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Bmal1-deficient mouse fibroblast cells do not provide premature cellular senescence in vitro

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ABSTRACT

Bmal1 is a core circadian clock gene. *Bmal1*$^{-/-}$ mice show disruption of the clock and premature aging phenotypes with a short lifespan. However, little is known whether disruption of *Bmal1* leads to premature aging at cellular level. Here, we established primary mouse embryonic fibroblast (MEF) cells derived from *Bmal1*$^{-/-}$ mice and investigated its effects on cellular senescence. Unexpectedly, *Bmal1*$^{-/-}$ primary MEFs that showed disrupted circadian oscillation underwent neither premature replicative nor stress-induced cellular senescence. Our results therefore uncover that *Bmal1* is not required for *in vitro* cellular senescence, suggesting that circadian clock does not control *in vitro* cellular senescence.

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Introduction

Circadian clock is an intrinsic time-keeping oscillator with an approximately 24-hr rhythm that exists in all tissues of the body and controls neuronal, endocrine, behavioral and physiological responses in mammals (Brown et al. 2012; Reppert and Weaver 2002). Molecular mechanisms that make up the circadian clock in mammals consist of transcriptional factors CLOCK/NPAS2, BMAL1, PERIOD1-2 (PER1-2), CRYPTOCHROME1-2 (CRY1-2), ROR and REV-ERB (Takahashi 2015). Briefly, CLOCK/NPAS2 and BMAL1 are bHLH-type transcriptional activators and heterodimerize to transcribe target genes by binding to E-box element on their promoters. *Pers* and *Crys* are two of BMAL1:CLOCK/NPAS2 target genes and PERs and CRYs also heterodimerize and repress their own transcription by binding to BMAL1:CLOCK/NPAS2 complex. However, this repression is soon alleviated due to the decline in *Pers* and *Crys* mRNAs and proteins, and again the next cycle starts. Since CLOCK/NPAS2 and BMAL1 are at the center of circadian clock mechanism, deficiency of *Clock/Npas2* or *Bmal1* gene completely disrupts circadian clock.

Disruption of circadian clock brings about many physiological abnormalities, leading to a wide variety of diseases and premature aging (Nakahata and Bessho 2016; Sahar and Sassone-Corsi 2012). One example is that deficiency of *Bmal1* accelerates premature aging and the average lifespan in *Bmal1*$^{-/-}$ mice is as short as 37.0 weeks (Kondratov et al. 2006). Furthermore, it has been reported that *Bmal1*$^{-/-}$ mice have a high level of reactive oxygen species (ROS) in some tissues (Kondratov et al. 2006; Kondratova et al. 2010) and the chronic oral administration of anti-oxidant reagent, N-acetylcysteine, partially mitigates the shortening of lifespan observed in *Bmal1*$^{-/-}$ mice (Kondratov et al. 2009). These reports suggest that the exposure to excessive ROS contributes to the short lifespan of *Bmal1*$^{-/-}$ mice.

Accumulation of ROS induces cellular senescence *in vivo* and *in vitro* (Bhatia-Dey et al. 2016; Rodier and Campisi 2011). Senescent cells are known to accumulate in tissues of aged animals and promote deterioration of tissue functions, causing a wide variety of diseases and also death (Bhatia-Dey et al. 2016; Lopez-Otin et al. 2013). Many groups have recently developed genetically modified mice or small molecules called "senolytic reagents" to eliminate senescent cells from aging mice, revealing that the elimination of senescent cells from organisms attenuates the

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progression of already established age-associated disorders and extends median lifespan (Baar et al. 2017; Baker et al. 2016; Chang et al. 2016; Schafer et al. 2017; Hashimoto et al. 2016; Yosef et al. 2016). Interestingly, it has been shown that senescent cells also accumulate in some tissues of *Bmal1*$^{-/-}$ mice (Khapre et al. 2011).

These reports prompted us to investigate whether cells in *Bmal1*$^{-/-}$ mice are vulnerable and prone to senesce, causing the shortening of lifespan. In this study, we established primary mouse embryonic fibroblast (MEF) cells derived from *Bmal1*$^{-/-}$ and littermate wild type mice to examine whether *Bmal1*$^{-/-}$–MEF cells show premature cellular senescence. However, our results indicated that deficiency of *Bmal1*, which indicates the disruption of circadian clock, has no effect on replicative and stress-induced cellular senescence *in vitro*. These results further suggest that accelerated *in vivo* cellular senescence in animals with disrupted circadian clock occurs presumably due to impairments of systemic regulations by interorgan communication network.

Materials and methods

Animals

Bmal1$^{+/-}$ mice, originally generated by Dr. Shimba at Nihon University in Japan, (Shimba et al. 2011) were housed under 12-hr light/12-hr dark cycles. *Bmal1*$^{+/-}$ male mice were crossed with *Bmal1*$^{+/-}$ female mice to generate primary *Bmal1*$^{-/-}$ and littermate wild type MEF cells. Our experiments were approved by the Animal Care Committee of Nara Institute of Science and Technology and conducted in accordance with guidelines that were established by the Science Council of Japan.

Isolation and culture of primary MEF cells

Primary MEF cells were derived from embryonic day 14.5 embryos. Isolation and culture of primary MEF cells were performed as described previously (Khaidizar et al. 2017).

Synchronization of circadian clocks in MEF cells, RNA extraction and qPCR

MEF cells at confluent conditions were treated with 1 μM dexamethasone (Dex) for 1 hr. Then the medium was replaced with normal culture medium and cells were cultured until indicated time points. RNA extraction and qPCR were performed as described previously (Khaidizar et al. 2017). The sequences of the forward and reverse primers were as follows. *Nrf2* FW: 5′-CCA GAC AGA CAC CAG TGG AT-3′, *Nrf2* RV: 5′-GCA AGC GAC TCA TGG TCA TC-3′; *Sod2* FW: 5′-GGA GCA AGG TCG CTT ACA GA-3′, *Sod2* RV: 5′-GTG CTC CCA CAC GTC AAT C-3′; *Catalase* FW: 5′-AAA TGC TTC AGG GCC GCC TT-3′, *Catalase* RV: 5′-GTA GGG ACA GTT CAC AGG TA-3′; *Gpx1* FW: 5′-AGT TCG GAC ACC AGG AGA AT-3′, *Gpx1* RV: 5′-GAA GGT AAA GAG CGG GTG AG-3′; *Gpx4* FW: 5′-TAA GAA CGG CTG CGT GGT-3′, *Gpx4* RV: 5′-GTA GGG GCA CAC ACT TGT AGG-3′; *Prx3* FW: 5′-GTG TGT CCT ACA GAA ATT GTT-3′, *Prx3* RV: 5′-AAC CAC CAT TCT TTