is largely unknown. My aim was therefore to understand the relationship between breast cancer and circadian genes, and to identify tumor-mediated factors that reduce circadian genes.

In the previous study, it was reported that when melanoma cells were injected into mice skin, the rhythm patterns of clock genes were disrupted in the adjacent-tumor as well as the tumor, and it was suggested that the cause of this phenomenon is a change of the environment around the tumor [24]. Since it is also well known that the circadian gene is expressed at a lower level in most cancer tissues than in surrounding normal tissues, it can be expected that the tumor microenvironments will be significantly related to the disrupted circadian rhythms in the tumor. However, which tumor microenvironment caused the disruption of circadian rhythms is not well known yet. Therefore, I want to find out which tumor microenvironment reduces circadian genes and disrupts the circadian rhythms.

Hypoxia is a representative tumor microenvironment present in almost all solid tumors, including breast cancer, which forms a mass through abnormally rapid growth, and promotes cancer progression by regulation of angiogenesis, signaling molecules, and increased metabolism, and by changing the behavior of stromal cells surrounding the tumor [25–27]. Under conditions of sufficient oxygen, metabolic glycolysis generally relies on mitochondrial oxidative phosphorylation to generate ATP. However, during hypoxia, cancer cells increase in the presence of inefficient glycolysis because large amounts of ATP and building blocks are needed for cell proliferation. As a result, many byproducts such as lactic acid are produced and released from cells through plasma membrane transporters [28,29]. Finally, extracellular pH of cancer cells becomes acidic. Tumor

acidosis, another major tumor microenvironment, promotes metastatic potency in MDA-MB-231 via the LAMP2 and ROS-AKT-NF-κB pathways [30–32]. Based on these studies, I speculated that tumor hypoxia and acidosis cause genetic alterations and it is closely related to the disruption of circadian rhythms in breast cancer.

Since the circadian rhythms were disrupted in most cancers, I hypothesized that common tumor microenvironments are the cause. In the previous study, the hypoxia-induced acidosis disrupts the circadian rhythms by mTOR signaling pathway [33]. However, in breast cancer, the relationship between the circadian gene *BMAL1* and the tumor microenvironment is still largely unknown, and it is insufficient to explain only mTOR signaling pathway. In this study, I focused this phenomenon more on breast cancer and I aimed to determine the new pathway for decreasing BMAL1 and recovering reduced BMAL1 in the breast cancer microenvironment.

MATERIALS AND METHODS

1. Reagents and Antibodies

Anti-BMAL1, anti-CLOCK, and anti-α-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-HIF-1α, anti-ZO-1, and anti-LDH-A antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). HRP-tagged anti-rabbit and anti-mouse antibodies were purchased from Enzo Life Science (Farmingdale, NY, USA). Lactic acid, cycloheximide (CHX), G418 disulfate salts (G418), sodium oxamate, melatonin, and sodium bicarbonate ($NaHCO_3$) were purchased from Sigma Aldrich (St. Louis, MO, USA).

2. Cell Lines and Culture Conditions

The human normal breast cell line MCF-10A was purchased from the American Type Culture Collection (ATCC) and maintained in DMEM/F-12 (Welgene, Gyeongsan, Republic of Korea) supplemented with 5% horse serum (GIBCO, Waltham, MA, USA), 100 ng/mL cholera toxin, 20 ng/mL EGF, 0.5 mg/mL hydrocortisone, 10 μg/mL insulin, and 1% penicillin/streptomycin (Capricorn Scientific GmbH, Ebsdorfergrund, Germany). The human breast cancer cell lines MCF-7, T47D, ZR-75-1, MDA-MB-231, MDA-MB-468, and Hs578T were purchased from ATCC, and maintained in DMEM (Capricorn Scientific GmbH) supplemented with 10% fetal bovine serum (FBS, Capricorn Scientific GmbH) and 1%

penicillin/streptomycin (Capricorn Scientific GmbH). The mouse mammary tumor cell line TUBO and TUBO-P2J were kindly provided from Professor SaeGwang Park (INJE University College of Medicine, Republic of Korea) and maintained in DMEM (Capricorn Scientific GmbH) supplemented with 10% FBS (Capricorn Scientific GmbH), 10% NCTC-109 medium (GIBCO), 2 mmol/L L-glutamine, 0.1 mmol/L MEM nonessential amino acids (GIBCO), and 1% penicillin/streptomycin (Capricorn Scientific GmbH). BMAL1 overexpressing MDA-MB-231 cell lines were established from G418-resistant clones. For hypoxia stimulation, the oxygen tension in incubator (Vision Science, Seoul, Republic of Korea) was 21% O_2 normoxic condition and 2% O_2 hypoxic condition respectively. These cells were maintained in humidified atmosphere containing 5% CO_2 at 37℃.

3. Conditioned Media

MCF-7 and MDA-MB-231 cells were seeded onto the plate. When cells were attached, growth media was exchanged for serum free media, and then cultured under normoxia (21% O_2) or hypoxia (2% O_2) for 48 h, respectively. Normoxic conditioned media (NCM) were obtained after culturing cells under normoxia, and hypoxic conditioned media (HCM) were obtained after culturing cells under hypoxia. The heat-inactivated HCM were obtained by boiling HCM at 100℃ for 5 min to degrade all secretory proteins in cultured media, and the neutralized HCM were obtained by neutralizing acidified pH of HCM using 1 M NaOH dose-dependently. These conditioned media were treated in each cell line for 24

h, respectively.

4. pH Regulation

To acidify the media pH, 1 M HCl and lactic acid were treated in the media dose-dependent manner and incubated at 37℃ 5% CO_2 condition for 24 h. After stabilization, pH of the media was immediately measured using a SevenEasy pH meter (Mettler Toledo, Columbus, OH, USA). The pH of the cultured media was measured immediately after the experiments using a SevenEasy pH meter, and the analyzed pH of the cultured media was summarized in Table 4–12.

5. Cell Viability Assay

Cells were grown in 96-well culture plates with each condition, and incubated with MTT reagent for 4 h. Blue formazan crystals were solubilized with DMSO, and formazan levels were determined at 570 nm using an Infinite M200 PRO plate reader (Tecan Group Ltd., Männedorf, Switzerland).

6. DNA and siRNA Transfection

For overexpression of genes, cells were transfected with the following constructs: pEGFP-C3-vector and pEGFP-C3-BMAL1. pEGFP-C3-vector and pEGFP-C3-BMAL1 were kindly provided from Professor Sang Ki Park (Pohang University of Science and Technology, Republic of Korea). *BMAL1*, *CLOCK*, and *LDH-A* were knocked down by transfection

with human si-BMAL1(SI00023016 and SI00023037; Qiagen) mouse si-BMAL1 (SI02685865 and SI00166719; Qiagen), human si-CLOCK (SI00069769 and SI00069776; Qiagen), human si-LDH-A (siRNA no.3939-1; Bioneer), and a negative control si-RNA (1028290; Qiagen). Transfection was performed using the Lipofectamine 3000 and Lipofectamin RNAiMax reagent (Invitrogen), according to the manufacturer's protocol.

7. Stable cell line

Cells were transfected with the pEGFP-C3 and pEGFP-C3-BMAL1 using Lipofectamine 3000 according to the manufacturer's protocol. After 2 days, G418 was dose-dependently treated to select transfected cells at least 1-2 weeks. pEGFP-C3 and pEGFP-C3-BMAL1 expressing MDA-MB-231 stable cell lines were established from four G418-resistant clones.

8. Immunoblotting

Cells were washed cold PBS, and then lysed in the triton lysis buffer containing protease and phosphatase inhibitors (2 mM phenylmethanesulfonyl fluoride, PMSF; 1 mM sodium fluoride, NaF; 2 mM ethylenediaminetetraacetic acid, EDTA; 0.5 mM sodium orthovanadate, Na_3VO_4; 10 μg/mL leupeptin). After incubation for 30 min on ice, lysates were centrifuged at 13,000 RPM (15,700 RCF) for 20 min at 4°C, and supernatants were collected. Lysates were separated on 7%–12% SDS-

polyacrylamide gels, and transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Chicago, Illinois). Membranes were blocked in 5% skim-milk for 1 h and incubated with primary antibodies overnight at 4°C. Membranes were incubated with a horseradish peroxidase-conjugated secondary antibodies for 1 h, and then visualized using the ECL detection kit (Young In Frontier Co., Ltd, Seoul, Republic of Korea).

9. RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated using the RNAiso Plus reagent (Takara, Shiga, Japan) and cDNA was synthesized using a ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). Quantitative real-time PCR was performed using the EvaGreen qPCR Mastermix (Applied Biological Materials, Richmond, Canada), and fluorescence was detected by CFX Connect Real-Time PCR Detection System (Bio-Rad). Data were analyzed with the CFX Manager Software (Bio-Rad), and the mRNA values of targeted genes were normalized to tubulin expression. The sequences of PCR primers are summarized in Supplementary Table 3.

10. Trans-Well Migration Assay

Cells were cultured in cell culture inserts with an 8 μm pore size polycarbonate membrane (Corning Life Sciences, MA, USA). Cells were seeded in the upper trans-well chamber containing serum-free media and the lower chamber contained complete media to induce cell migration. After incubation, cells on the upper chamber membrane were fixed and

stained with Diff-Quick solution kit (Sysmex Corporation, Kobe, Japan). The stained cells on the upper side of the interface membrane were wiped with a cotton swab and migration cells on the lower side of the membrane were counted using ECLIPSE TS100 inverted microscope (Nikon Instruments Inc., Melville, NY, USA).

11. Wound-Healing Assay

Cells were seeded onto 6-well plate. When the cell confluence reached about 80%-90%, detached cells were scratched using a 200 μL plastic tip, and then the debris was removed by washing with PBS. The cells were incubated in serum-free medium. The cell migration ability was analyzed by visualizing the edge of cells using ECLIPSE TS100 inverted microscope (Nikon Instruments Inc).

12. Public Datasets

Breast cancer gene expression omnibus (GEO) datasets (GSE3744 and GSE5364) used in this study were obtained national center for biotechnology information (NCBI, https://www.ncbi.nlm.nih.gov/geo/). An online Kaplan–Meier plotter database was used to analyze recurrence free survival (RFS) of breast cancer patients (http://kmplot.com/analysis) [34].

13. Statistical Analysis

All data were statistically analyzed using Microsoft Excel 2017 software and Graph pad Prism 5 software. Results are presented as means ±

standard deviation (SD) of three independent experiments. The statistical significance was determined by an unpaired Student's t−test. All statistical significances were considered when a p−value was less than 0.05.

RESULTS

1. Chronic hypoxia reduces the BMAL1 expression in breast cancer cells

Since previous studies have reported that circadian genes including *BMAL1* are reduced in almost cancers, I hypothesized that tumor hypoxia, a common and predominant occurrence in cancer, plays a critical role in decrease of the *BMAL1* circadian gene. Hypoxia significantly reduces BMAL1 protein expression in MCF-7, T47D (luminal A), ZR-75-1 (luminal B), MDA-MB-231, MDA-MB-468, Hs578T (basal-like) human breast cancer, and TUBO and TUBO-P2J (TUBO metastatic variant) mouse breast cancer cells (Figure 1a, b and Table 1). In addition, BMAL1 was reduced under hypoxia in MCF-10A human normal breast cells (Figure 1c). Since MCF-7 and MDA-MB-231 cell lines have been used for decades as in vitro breast cancer models, I used for these representative two cell lines. BMAL1 is only reduced in chronic and deep hypoxia for 48 h in 2% O_2, and not in acute and mild hypoxia for 24 h in 2% O_2 or 48 h in 10% O_2 (Figure 1d, e). In addition, hypoxia also reduces *BMAL1* mRNA expression (Figure 1f). *CLOCK* is also the circadian gene and forms a complex with BMAL1. This heterodimer complex binds to E-box elements in promoters and drives circadian rhythms [8]. The circadian genes are linked to other circadian genes expression because these are composed of transcription–translation feedback loops [9]. Interestingly, protein and mRNA levels of *CLOCK* were also reduced under chronic hypoxia (Figure 2a, b). In this study, I focused more on *BMAL1* among

several clock genes. Hypoxia-inducible factors (HIFs) are transcription factors that are activated in low oxygen conditions. The previous studies have reported that not only hypoxia but also HIF-1α reciprocally regulates circadian rhythms in human osteosarcoma cells and mouse myoblast cells [33,35]. However, in breast cancer cells, I found that the hypoxia-mimetic agent $CoCl_2$ did not affect BMAL1 protein expression (Figure 3a). In addition, both overexpression of wild-type (WT) and constitutively stable HIF-1α (P405A and P564A) did not affect the BMAL1 protein expression (Figure 3b). Moreover, when HIF-1α was silenced during hypoxia, it did not prevent the decrease of BMAL1 protein expression (Figure 3c). Together, these results suggested that BMAL1 was reduced by chronic hypoxia independently of HIF-1α in breast cancer cells.

2. Hypoxia-mediated acidosis reduces circadian BMAL1 expression in breast cancer cells

In hypoxia, many cells secrete pro-tumorigenic cytokines, chemokines, and growth factors, which play important roles in tumorigenesis, such as recruiting diverse types of immune cells and accelerating angiogenesis [26,28]. I hypothesized that hypoxia-mediated secretory factors reduce BMAL1 expression in breast cancer cells. When MCF-7 and MDA-MB-231 breast cancer cells were exposed to hypoxic conditioned media (HCM), BMAL1 was reduced (Figure 4a). However, BMAL1 was also reduced in heat-inactivated HCM, in which all secretory proteins were degraded (Figure 4a). These results suggested that the hypoxia-mediated

secretory proteins had no effect on the regulation of BMAL1, but other sources of HCM may be involved. I then observed that media containing phenol red was yellowish in HCM-treated MCF-7 cells, normoxic conditioned media (NCM), and HCM-treated MDA-MB-231 cells (Figure 4a bottom). This phenomenon exhibited a similar pattern as the decrease of BMAL1 expression. Since yellowish medium indicates that acidosis has occurred and in the previous study, the BMAL1 was disrupted under hypoxia-induced acidosis in osteosarcoma cells [33], I expected that hypoxia-induced acidosis also reduced BMAL1 expression in breast cancer cells. BMAL1 was reduced by HCM, but was not reduced when the pH of the medium was neutralized by NaOH in a dose-dependent manner (Figure 4b-d). Many studies have reported that tight junction proteins are reduced in acidic conditions [36,37]. Since the tight junction protein ZO-1 was significantly reduced under acidic conditions, it was an indication that the breast cancer cells were involved in acidosis. In addition, the protein and mRNA levels of *BMAL1* in MCF-7 and MDA-MB-231 breast cancer cells were reduced in chronic hypoxia but unchanged when the pH of the medium was neutralized by NaOH (Figure 4e-g) or buffered by $NaHCO_3$ (Figure 4h-k). Together, these results showed that hypoxia-induced acidosis reduced BMAL1 expression, which could be prevented by selectively targeting the acidic pH in breast cancer cells.

3. Tumor acidosis reduces BMAL1 via inhibition of transcription activity and protein stability in breast cancer cells

In hypoxia, most cells including cancer cells release large amounts of lactic acid via anaerobic glycolysis and become acidified, which is a hallmark of tumor malignancy [30]. According to previous studies, in normal cells, intracellular pH (pHi) is lower than extracellular pH (pHe; pHi = 7.2 and pHe = 7.4). However, in cancer cells, pHi is higher than pHe (pHi ≥ 7.2 and pHe = 6.7–7.1) (Table 2) [38–43]. I assumed the tumor acidic pHe to be < 7.0 according to several studies (Figure 5a).

To determine whether BMAL1 was reduced only by tumor acidosis, I adjusted the pH of the cell culture medium using HCl (Figure 5b). When the acidic media were treated in MCF-7 and MDA-MB-231 cells for 24 h, media with pH < 7.0 reduced BMAL1 expression, and the cultured media of MCF-7 and MDA-MB-231 cells had pH values less than 6.7 (Figure 5c-e). Similar results were also obtained for T47D, ZR-75-1, MDA-MB-468, Hs578T human breast cancer, and TUBO and TUBO-P2J mouse breast cancer cell lines (Figure 5f). Since reduced circadian genes in all organs can directly cause many diseases including cancer, continuously oscillating circadian genes are important for the treatment and prevention of diseases [11]. Notably, decrease of BMAL1 by tumor acidosis was recovered by the exchanging acidic cultured media to fresh media (Figure 6a-c) or adding $NaHCO_3$ to the acidic cultured media (Figure 6d-g). I also found that BMAL1 was reduced by HCl-mediated acidosis in MCF-10A normal breast cells (Figure 7a-d) and decrease of BMAL1 by tumor acidosis was recovered by adding $NaHCO_3$ to the acidic cultured media (Figure 7e, f). Additionally, I found that CLOCK was also reduced by

tumor acidosis (Figure 8a, b). The metabolic byproduct lactic acid is a major metabolite that induces hypoxia-mediated acidosis. BMAL1 is also reduced by lactic acidosis and recovered by exchanging media (Figure 9a-d) or adding $NaHCO_3$ (Figure 8e-g). These results suggested that circadian BMAL1 was only reduced by acidosis and recovered by neutralizing and buffering the acidic pH in breast cancer cell lines. Under hypoxia and acidic conditions, while the protein level of BMAL1 was almost reduced (Figures 1a and 5f), the mRNA level was not completely reduced in breast cancer cells (Figure 1f and 6c, g). Since previous studies have reported that circadian genes including BMAL1 sustaining their circadian rhythms through transcription, protein post-translational modification, and protein degradation [8], I hypothesized that other mechanisms may reduce BMAL1 protein levels by acidosis besides transcriptional inhibition. Cycloheximide (CHX), an inhibitor of de novo protein synthesis, decreased the half-life of BMAL1 protein expression in MCF-7 and MDA-MB-231 breast cancer cells. Notably, I found that the half-life of BMAL1 was shortened even further by acidic conditions and treatment with CHX (Figure 10a). Together, the results suggested that tumor acidosis reduced BMAL1 via inhibition of transcription and decrease of protein stability (Figure 11a).

4. Tumor acidosis-mediated decrease of BMAL1 promotes metastatic potency in breast cancer cells

Breast cancer can be successfully treated by surgery and therapeutic

strategies, but when metastasis occurs, the survival rate falls dramatically despite many previous studies to prevent breast cancer metastasis [5]. A novel mechanism is therefore needed to prevent tumor metastasis. Since tumor hypoxia and acidosis are well-known to promote tumor metastasis in cancers including MDA-MB-231 cells, targeting the tumor hypoxia and acidosis is likely important in treating tumors [44–47]. Interestingly, the previous study reported that knocked-out BMAL1 promotes metastasis in MDA-MB-231 cells, and previously my results showed that BMAL1 was reduced by tumor hypoxia-induced acidosis and recovered by selectively targeting the acidic pH in breast cancer cells [23]. Based on these references and my results, I hypothesized that promoted metastasis under acidosis is caused by a decrease of BMAL1, and maintaining BMAL1 by buffering acidic pH is a therapeutic approach to prevent metastasis. I used MDA-MB-231 and TUBO-P2J breast cancer cell lines, which have metastatic potencies, to investigate the relationship between reduced BMAL1 by acidosis and breast cancer metastasis. MDA-MB-231 and TUBO-P2J breast cancer cells increase metastasis during acidosis. However, when the acidic pH was buffered, increased migration by acidosis was alleviated (Figure 12a, b). This tendency was the same as the expression pattern of BMAL1 (Figure 12c). To characterize the role of BMAL1 in acidosis-mediated breast cancer metastasis, I used an overexpression system to maintain BMAL1 expression levels during acidosis. Importantly, increased migration by acidosis was alleviated in the green fluorescent protein (GFP)-tagged BMAL1-transfected MDA-MB-

231 and TUBO-P2J breast cancer cells because GFP-BMAL1 was overexpressed despite decrease of endogenous BMAL1 by acidosis (Figure 13a-c). To confirm these results, I established GFP-tagged BMAL1 stably overexpressed MDA-MB-231 cell lines. When GFP-tagged BMAL1 was consistently overexpressed, tumor migration was significantly alleviated, and increased migration by acidosis was also alleviated in GFP-tagged BMAL1 stable MDA-MB-231 cell lines (Figure 14a–d). By contrast, when BMAL1 was knocked down using small interfering RNA, tumor migration was significantly promoted in MDA-MB-231 and TUBO-P2J breast cancer cell lines (Figure 15a–d). Additionally, I found that knock-down of *CLOCK* promotes metastasis, and double knock-down of *BMAL1* and *CLOCK* further promotes metastasis in MDA-MB-231 breast cancer cells (Figure 16a–c). Together, these results suggest that tumor acidosis-mediated decrease of the BMAL1 promotes tumor metastatic potency in breast cancer cell lines, and selectively targeting tumor acidosis to maintain BMAL1 prevents breast cancer metastasis.

5. Melatonin attenuates decrease of BMAL1 by inhibiting hypoxia-mediated LDH-A in breast cancer cells

Since BMAL1 was reduced by tumor acidosis, I wanted to identify a biological mechanism to prevent the decrease of BMAL1 during tumor acidosis. Melatonin, a hormone produced in the pineal gland, is responsible for the oscillation of overall circadian rhythms in humans [48]. Melatonin

regulates the sleep–wake cycle, as well as blood pressure and body temperature [49–51], and has been also reported to be a potential effector of antioxidant, anti-inflammatory, and anticancer activities in many diseases [52–54]. I hypothesized that melatonin might prevent the decrease of BMAL1 by tumor acidosis. To investigate a possible mechanism, MCF-7 and MDA-MB-231 cells were treated with melatonin in hypoxic conditions. Remarkably, with melatonin treatment, hypoxia-mediated decrease of BMAL1 was significantly prevented (Figure 17a-c). However, in HCl-induced acidic conditions, decrease of BMAL1 was not prevented by melatonin because the HCl-induced acidic pH was not controlled by melatonin in MCF-7 and MDA-MB-231 cells (Figure 18a, b) and without cells (Figure 18c). These results suggest that melatonin prevented hypoxia-induced acidosis and a decrease of BMAL1 in breast cancer cells. During anaerobic glycolysis, pyruvate is converted into lactic acid by LDH, the primary enzyme of hypoxia-mediated acidosis [55]. Previous studies reported that melatonin inhibits LDH expression and activity [56–58]. Based on these results, I hypothesized that increased LDH in hypoxia was inhibited by melatonin, which might prevent hypoxia-mediated decrease of BMAL1 by inhibiting acidosis. As expected, increased LDH-A in hypoxia was reduced by melatonin in MCF-7 and MDA-MB-231 breast cancer cells (Figure 19a). Additionally, I confirmed that hypoxia-mediated decrease of BMAL1 was prevented by inhibiting LDH-A and tumor acidosis using oxamate (Figure 19b, c), non-competitive LDH inhibitor, and small interfering RNA (Figure 19d, e).

LDH-A is known as HIF-1α target gene for decades. However, in a previous study, overexpression of WT HIF-1α did not adequately increase the several HIF-1α target genes including LDH-A compared to hypoxia [59]. I additionally confirmed that HIF-1α alone could not regulate LDH-A and cultured media pH (Figure 20a, b). My results suggest that the hypoxia-induced acidosis reduced BMAL1 independently of HIF-1α in breast cancer cells. Together, these results show that melatonin maintained BMAL1 by inhibiting the expression of LDH-A to prevent hypoxia-induced acidosis in MCF-7 and MDA-MB-231 breast cancer cells (Figure 21a). Therefore, I proposed a new mechanism for melatonin, which regulates BMAL1 expression during hypoxia-mediated tumor acidosis by inhibiting LDH-A.

6. Decrease of *BMAL1* is clinically related to poor prognoses in breast cancer patients

I then investigated the possible clinical relevance of *BMAL1* expression between normal and breast cancer tissues using the GSE database. *BMAL1* was significantly decreased in breast cancer compared with normal breast tissue in GSE5364 and GSE3744 (Figure 22a). In the same GSE databases, *LDH-A*, which induces hypoxia-mediated acidosis, was also higher in cancer tissues (Figure 22b). I additionally investigated whether the *BMAL1* gene was associated with survival in breast cancer patients using the Kaplan–Meier (KM) database [34]. When breast cancer was divided into *ARNTL* and *LDH-A* low or high groups by the mean median value,

recurrence free survival (RFS) was higher in the *BMAL1* high group than the *BMAL1* low group and lower in the *LDH-A* high group than the *LDH-A* low group (Figure 23a, b). Furthermore, RFS was higher in the *CLOCK* high group than the *CLOCK* low group (Figure 24a). These databases predicted that breast cancer involves hypoxia-induced acidosis, which reduces *BMAL1* and *CLOCK*. As a result, expression of *BMAL1* and *CLOCK* was associated with poor prognoses in breast cancer patients. Overall, my results demonstrated that chronic hypoxia induced acidosis, one of the most obvious tumor microenvironments, which reduced the *BMAL1* circadian clock gene via inhibition of transcriptional activity and decreased protein stability in breast cancer, and reduced *BMAL1* promoted metastatic potency, which could be prevented by targeting tumor acidosis using melatonin via inhibition of *LDH-A* (Figure 25a). I additionally suggest a possibility that *CLOCK* is also reduced under hypoxia-mediated acidosis and reduced *CLOCK* promotes breast cancer metastasis.

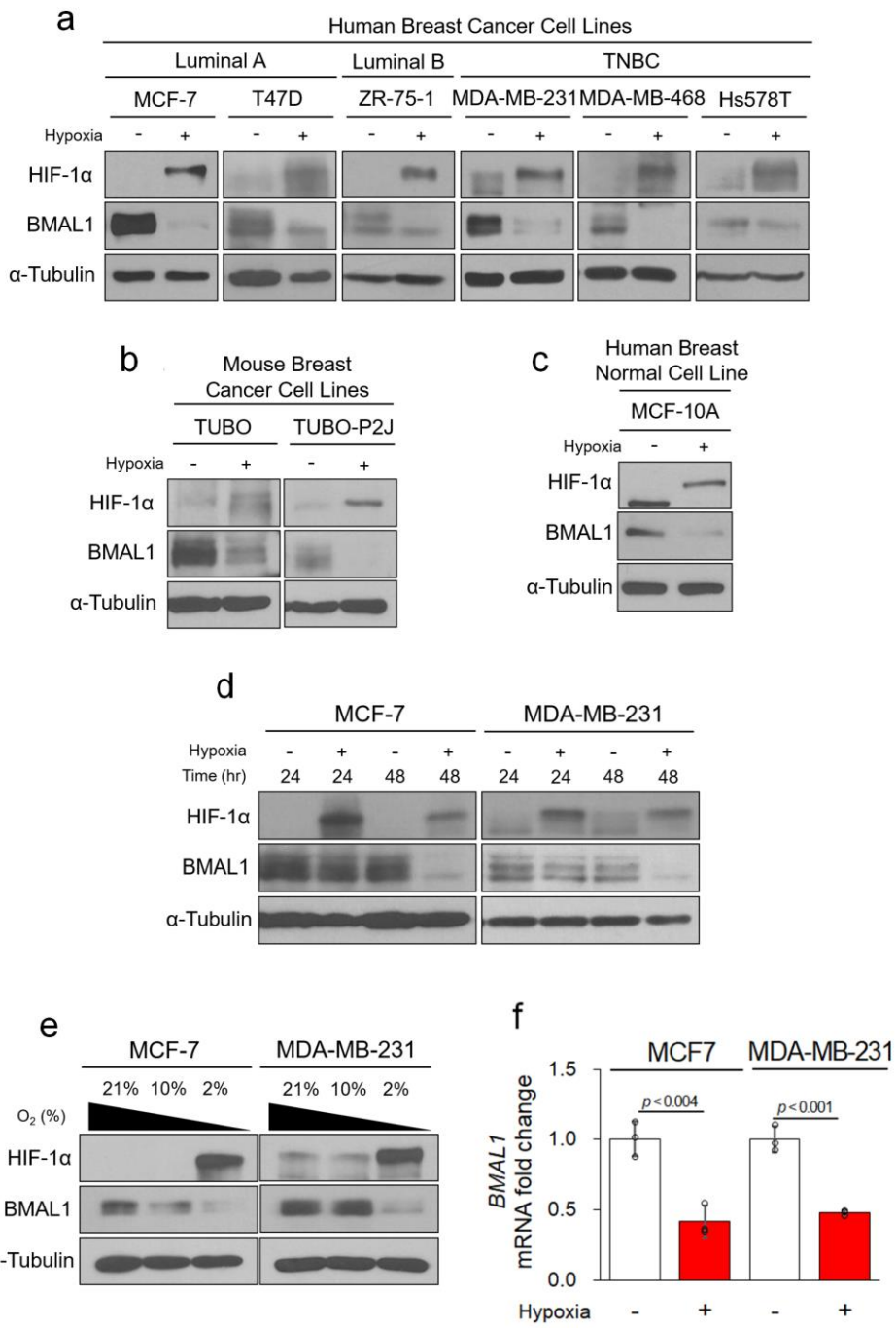


Figure 1. Chronic hypoxia reduces circadian BMAL1 expression in breast cancer cells

(a, b) Breast cancer cell lines (a) and normal breast cell line (b) were

incubated in normoxia or 2% O_2 hypoxia for 48 h. Cell lysates were analyzed by immunoblotting. (c) MCF-10A was incubated in normoxia or 2% O_2 hypoxia for 48 h. Cell lysates were analyzed by immunoblotting. (d) MCF-7 and MDA-MB-231 were incubated in normoxia or 2% O_2 hypoxia for 24 and 48 h. Cell lysates were analyzed by immunoblotting. (e) MCF-7 and MDA-MB-231 were incubated in normoxia, 2% or 10% O_2 hypoxia for 48 h. Cell lysates were analyzed by immunoblotting. (f) MCF-7 and MDA-MB-231 were incubated in normoxia or 2% O_2 hypoxia for 48 h. Cell lysates were analyzed by qRT-PCR. All experiments were performed at least three times in independent experiments. The statistical significance was determined by an unpaired Student's *t*-test.

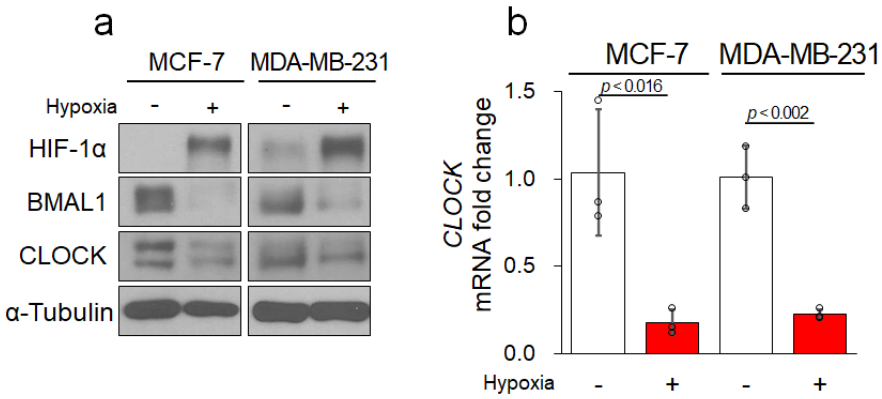


Figure 2. *CLOCK* was also reduced by chronic hypoxia in breast cancer cells

(a, b) MCF-7 and MDA-MB-231 were incubated in normoxia or 2% O_2 hypoxia for 48 h. Cell lysates were analyzed by immunoblotting (a) and qRT-PCR (b). All experiments were performed at least three times in independent experiments. Data represent the mean ± SD. The statistical significance was determined by an unpaired Student's *t*-test.

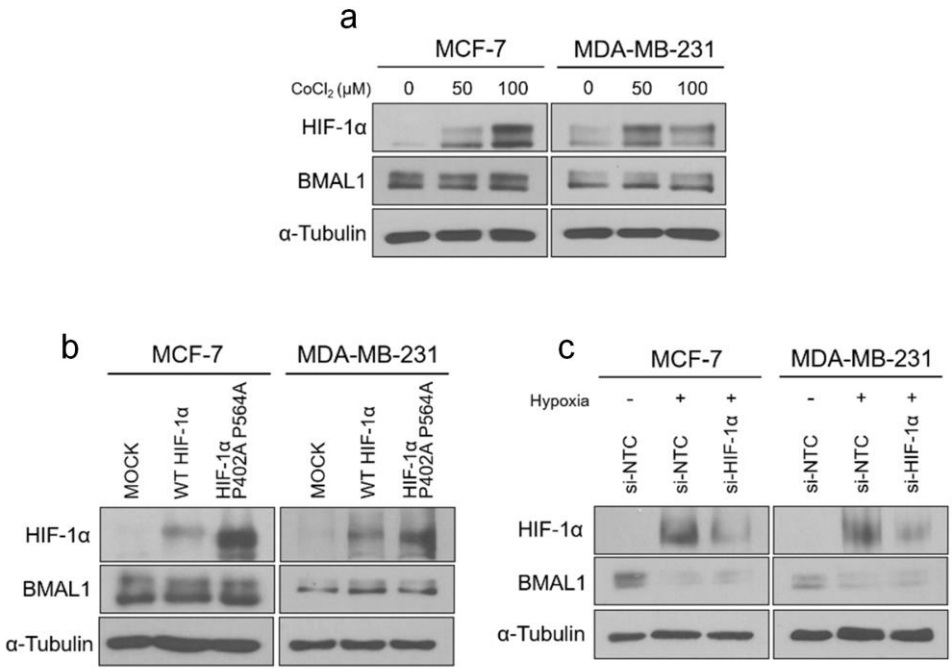
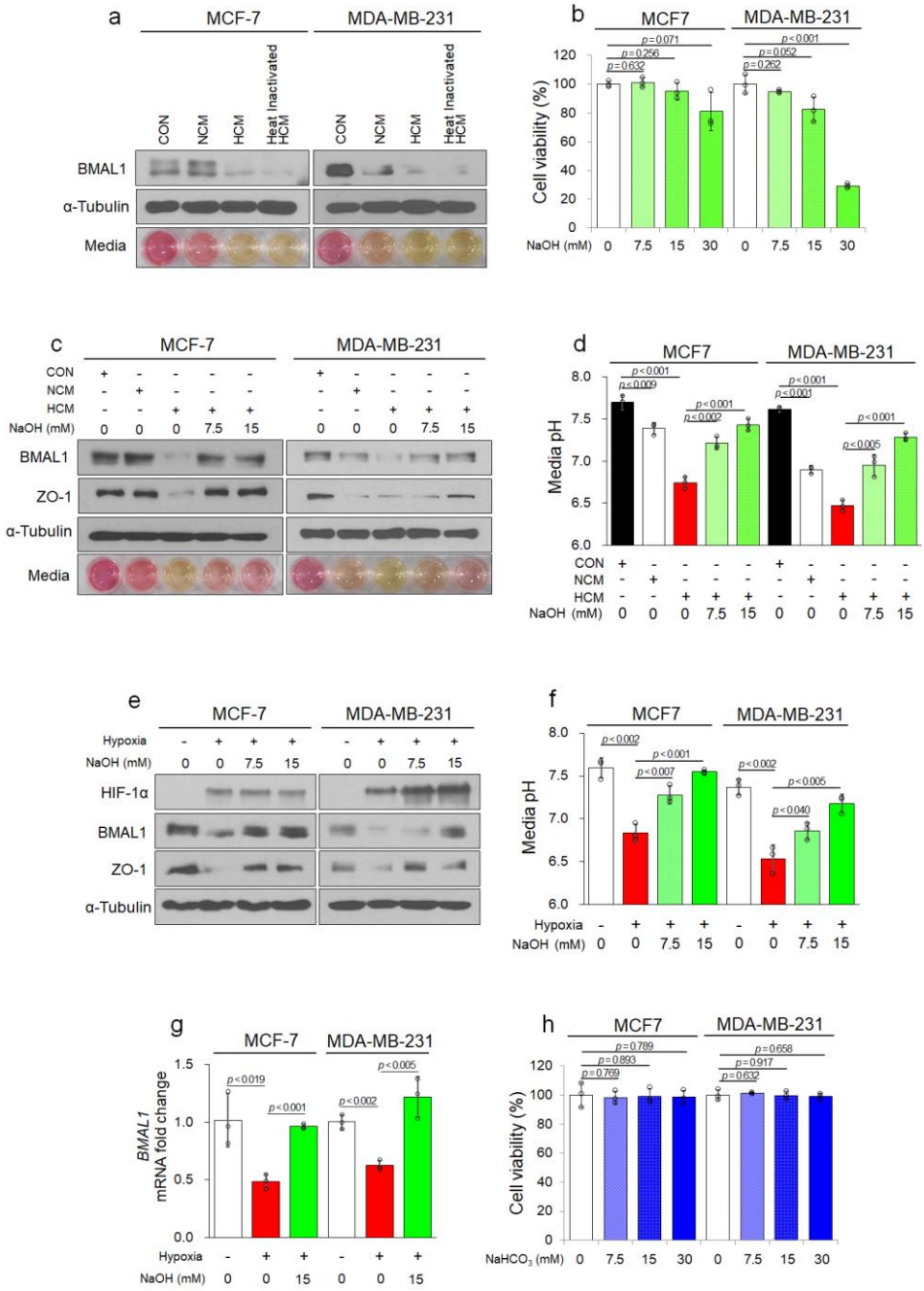


Figure 3. BMAL1 was reduced by chronic hypoxia independently of HIF-1α in breast cancer cells

(a) MCF-7 and MDA-MB-231 were incubated in normoxia with $CoCl_2$ for 48 h. Cell lysates were analyzed by immunoblotting. (b) MCF-7 and MDA-MB-231 were transfected with HIF-1α^{WT} or HIF-1$\alpha^{P402A/P564A}$ plasmid, and incubated in normoxia for 48 h. Cell lysates were analyzed by immunoblotting. (c) MCF-7 and MDA-MB-231 were transfected with si-HIF-1α, and incubated in 2% O_2 hypoxia for 48 h. Cell lysates were analyzed by immunoblotting. All experiments were performed at least three times in independent experiments.


a
MCF-7
MDA-MB-231
CON
NCM
HCM
Heat Inactivated HCM
BMAL1
α-Tubulin
Media
b
MCF7
MDA-MB-231
Cell viability (%)
NaOH (mM)
c
ZO-1
d
Media pH
e
Hypoxia
HIF-1α
f
g
BMAL1 mRNA fold change
h
NaHCO3 (mM)

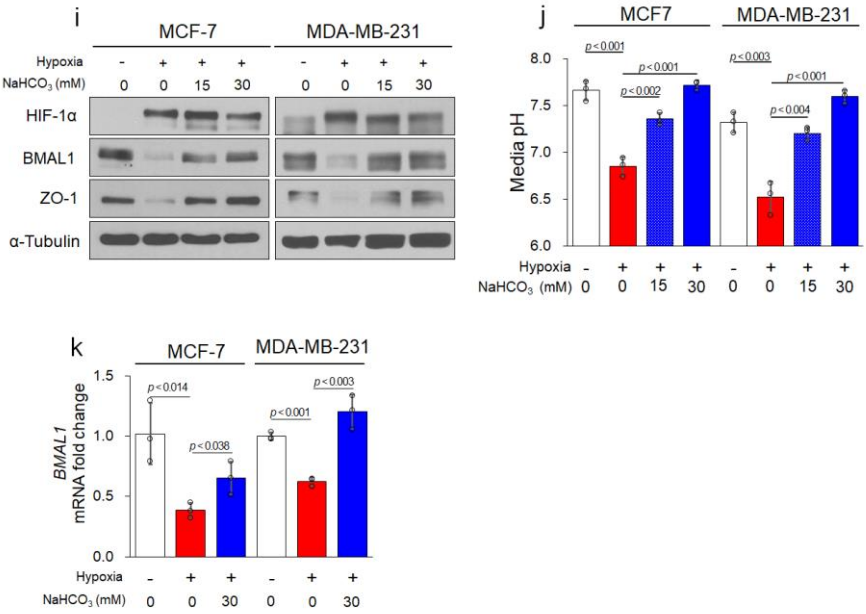


Figure 4. Hypoxia-mediated acidosis reduces circadian BMAL1 expression in breast cancer cells

(a) MCF-7 and MDA-MB-231 were treated with fresh media (control; CON), normoxic conditioned media (NCM), hypoxic conditioned media (HCM), or heat inactivated HCM for 24 h. Cell lysates were analyzed by immunoblotting. Representative images of cultured media are shown (bottom panel). (b) MCF-7 and MDA-MB-231 were treated with NaOH for 24 h. Cell viability was measured by MTT assay. (c, d) MCF-7 and MDA-MB-231 were treated with fresh media, NCM, HCM, or NaOH treated HCM for 24 h. Cell lysates were analyzed by immunoblotting (c) and pH of the cultured media was immediately measured using a pH meter (d). Representative images of cultured media are shown (Figure 4c bottom panel). (e-g) MCF-7 and MDA-MB-231 were incubated in normoxia or 2% O_2 hypoxia with NaOH for 48 h. Cell lysates were analyzed by

immunoblotting (e) and pH of the cultured media was immediately measured using a pH meter (f). Cell lysates were analyzed by qRT−PCR (g). (h) MCF-7 and MDA-MB-231 were treated with $NaHCO_3$ for 24 h. Cell viability was measured by MTT assay. (i−k) MCF−7 and MDA−MB−231 were incubated in normoxia or 2% O_2 hypoxia with $NaHCO_3$ for 48 h. Cell lysates were analyzed by immunoblotting (i) and pH of the cultured media was immediately measured using a pH meter (j). Cell lysates were analyzed by qRT−PCR (k). All experiments were performed at least three times in independent experiments. The statistical significance was determined by an unpaired Student's *t*−test.

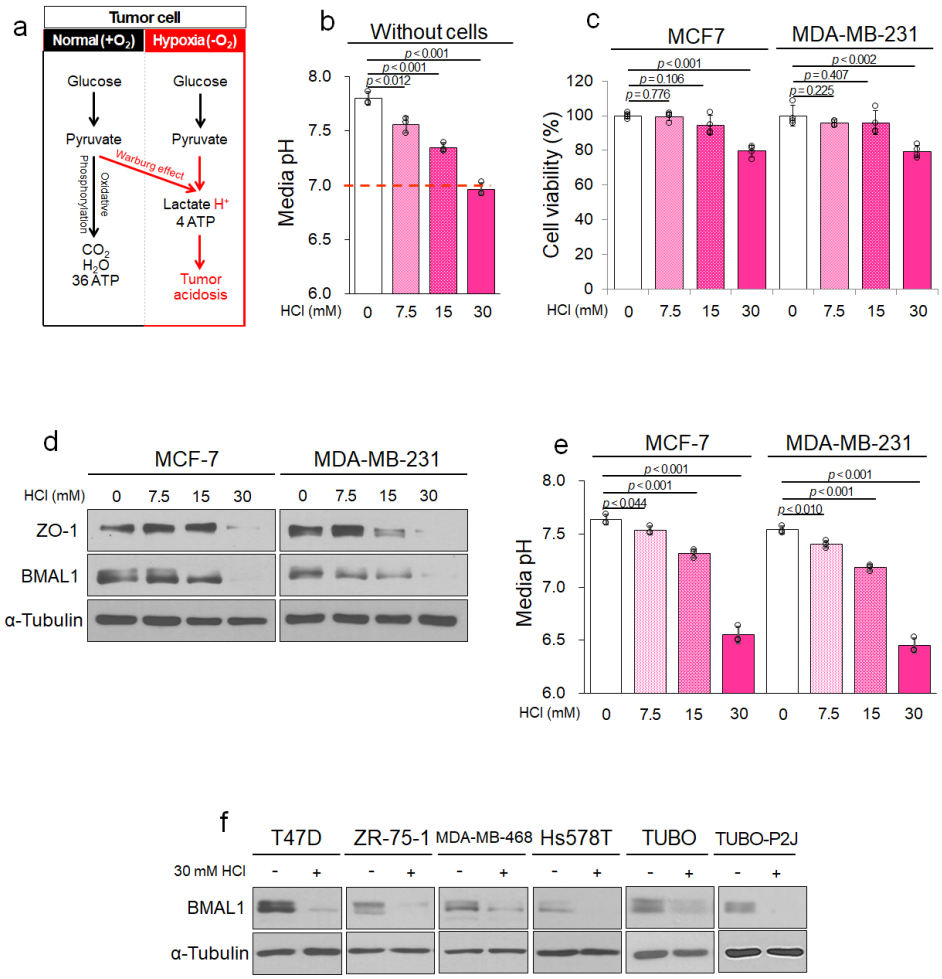


Figure 5. Tumor acidosis reduces BMAL1 expression in breast cancer cells

(a) The summarization of the glycolysis pathway in normoxia and hypoxia. (b) HCl-mediated acidic DMEM media were incubated for 24 h without cells, and media pH was immediately measured using a pH meter. (c) MCF-7 and MDA-MB-231 were treated with HCl for 24 h. Cell viability was measured by MTT assay. (d, e) MCF-7 and MDA-MB-231 were treated with HCl-induced acidic media for 24 h. Cell lysates were analyzed by immunoblotting (c) and pH of the cultured media was

immediately measured using pH meter (d). (f) Breast cancer cell lines were treated with HCl−mediated acidic media for 24 h. Cell lysates were analyzed by immunoblotting. All experiments were performed at least three times in independent experiments. The statistical significance was determined by an unpaired Student's *t*−test.

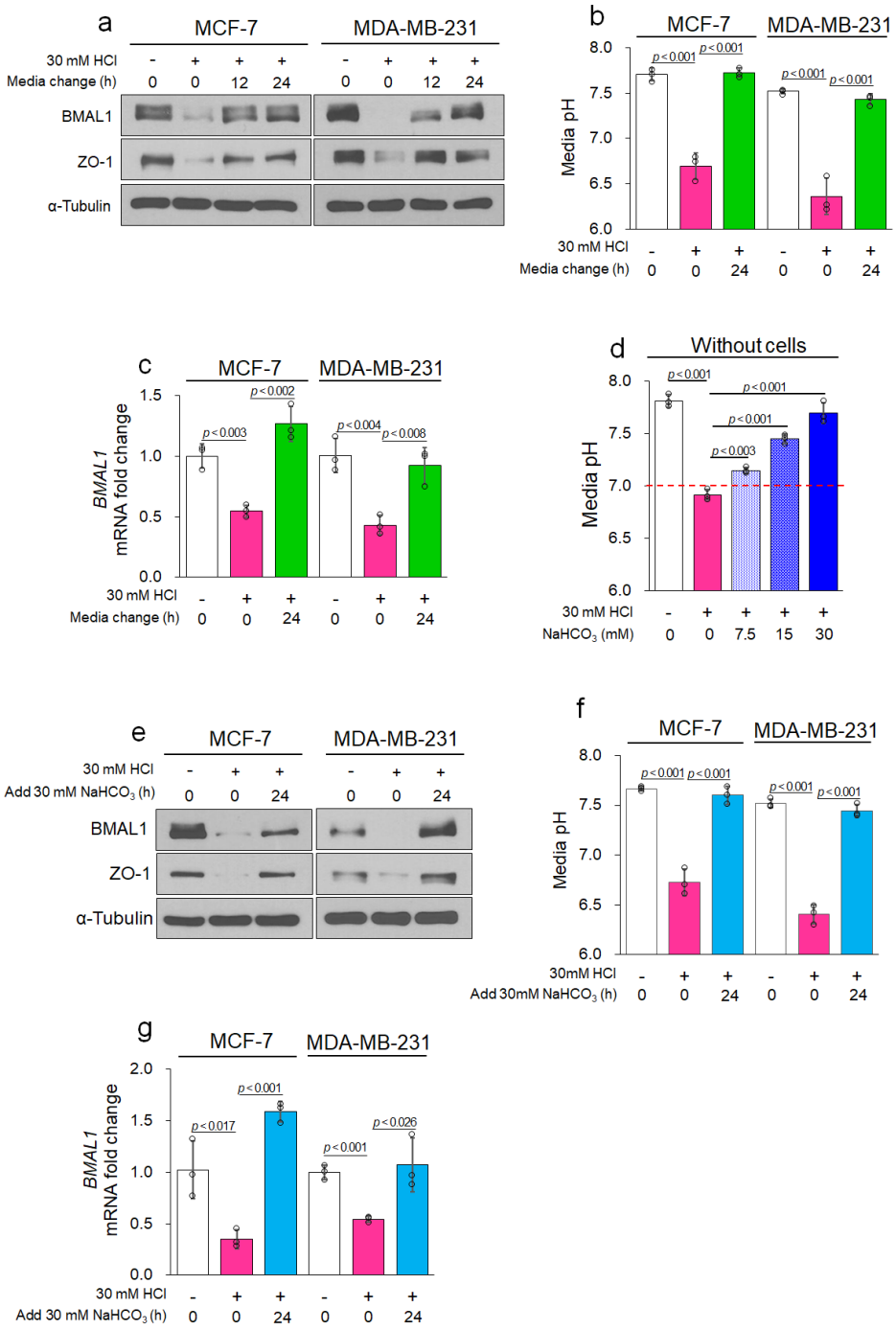


Figure 6. Decrease of BMAL1 by tumor acidosis was restored by neutralizing acidic pH in breast cancer cells

(a–c) MCF–7 and MDA–MB–231 were treated with HCl–mediated acidic media for 24 h. The acidic cultured media were exchanged to fresh media and then incubated for 12 and 24 h. Cell lysates were analyzed by immunoblotting (a) and pH of the cultured media was immediately measured using a pH meter (b). Cell lysates were analyzed by qRT–PCR (c). (d) HCl–mediated acidic DMEM media were incubated for 24 h without cells. The acidic media were added to $NaHCO_3$ and then incubated for 24 h. media pH was immediately measured using a pH meter. (e–g) MCF–7 and MDA–MB–231 were treated with HCl–mediated acidic media for 24 h. The acidic cultured media were added to $NaHCO_3$ and then incubated for 24 h. Cell lysates were analyzed by immunoblotting (e) and pH of the cultured media was immediately measured using a pH meter (f). Cell lysates were analyzed by qRT–PCR (g). All experiments were performed at least three times in independent experiments. The statistical significance was determined by an unpaired Student's *t*–test.

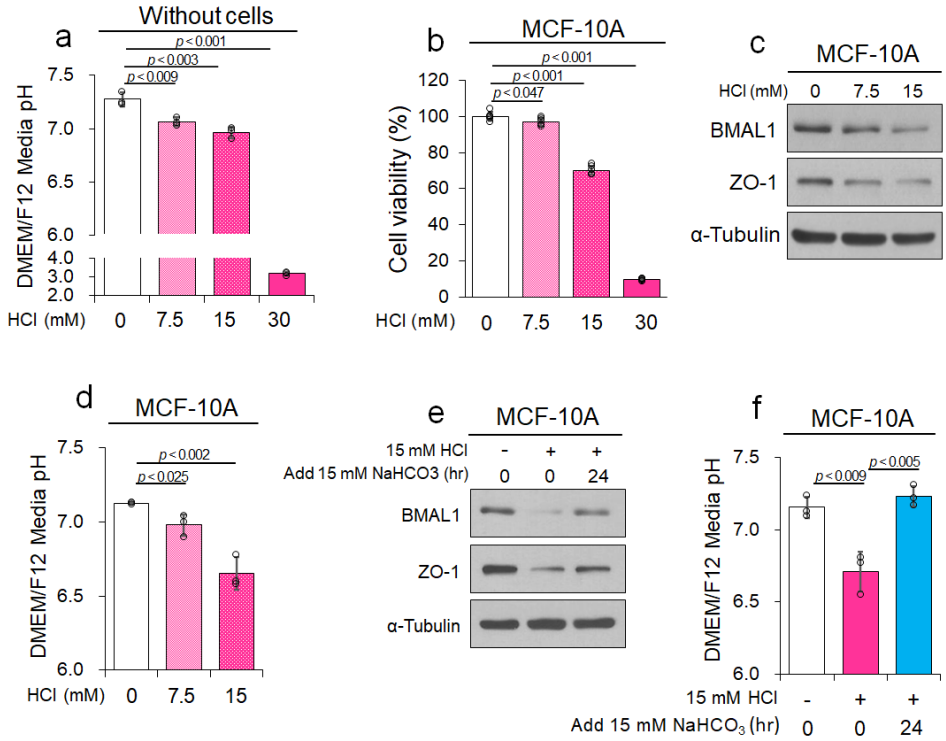


Figure 7. Tumor acidosis decreases BMAL1 expression in normal breast cells as well as breast cancer cells.

(a) HCl-mediated acidic DMEM/F12 media were incubated for 24 h without cells. pH of the cultured media was immediately measured using a pH meter. (b) MCF-10A was treated with HCl for 24 h. Cell viability was measured by MTT assay. (c, d) MCF-10A was treated with HCl-induced acidic media for 24 h. Cell lysates were analyzed by immunoblotting (c) and pH of the cultured media was immediately measured using pH meter (d). (e, f) MCF-10A was incubated in normoxia or 2% O_2 hypoxia with $NaHCO_3$ for 48 h. Cell lysates were analyzed by immunoblotting (e) and qRT-PCR (f). All experiments were performed at least three times in independent experiments. The statistical significance was determined by

an unpaired Student's t−test.

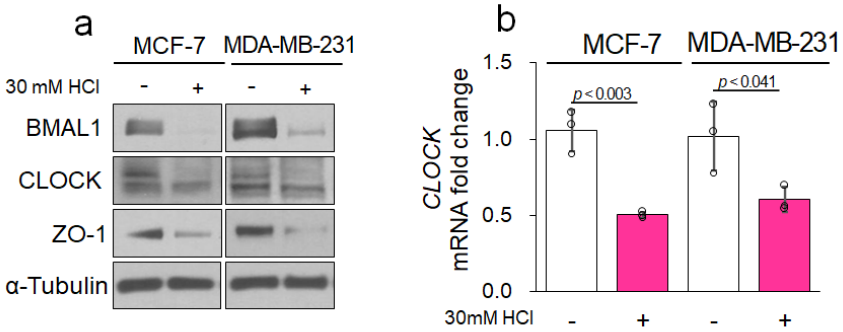


Figure 8. Tumor acidosis also reduces *CLOCK* expression as well as BMAL1 in breast cancer cells

(a, b) MCF-7 and MDA-MB-231 were treated with HCl-mediated acidic media for 24 h. Cell lysates were analyzed by immunoblotting (a) and qRT-PCR (b). All experiments were performed at least three times in independent experiments. The statistical significance was determined by an unpaired Student's *t*-test.

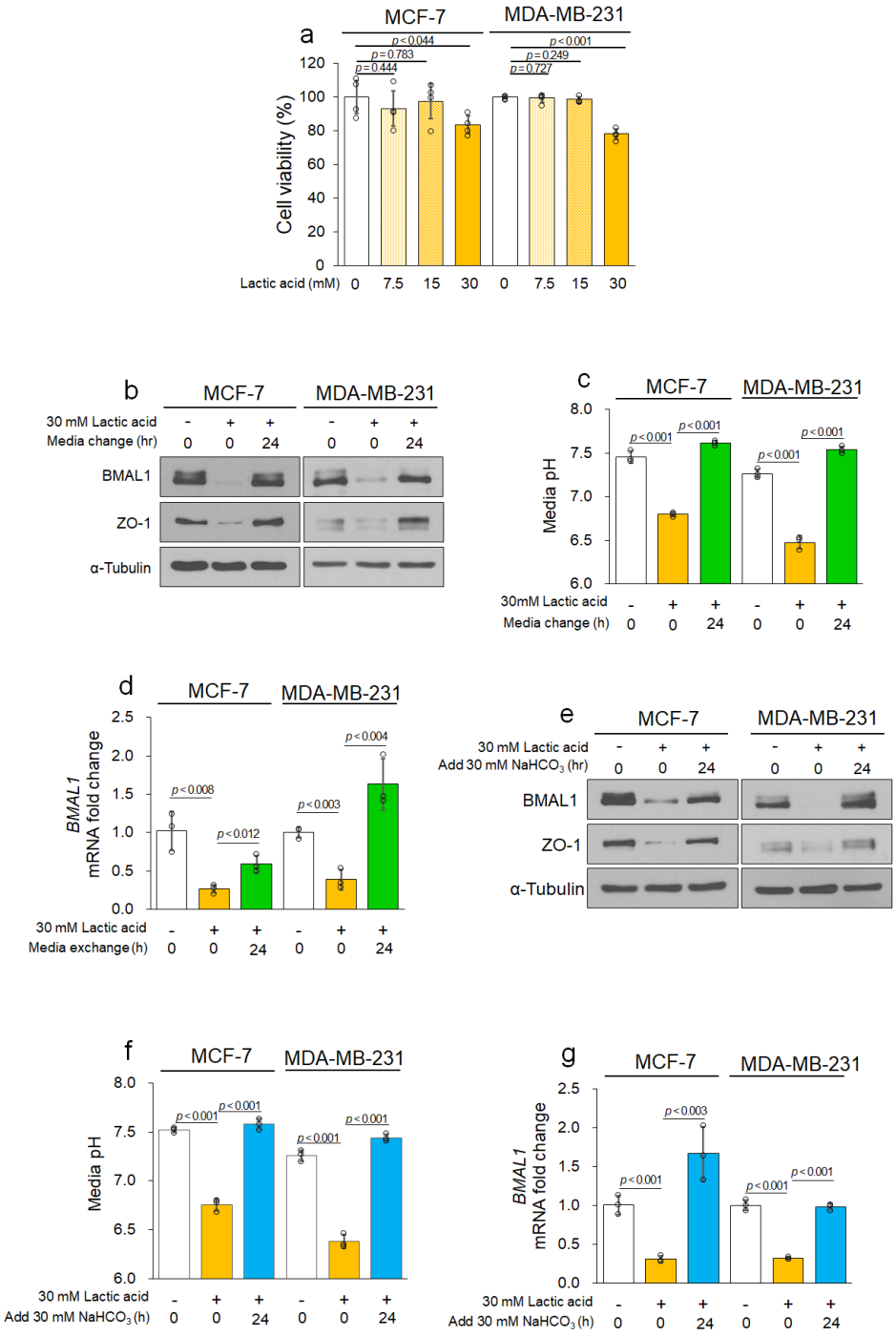


Figure 9. Decrease of BMAL1 by lactic acidosis was also restored by neutralizing acidic pH in breast cancer cells

(a) MCF-7 and MDA-MB-231 were treated with lactic acid for 48 h. Cell viability was measured by MTT assay. (b-d) MCF-7 and MDA-MB-231 were treated with lactic acid-induced acidic media for 48 h. The acidic cultured media were exchanged to fresh media and then incubated for 24 h. Cell lysates were analyzed by immunoblotting (b) and pH of the cultured media was immediately measured using a pH meter (c). Cell lysates were analyzed by qRT-PCR (d). (e-g) MCF-7 and MDA-MB-231 were treated with lactic acid induced-acidic media for 48 h. The acidic cultured media were added to $NaHCO_3$ and then incubated for 24 h. Cell lysates were analyzed by immunoblotting (e) and pH of the cultured media was immediately measured using a pH meter (f). Cell lysates were analyzed by qRT-PCR (g). All experiments were performed at least three times in independent experiments. The statistical significance was determined by an unpaired Student's *t*-test.

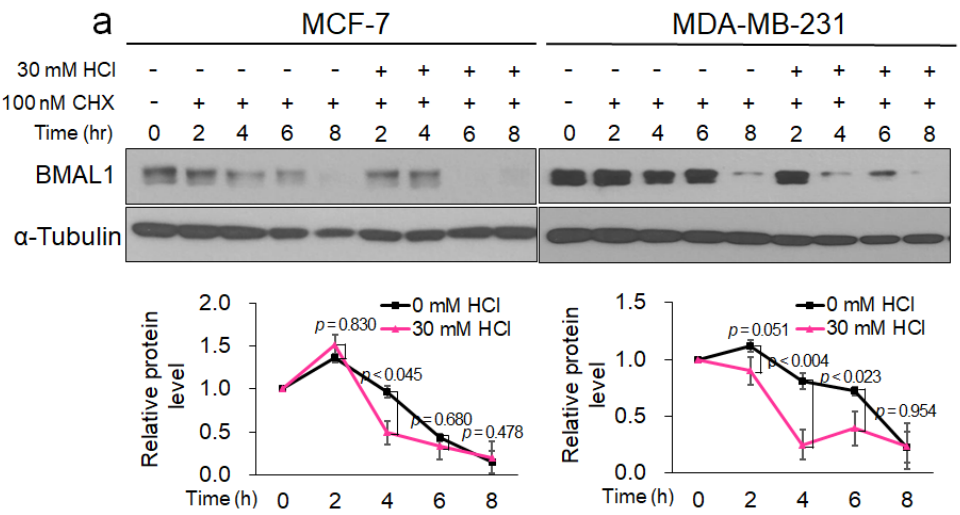


Figure 10. Tumor acidosis reduces BMAL1 protein expression via inhibition of protein stability in breast cancer cells

(a) MCF-7 and MDA-MB-231 were treated with CHX in acidic condition for the indicated periods. Cell lysates were analyzed by immunoblotting. The blots of BMAL1 were quantified using ImageJ (bottom panel). All experiments were performed at least three times in independent experiments. The statistical significance was determined by an unpaired Student's *t*-test.

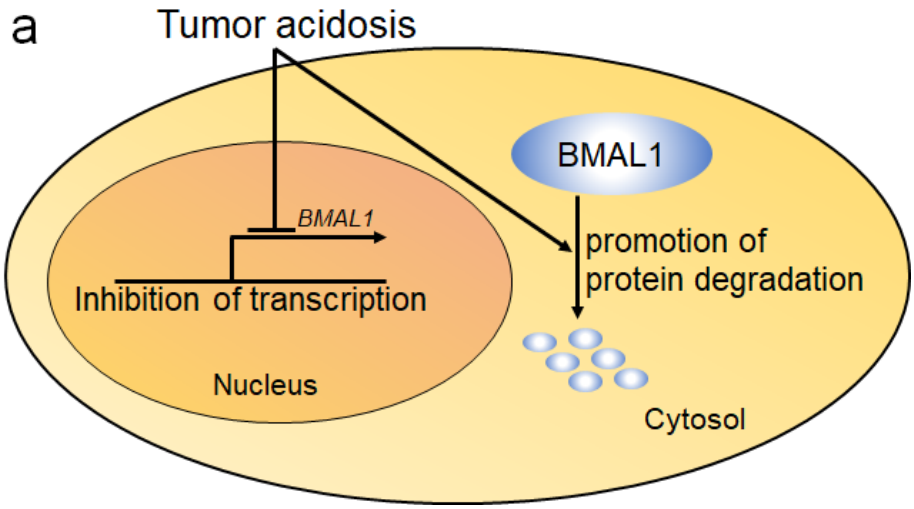


Figure 11. Graphical summarization of the dual pathways that reduce BMAL1.

Tumor acidosis reduces protein and mRNA levels of BMAL1 via inhibition of transcription activity and promotion of protein degradation in breast cancer cells.

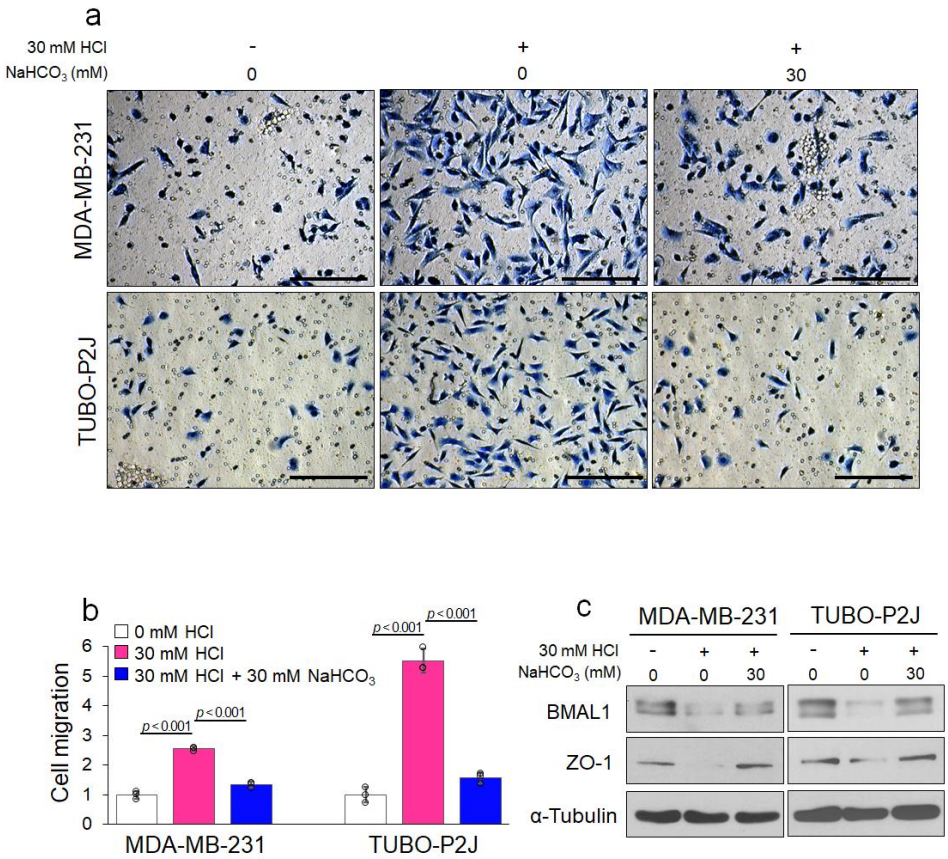


Figure 12. Tumor acidosis promotes metastatic potency in breast cancer cells

(a, b) MDA-MB-231 and TUBO-P2J were subjected to trans-well migration assay in HCl-mediated acidic condition with $NaHCO_3$ for 24 h as indicated. Representative images of migrated cells are shown. Scale bars:250 μm (a). The average number of migrated MDA-MB-231 cells and TUBO-P2J cells was counted in three random microscopic fields (b). (c) MDA-MB-231 and TUBO-P2J were incubated in HCl-mediated acidic condition with $NaHCO_3$ for 24 h as indicated. Cell lysates were determined by immunoblotting. All experiments were performed at least

three times in independent experiments. The statistical significance was determined by an unpaired Student's t−test.

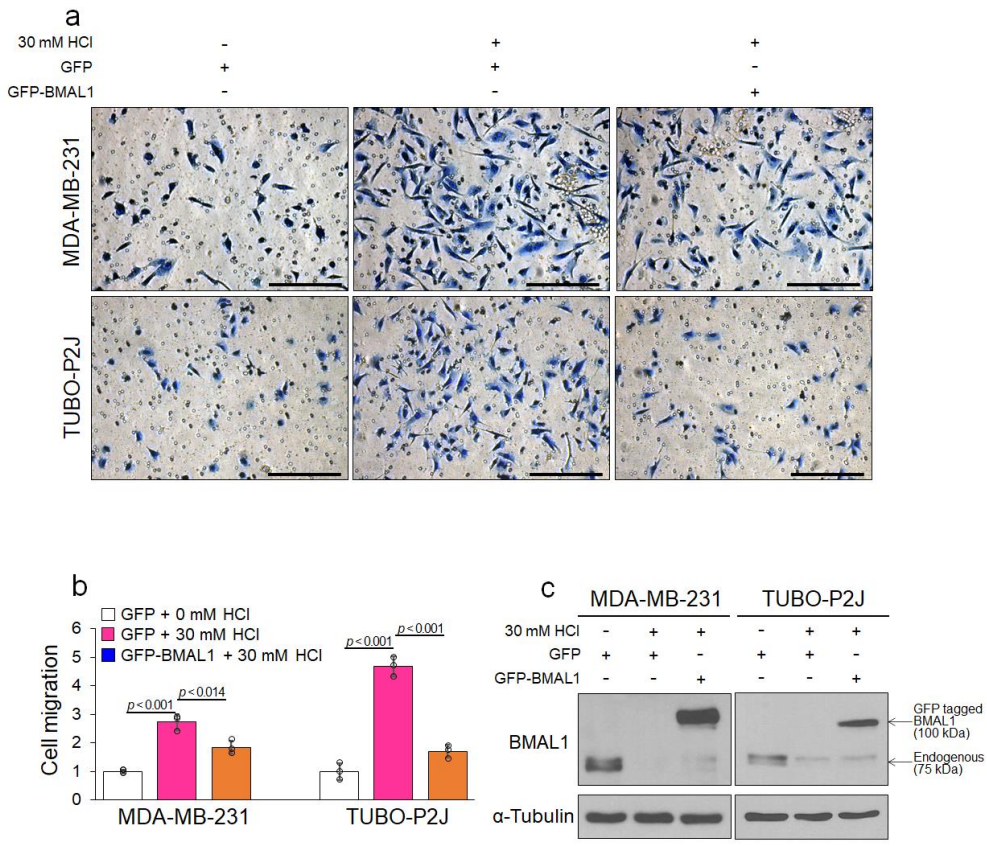
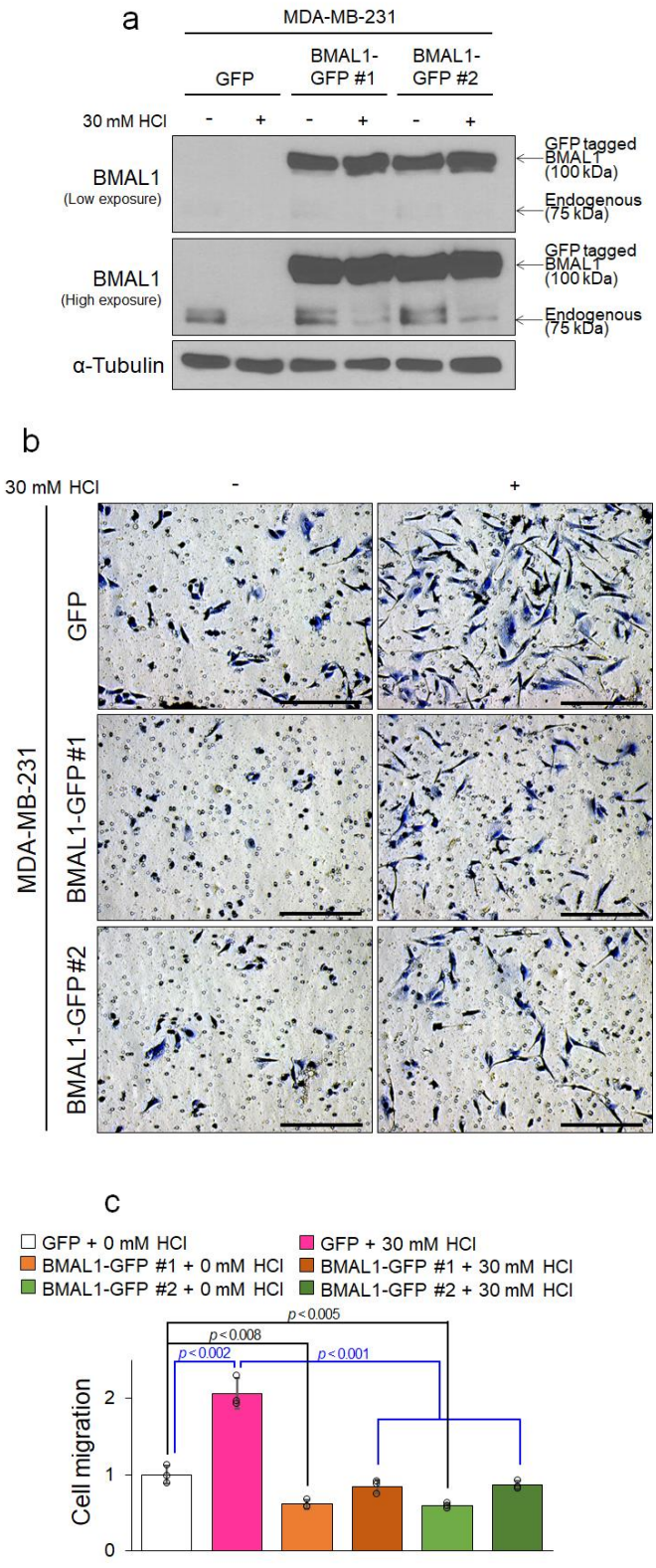


Figure 13. BMAL1 inhibits breast cancer metastasis

(a, b) MDA-MB-231 and TUBO-P2J were transfected with GFP or GFP-BMAL1, and subjected to trans-well migration assay in acidic condition for 24 h as indicated. Representative images of migrated cells are shown. Scale bars:250 μm (a) and the average number of migrated MDA-MB-231 and TUBO-P2J cells was counted in three random microscopic fields (b). (c) MDA-MB-231 and TUBO-P2J were transfected with GFP or GFP-BMAL1, and incubated in acidic condition for 24 h as indicated. Cell lysates were analyzed by immunoblotting. All experiments were performed at least three times in independent

experiments. The statistical significance was determined by an unpaired Student's t−test.


a
MDA-MB-231
GFP
BMAL1-GFP #1
BMAL1-GFP #2
30 mM HCl
- + - + - +
BMAL1
(Low exposure)
BMAL1
(High exposure)
α-Tubulin
GFP tagged BMAL1 (100 kDa)
Endogenous (75 kDa)
b
30 mM HCl
-
+
MDA-MB-231
GFP
BMAL1-GFP #1
BMAL1-GFP #2
c
GFP + 0 mM HCl
BMAL1-GFP #1 + 0 mM HCl
BMAL1-GFP #2 + 0 mM HCl
GFP + 30 mM HCl
BMAL1-GFP #1 + 30 mM HCl
BMAL1-GFP #2 + 30 mM HCl
p < 0.005
p < 0.008
p < 0.002
p < 0.001
Cell migration
0
1
2

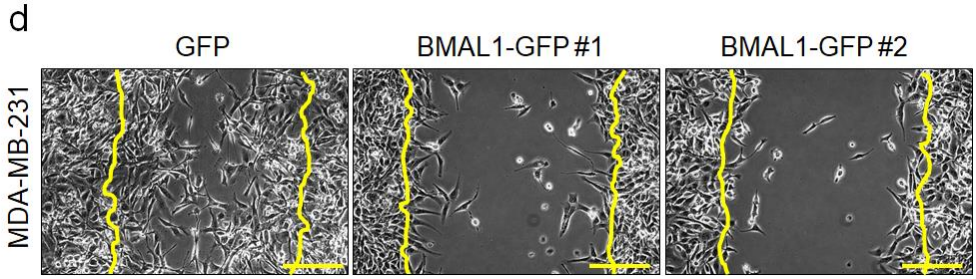


Figure 14. Stable overexpression of BMAL1 inhibits breast cancer metastatic potency

(a) The green fluorescent protein (GFP) or GFP-BMAL1 stably overexpressed MDA-MB-231 cell lines were incubated in HCl-mediated acidic condition for 24 h as indicated, and cell lysates were determined by immunoblotting. **(b, c)** GFP or GFP-BMAL1 stably overexpressed MDA-MB-231 cell lines were subjected to trans-well migration assay in HCl-mediated acidic condition for 24 h as indicated. Representative images of migrated cells are shown. Scale bars: 250 μm **(b)**. The average number of migrated GFP or GFP-BMAL1 stably overexpressed MDA-MB-231 cell lines was counted in three random microscopic fields **(c)**. **(d)** GFP or GFP-BMAL1 stably overexpressed MDA-MB-231 cell lines were subjected to wound-healing assay for 48 h. Representative images of migrated cells are shown. Scale bars:250 μm. All experiments were performed at least three times in independent experiments. The statistical significance was determined by an unpaired Student's t-test.

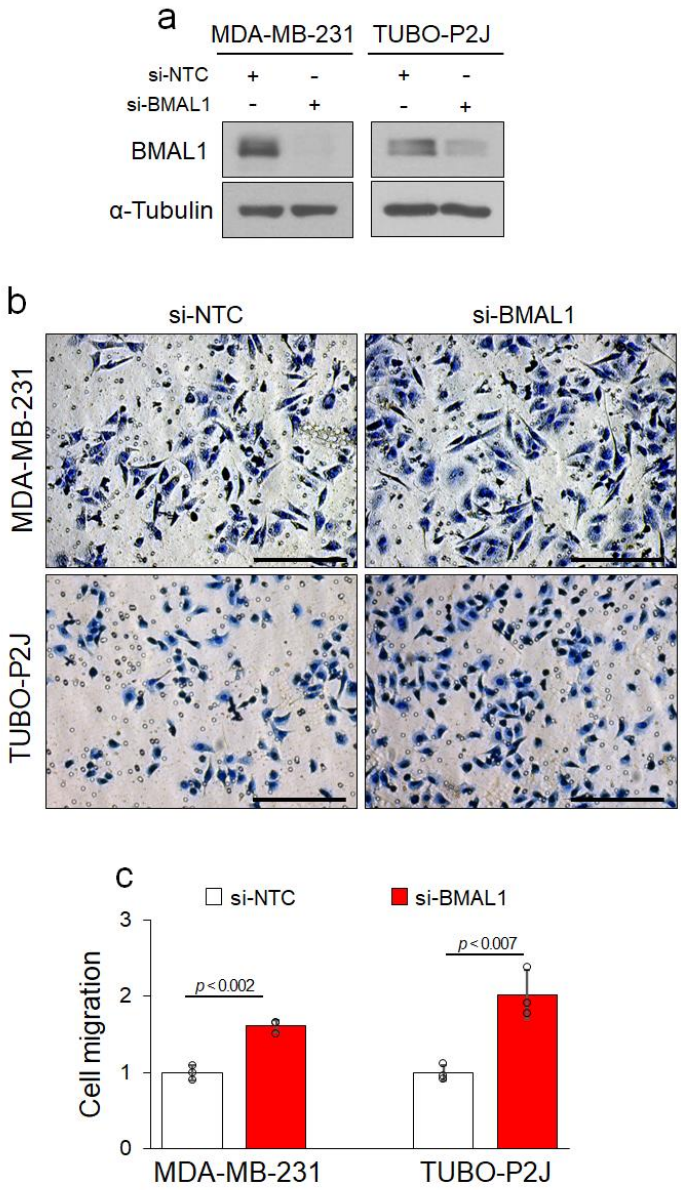


Figure 15. Knock-down of BMAL1 promotes breast cancer metastatic potency

(a) MDA-MB-231 and TUBO-P2J were transfected with si-NTC or si-BMAL1. Cell lysates were determined by immunoblotting. (b-d) MDA-

MB-231 and TUBO-P2J were transfected with si-NTC or si-BMAL1, and was subjected to trans-well migration assay for 24 h. Representative images of migrated cells are shown. Scale bars: 250 μm (b). The average number of migrated MDA-MB-231 was counted in three random microscopic fields (c). All experiments were performed at least three times in independent experiments. The statistical significance was determined by an unpaired Student's t-test.

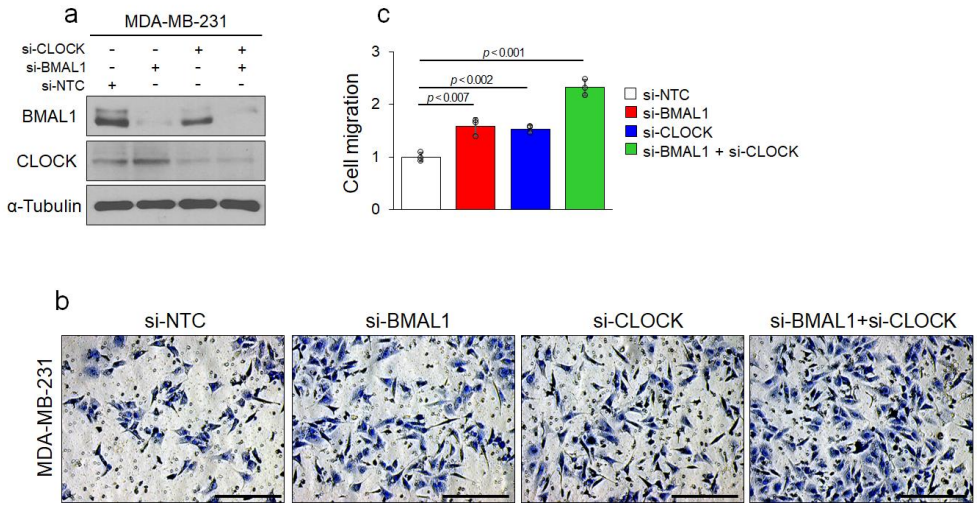
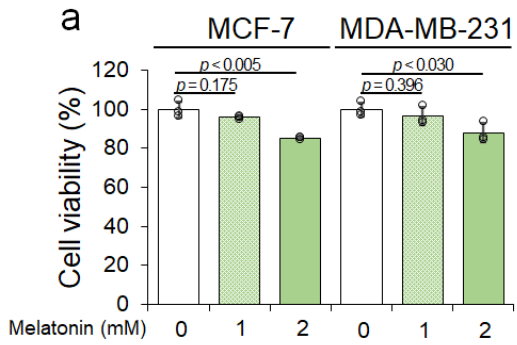
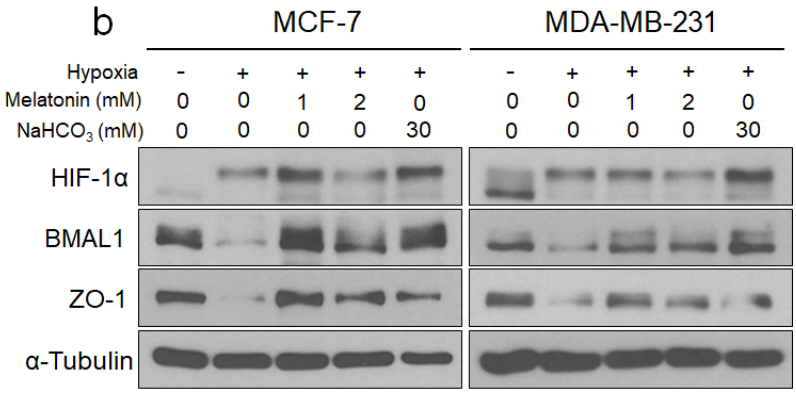


Figure 16. Reduced BMAL1/CLOCK promotes metastasis in breast cancer cells

(a) MDA-MB-231 was transfected with si-BMAL1 and/or si-CLOCK. Cell lysates were analyzed by immunoblotting. (b, c) MDA-MB-231 was transfected with si-NTC, si-BMAL1 and/or si-CLOCK, and subjected to trans-well migration assay for 24 h. Representative images of migrated cells are shown. Scale bars:250 μm (b) and the average number of migrated MDA-MB-231 cells was counted in three random microscopic fields (c). All experiments were performed at least three times in independent experiments. The statistical significance was determined by an unpaired Student's t-test.





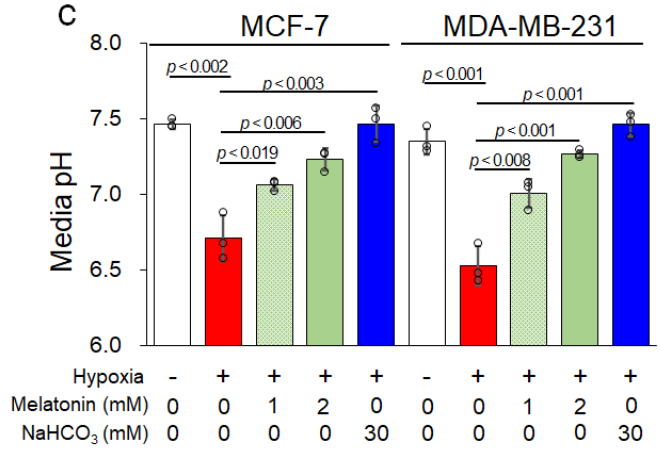


Figure 17. Melatonin attenuates hypoxia-mediated decrease of BMAL1 in breast cancer cells

(a) MCF-7 and MDA-MB-231 were treated with melatonin for 48 h. Cell

viability was measured by MTT assay. (b, c) MCF-7 and MDA-MB-231 were incubated in normoxia or 2% O_2 hypoxia with melatonin or $NaHCO_3$ for 48 h. Cell lysates were analyzed by immunoblotting (b) and pH of the cultured media was immediately measured using a pH meter (c). All experiments were performed at least three times in independent experiments. The statistical significance was determined by an unpaired Student's *t*-test.

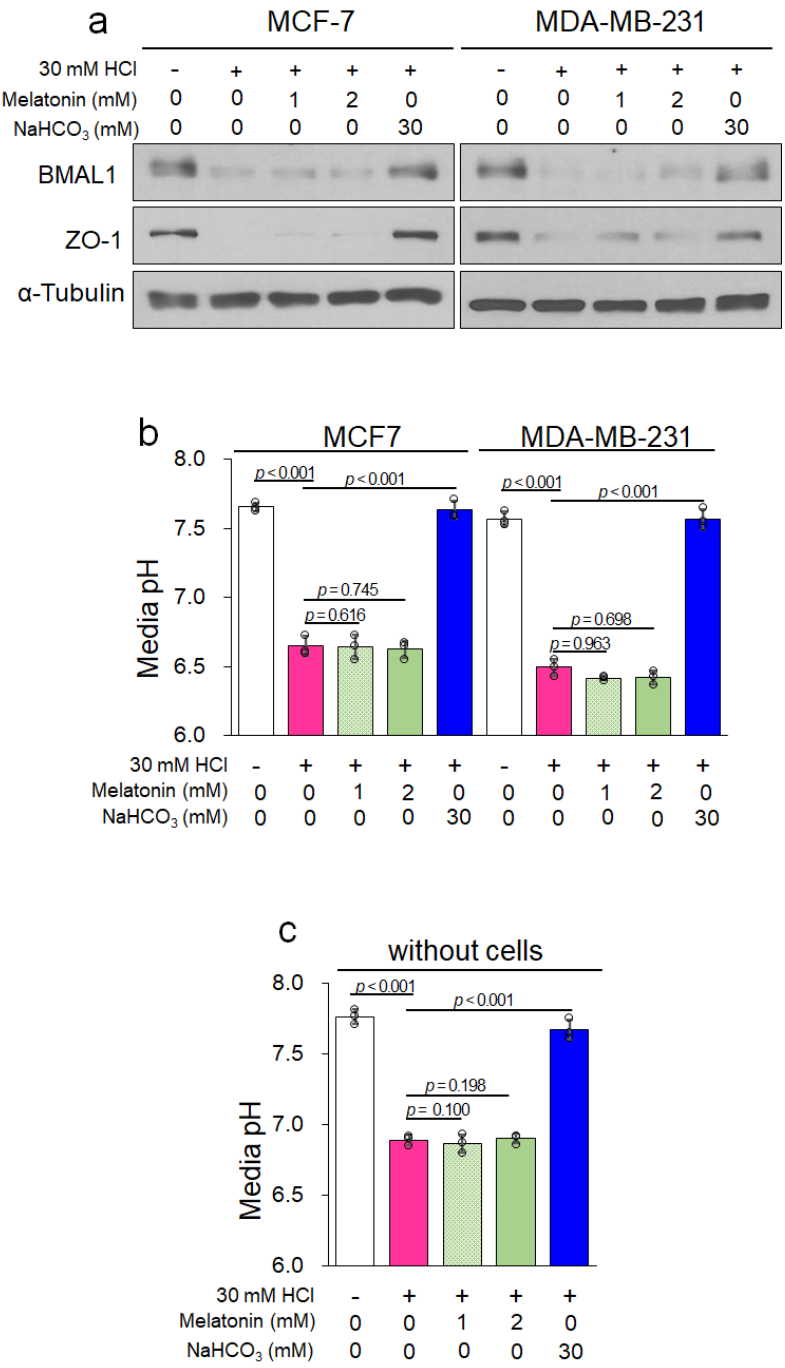


Figure 18. Melatonin has no effects on acidosis-mediated decrease of BMAL1 in breast cancer cells

(a, b) MCF-7 and MDA-MB-231 were incubated in acidic condition with

melatonin or $NaHCO_3$ for 24 h. Cell lysates were analyzed by immunoblotting (a) and pH of the cultured media was immediately measured using a pH meter (b). (c) HCl-mediated acidic DMEM media were incubated with melatonin or $NaHCO_3$ for 24 h without cells, and media pH was immediately measured using a pH meter. All experiments were performed at least three times in independent experiments. The statistical significance was determined by an unpaired Student's *t*-test.

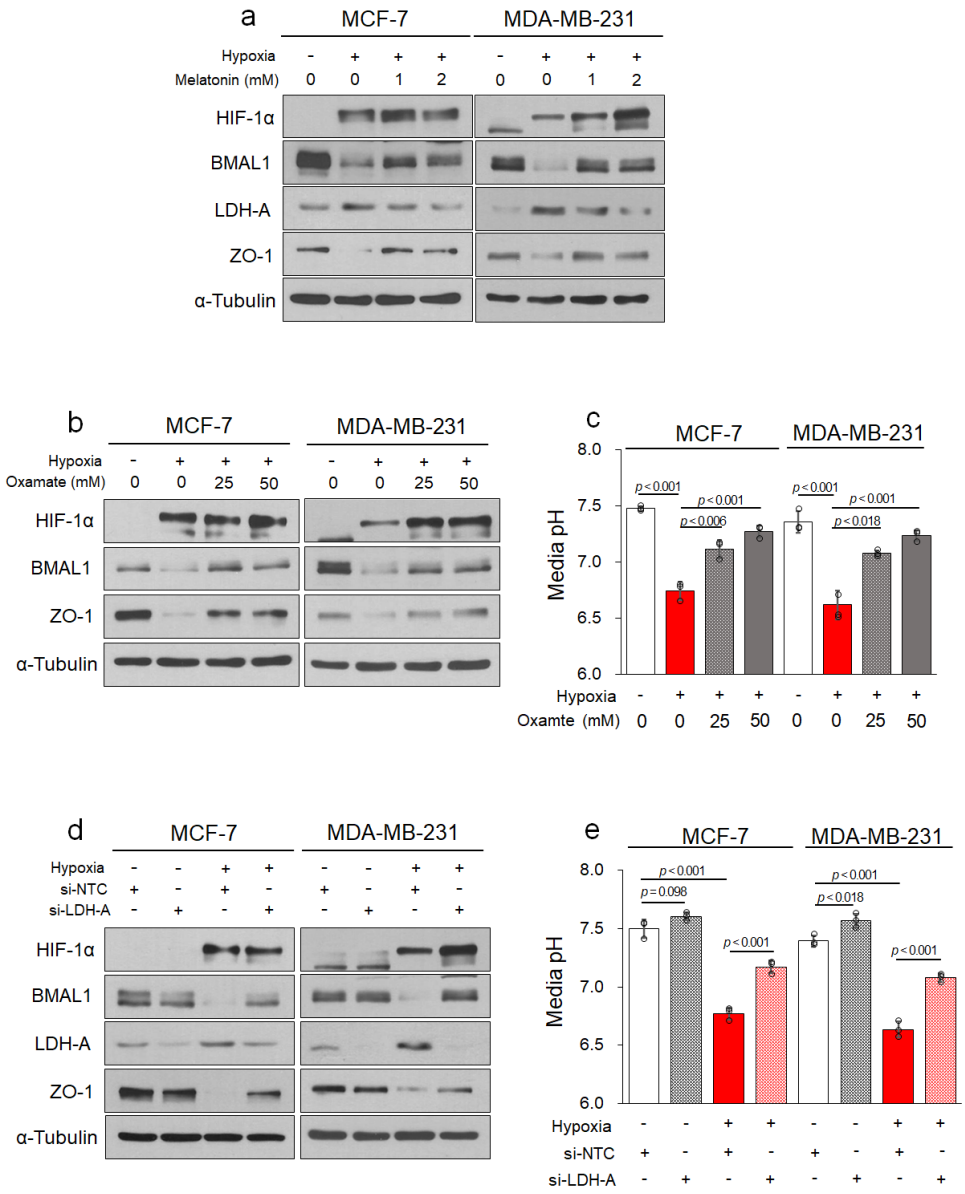


Figure 19. Melatonin attenuates decrease of BMAL1 by inhibiting hypoxia-mediated LDH-A in breast cancer cells

(a) MCF-7 and MDA-MB-231 were incubated in normoxia or 2% O_2 hypoxia with melatonin for 48 h. Cell lysates were analyzed by immunoblotting. (b, c) MCF-7 and MDA-MB-231 were incubated in normoxia or 2% O_2 hypoxia with oxamate for 48 h. Cell lysates were

analyzed by immunoblotting (b) and pH of the cultured media was immediately measured using pH meter (c). (d, e) MCF-7 and MDA-MB-231 were transfected with si-NTC or si-LDH-A, and cells were incubated in normoxia or 2% O_2 hypoxia for 48 h. Cell lysates were determined by immunoblotting (d) and pH of the cultured media was immediately measured using a pH meter (e). All experiments were performed at least three times in independent experiments. The statistical significance was determined by an unpaired Student's *t*-test.

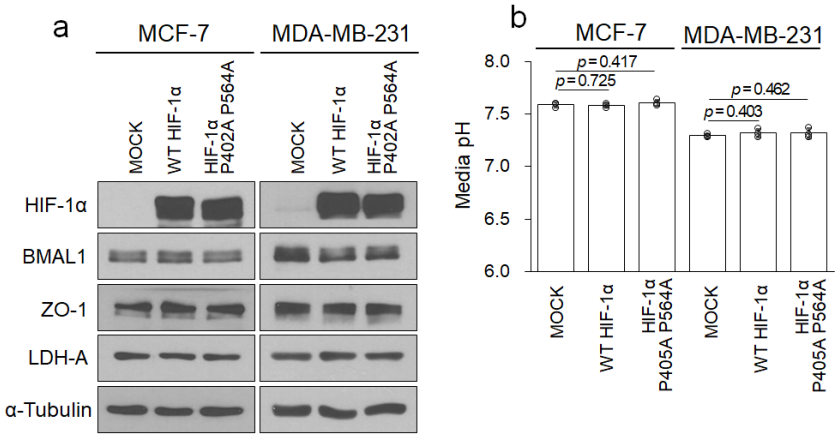


Figure 20. HIF-1α has no effects on hypoxia-mediated decrease of BMAL1 in breast cancer cells

(a, b) MCF-7 and MDA-MB-231 were transfected with HIF-1α^{WT} or HIF-1$\alpha^{P402A/P564A}$ plasmid, and incubated in normoxia for 48 h. Cell lysates were analyzed by immunoblotting (a) and pH of the cultured media was immediately measured using pH meter (b). All experiments were performed at least three times in independent experiments. Data represent the mean ± SD. The statistical significance was determined by an unpaired Student's t-test.

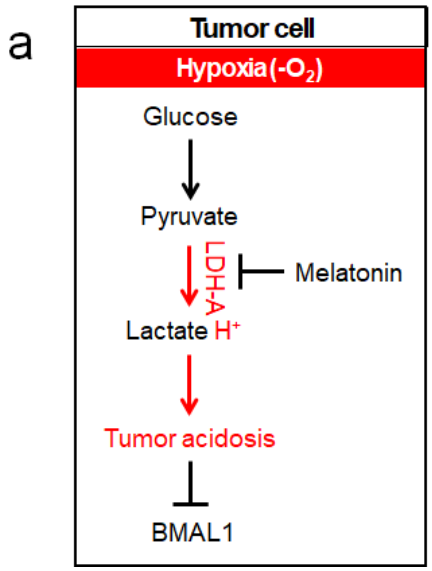


Figure 21. The summary of pathway that melatonin attenuates hypoxia-mediated decrease of BMAL1 by inhibiting LDH-A in hypoxia.

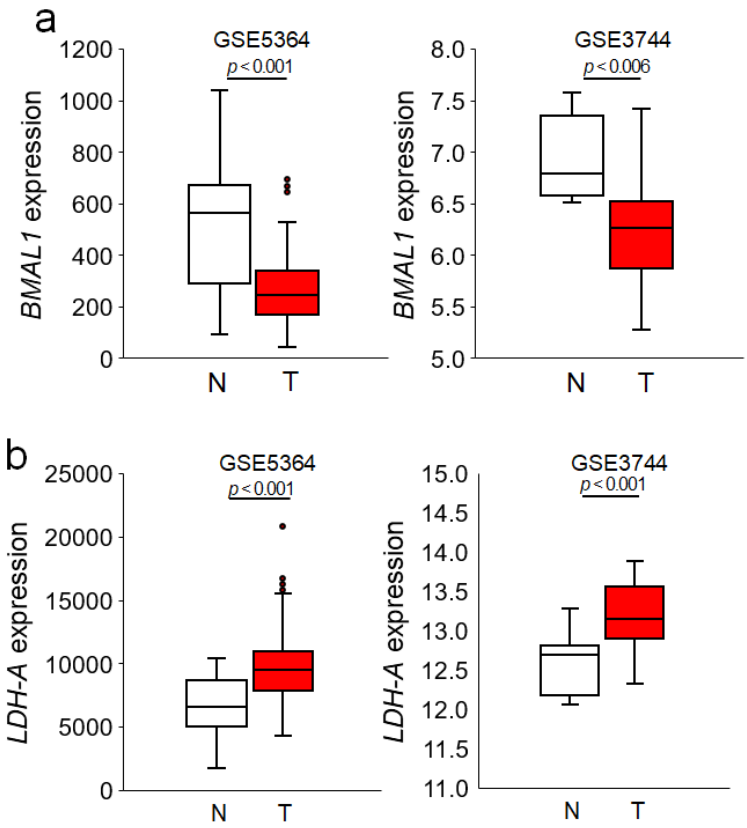


Figure 22. Clinical relevance of *BMAL1* and *LDH-A* expression in breast cancer patients

(a, b) BMAL1 (a) and LDH-A (b) mRNA expression in normal and cancer breast tissue samples from GSE5364(n = N 13 and T 183) and GSE3744(n = N 7 and T 40) database sets. N: normal breast tissue, T: breast cancer tissue. The statistical significance was determined by an unpaired Student's *t*-test.

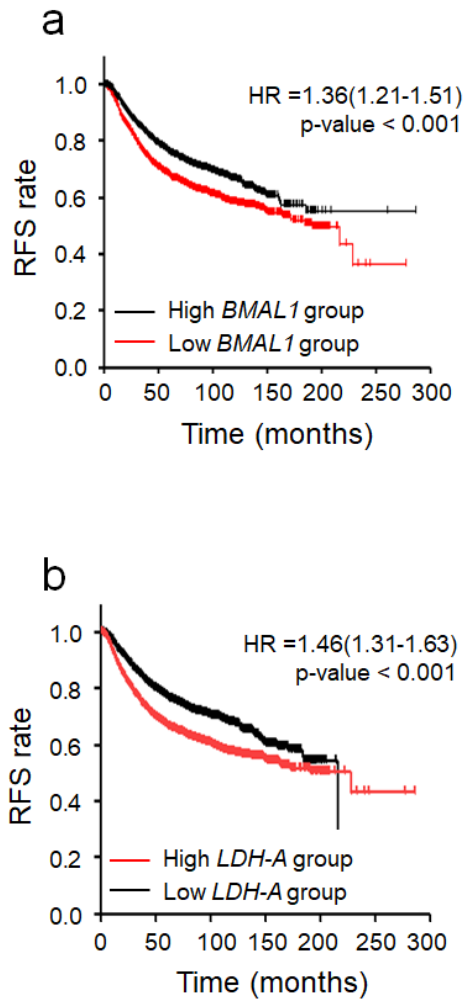


Figure 23. Decrease of *BMAL1* and *LDH-A* are clinically related to poor prognoses in breast cancer patients

(a, b) Relapse-free survival (RFS) analysis of *BMAL1* (a) and *LDH-A* (b) low and high breast cancer patients on the Kaplan-Meier plotter database. (p: log-rank, HR: hazard ratio, n = 4929).

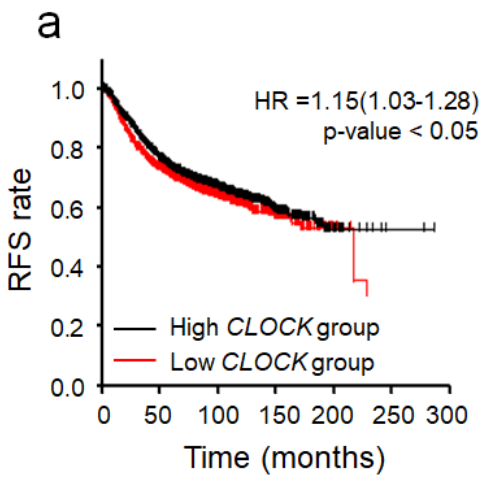


Figure 24. Decrease of *CLOCK* is slightly related to poor prognoses in breast cancer patients

(a) Relapse-free survival (RFS) analysis of *CLOCK* low and high breast cancer patients on the Kaplan-Meier plotter database. (p: log-rank, HR: hazard ratio, n = 4929).

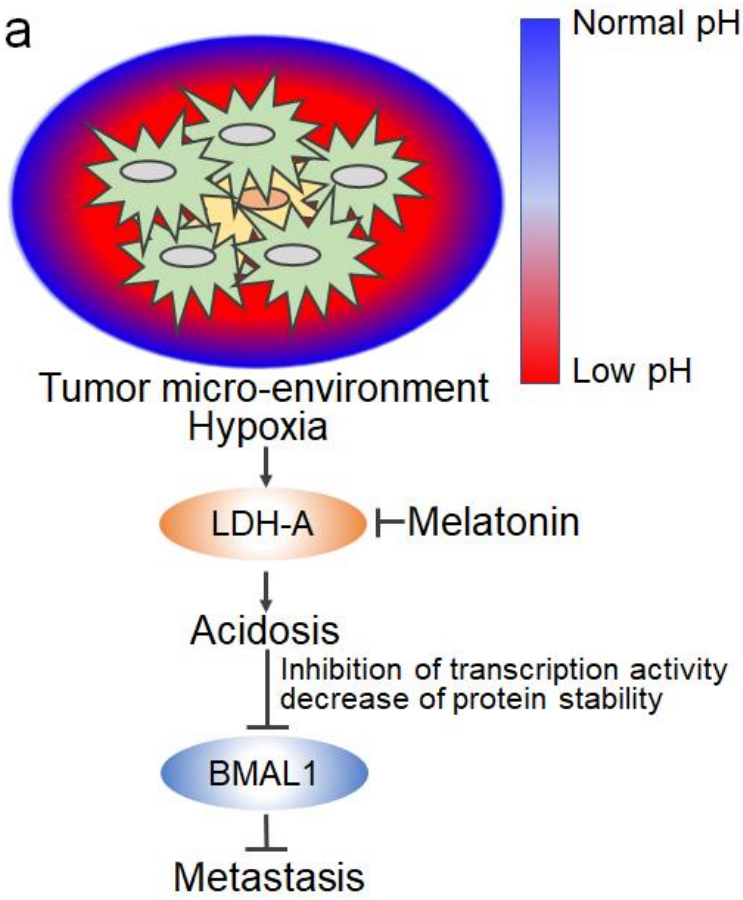


Figure 25. Graphical summary.

Tumor acidosis-mediated decrease of BMAL1 via inhibition of transcription activity and protein stability promotes metastatic potency, which could be prevented by melatonin that inhibits hypoxia-induced LDH-A in breast cancer

Table 1. Subtypes of human breast cancer cell lines.

Classification	Immunoprofile	Example cell lines
Luminal A	ER^{+}, $PR^{+/-}$, $HER2^{-}$	MCF-7, T47D
Luminal B	ER^{+}, $PR^{+/-}$, $HER2^{+}$	ZR-75-1
Basal-like	ER^{-}, PR^{-}, $HER2^{-}$	MDA-MB-468, MDA-MB-231, Hs578T

Table 2. Extracellular pH of normal and tumor.

Normal	Tumor	ref.
7.5	6.5-6.8	*Biomater Res,* **22,** 1-11 (2018) [38]
7.4	6.8	*Cell Rep,* **18,** 2228-2242 [39]
7.4	6.2-7.0	*Clin Cancer Res,* **21,** 4502-4504 (2015) [40]
7.4	6.7	*Front Physiol,* **4,** 370 (2013) [41]
7.2-7.4	6.5-6.9	*Cancer Res,* **73,** 1524-1535 (2013) [42]
7.4	6.8-7.1	*Nat Rev Cancer,* **11,** 671 (2011) [43]

Table 3. Oligonucleotide sequences for the quantitative RT−PCR.

Name		Sequence (5' -> 3')
BMAL1 (*ARNTL*, human)	Forward	TGCCACCAATCCATACACAG
	Reverse	TCGGTCACATCCTACGACAAAC
CLOCK (human)	Forward	CTCATCTGCTGGAAAGTGATTC
	Reverse	TGGCTCCTTTGGGTCTATTG
ACTB (human)	Forward	AAATCTGGCACCACACCTTC
	Reverse	GGGGTGTTGAAGGTCTCAAA
TUBA1A (human)	Forward	CTTCGTCTCCGCCATCAG
	Reverse	CGTGTTCCAGGCAGTAGAGC

Table 4. Media pH of indicated conditions in Figure 4

CON	+	−	−	−	−	Figure 4d
NCM	−	+	−	−	−	
HCM	−	−	+	+	+	
NaOH (mM)	0	0	0	7.5	15	
MCF-7	7.70 (±0.09)	7.39 (±0.07)	6.74 (±0.07)	7.21 (±0.08)	7.43 (±0.07)	
MDA-MB-231	7.43 (±0.07)	6.90 (±0.04)	6.47 (±0.07)	6.95 (±0.13)	7.29 (±0.05)	
Hypoxia	−	+	+	+		Figure 4f
NaOH (mM)	0	0	7.5	15		
MCF-7	7.59 (±0.12)	6.83 (±0.10)	7.28 (±0.11)	7.55 (±0.02)		
MDA-MB-231	7.37 (±0.09)	6.54 (±0.16)	6.86 (±0.10)	7.18 (±0.10)		
Hypoxia	−	+	+	+		Figure 4j
NaHCO3(mM)	0	0	15	30		
MCF-7	7.66 (±0.11)	6.85 (±0.10)	7.36 (±0.07)	7.72 (±0.05)		
MDA-MB-231	7.32 (±0.11)	6.52 (±0.17)	7.20 (±0.07)	7.60 (±0.07)		

Table 5. Media pH of indicated conditions in Figure 5

HCl (mM)	0	7.5	15	30	Figure 5b
Without cells (DMEM)	7.80 (±0.06)	7.56 (±0.07)	7.35 (±0.04)	6.96 (±0.06)	
HCl (mM)	0	7.5	15	30	Figure 5e
MCF-7	7.64 (±0.05)	7.53 (±0.04)	7.32 (±0.04)	6.55 (±0.06)	
MDA-MB-231	7.54 (±0.04)	7.40 (±0.04)	7.18 (±0.03)	6.45 (±0.07)	

Table 6. Media pH of indicated conditions in Figure 7

30 mM HCl	−		+		+	Figure 6b
Media change (hr)	0		0		24	
MCF−7	7.70 (±0.07)		6.69 (±0.14)		7.73 (±0.05)	
MDA−MB−231	7.52 (±0.09)		6.36 (±0.12)		7.43 (±0.06)	
30 mM HCl	−	+	+	+	+	Figure 6d
NaHCO3 (mM)	0	0	7.5	15	30	
Without cells (DMEM)	7.81 (±0.06)	6.91 (±0.05)	7.15 (±0.03)	7.45 (±0.05)	7.70 (±0.10)	
30 mM HCl	−		+		+	Figure 6f
Add 30 mM NaHCO3 (hr)	0		0		24	
MCF−7	7.67 (±0.03)		6.73 (±0.13)		7.60 (±0.09)	
MDA−MB−231	7.32 (±0.11)		6.52 (±0.17)		7.44 (±0.07)	

Table 7. Media pH of indicated conditions in Figure 6

HCl (mM)	0	7.5	15	30	Figure 7a
Without cells (DMEM/F12)	7.28 (±0.06)	7.07 (±0.04)	7.00 (±0.05)	3.17 (±0.11)	

HCl (mM)	0	7.5	15	Figure 7d
MCF-10A	7.12 (±0.005)	6.98 (±0.07)	3.07 (±0.08)	

Table 8. Media pH of indicated conditions in Figure 8

30 mM Lactic acid	−	+	+	Figure 8a
Media change (hr)	0	0	24	
MCF−7	7.45 (±0.07)	6.80 (±0.03)	7.61 (±0.03)	
MDA−MB−231	7.26 (±0.05)	6.48 (±0.08)	7.54 (±0.04)	
30m M Lactic acid	−	+	+	Figure 8c
Add 30 mM NaHCO3(hr)	0	0	24	
MCF−7	7.52 (±0.03)	6.75 (±0.06)	7.58 (±0.06)	
MDA−MB−231	7.26 (±0.06)	6.38 (±0.07)	7.44 (±0.04)	

Table 9. Media pH of indicated conditions in Figure 17

Hypoxia	−	+	+	+	+	Figure 17c
Melatonin (mM)	0	0	1	2	0	
NaHCO3 (mM)	0	0	0	0	30	
MCF−7	7.47 (±0.03)	6.71 (±0.15)	7.06 (±0.04)	7.23 (±0.07)	7.47 (±0.12)	
MDA−MB−231	7.35 (±0.09)	6.53 (±0.13)	7.01 (±0.10)	7.27 (±0.03)	7.46 (±0.08)	

Table 10. Media pH of indicated conditions in Figure 18

30 mM HCl	−	+	+	+	+	Figure 18b
Melatonin (mM)	0	0	1	2	0	
NaHCO3 (mM)	0	0	0	0	30	
MCF−7	7.66 (±0.03)	6.65 (±0.07)	6.64 (±0.09)	6.62 (±0.06)	7.63 (±0.07)	
MDA−MB−231	7.57 (±0.05)	6.49 (±0.06)	6.42 (±0.02)	6.42 (±0.05)	7.57 (±0.07)	
30 mM HCl	−	+	+	+	+	Figure 18c
Melatonin	0	0	1	2	0	
NaHCO3 (mM)	0	0	0	0	30	
Without cells (DMEM)	7.76 (±0.05)	6.89 (±0.04)	6.87 (±0.07)	6.90 (±0.03)	7.67 (±0.07)	

Table 11. Media pH of indicated conditions in Figure 19

Hypoxia	−	+	+	+	Figure 19c
Oxamate (mM)	0	0	25	50	
MCF−7	7.48 (±0.02)	6.74 (±0.08)	7.12 (±0.08)	7.27 (±0.06)	
MDA−MB−231	7.36 (±0.10)	6.62 (±0.13)	7.08 (±0.03)	7.24 (±0.05)	
Hypoxia	−	−	+	+	Figure 19e
si−NTC	+	−	+	−	
si−LDH−A	−	+	−	+	
MCF−7	7.55 (±0.05)	6.87 (±0.07)	6.90 (±0.03)	7.67 (±0.07)	
MDA−MB−231	7.76 (±0.05)	6.87 (±0.07)	6.90 (±0.03)	7.67 (±0.07)	

Table 12. Media pH of indicated conditions in Figure 20

	MOCK	WT HIF-1α	HIF-1α P405A P564A	
MCF-7	7.59 (±0.02)	7.58 (±0.02)	7.61 (±0.03)	Figure 20b
MDA-MB-231	7.29 (±0.02)	7.31 (±0.04)	7.31 (±0.05)	

DISCUSSION

The majority of people in the world have abnormal circadian rhythms due to irregular living patterns. The disruption of circadian rhythms and a decrease of genes are highly associated with various diseases, including cancer. For example, recent studies have shown that night workers such as nurses are more likely to suffer from hormone-dependent cancers such as breast cancer [60,61]. Therefore, it can be expected that maintaining circadian patterns or genes is a strategy to prevent and treat cancer. Breast cancer is a prevalent female cancer and can sometimes be successfully treated with chemotherapy, radiation therapy, and surgery. However, when the tumor migrates and invades peripheral tissues, the survival rate is dramatically reduced [5]. There has been extensive research to overcome breast cancer metastasis, but it has not been adequately solved. According to previous reports, circadian genes, which are significantly reduced in cancer, suppress tumor progression including metastasis [19–21]. For this reason, I wanted to find a way to recover the reduced circadian genes in cancer to increase the survival rate by preventing metastasis.

Some materials or protocols such as serum shock, heat shock, forskolin and dexamethasone are well known to oscillate or synchronize the disrupted circadian gene in various cells including cancer cells. Dexamethasone is a hormonal agent with immunosuppressive and anti-

inflammatory action, and is used for endocrine disorders or inflammatory diseases as well as for some cancer treatments. In a recent study, it was reported that increased circadian rhythm induced by dexamethasone induces cell cycle arrest of tumor cells [62]. This study shows that restoring circadian rhythm is a key way to suppress cancer. Therefore, it is very important to analyze the causes of disrupted circadian rhythms and genes in cancer and to understand the overall mechanism of action.

The previous study reported that the expression patterns of the circadian genes were disrupted in tumor or adjacent-tumor tissue compared to normal tissue, and it was suggested that tumor macro or/and microenvironments are the cause [24]. Tumor hypoxia and acidosis are a characteristic of the tumor microenvironment in all solid tumors, and is clinically associated with tumor progression and poor prognoses in breast cancer patients [63,64]. In the previous study, acidification reduces circadian genes through the mTOR signaling pathway [33], but it is not well known yet in breast cancer, and it is not enough to explain the mTOR signaling pathway alone. In the present study, I made effort to find a new mechanism by which BMAL1 was reduced by the cancer microenvironment, and I additionally found that tumor hypoxia-induced acidosis significantly reduced the BMAL1 circadian clock gene via inhibition of both transcriptional activity and protein stability. Therefore, I suggested that circadian genes and rhythms were greatly influenced by pH.

HIFs are transcription factors that are activated in the hypoxic conditions. In the previous studies, HIF-1α reciprocally regulates

circadian genes, both *in vitro* and *in vivo* models [33,35,65]. However, in breast cancer cells, both overexpression of HIF-1α and hypoxia-mimetic agent $CoCl_2$ did not affect the BMAL1 protein expression. Therefore, I suggested that in breast cancer cells, a hypoxia-mediated decrease of BMAL1 protein expression is pH dependent and HIF-1 α independent.

Melatonin is mainly produced and secreted by the pineal gland, and plays a central role in the generation and regulation of circadian rhythm in humans [48]. In previous studies, melatonin has been shown to suppress cancer progression by inducing apoptosis and inhibiting angiogenesis, metastasis, and cell proliferation via modulating miRNAs (such as miRNA 10a, 148b and 210), growth factors (such as VEGF), epithelial mesenchymal transition (EMT) markers (such as fibronectin, E-cadherin, vimentin and snail) and MMPs (matrix metallopeptidases) [54, 66-70]. Melatonin also prevented disruption of the circadian rhythm in melanoma-bearing mice [71]. However, the relationship between reduced BMAL1 in acidosis and melatonin remains unclear. Interestingly, I found that hypoxia-induced acidic pH was buffered by melatonin through inhibition of LDH-A. I therefore suggest that melatonin is a way to recover the reduced circadian genes in cancer. I expect that other drugs and substances that maintain the acidified pH at the normal pH in cancer, or inhibit the tumor acidification process, can potentially recover circadian genes that are reduced under tumor acidosis.

In summary, I showed that tumor hypoxia-induced acidosis reduced the BMAL1 circadian clock gene in breast cancer. BMAL1 could be maintained

in a tumor acidic pH by selectively targeting for acidosis via buffering the increased protons using $NaHCO_3$ or inhibiting anaerobic glycolysis enzymes such as LDH-A using melatonin. These treatments provide a novel mechanism for inhibiting breast cancer metastasis by maintaining circadian gene BMAL1 in tumor hypoxia-induced acidosis [72].

In my opinion, it is important which drug is used to treat cancer, but it will be difficult to expect a good prognosis without solving the fundamental cause that makes cancer worse. Through this study, I confirmed that the circadian gene is disrupted by the acidic tumor microenvironment. Even if substances (drugs, natural products or chemicals etc.) that increases the circadian genes or circadian rhythms are used for cancer treatment, it is expected that if the tumor acidosis (fundamental cause) was not targeted first, it would be like pouring water on duck's back. It is important to develop drugs that target an increasing oncoproteins or genes in tumors, but I would like to suggest that finding and solving the root cause of increasing oncoproteins or genes is the first priority.

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국문 초록

일주기 진동은 많은 생리학적 및 생물학적 메커니즘에 영향을 미치는 필수 과정이다. 붕괴된 일주기 리듬과 감소된 일주기 유전자는 암과 같은 많은 질병과 관련이 있다. 암에서 일주기 유전자 감소 및 감소된 일주기 유전자 회복에 대한 상세한 기전을 밝히기 위한 많은 노력에도 불구하고 여전히 크게 알려지지 않았다.

본 연구를 통해 BMAL1의 발현이 종양 저산소증으로 인한 산성화에 의해 감소했고, 산성 pH를 선택적으로 표적화함으로써 회복되었음을 유방암 세포주에서 확인했다. BMAL1의 발현은 산성 종양 미세환경에서 전사 억제와 단백질 안정성의 감소로 감소되었다. 또한, 멜라토닌은 저산소 상태에서 젖산 탈수소효소-A(LDH-A)를 억제하여 산성화에 의한 BMAL1의 발현 감소를 유의하게 억제했다. 산성화에 의한 암전이는 유방암 세포에서 BMAL1 과발현에 의해 유의하게 완화되었다.

따라서 본 연구를 통해 종양 저산소증으로 인한 산성화가 BMAL1의 발현을 감소시켜 전이 능력을 촉진하고 산성 종양 미세환경이 BMAL1을 유지함으로써 유방암 전이를 예방하는 표적이 될 수 있음을 제안한다.

주요어 : 일주기 시계; BMAL1; 저산소증; 산성 종양 미세환경; 유방암; 전이; 젖산 탈수소효소-A; 멜라토닌

학 번 : 2016-21972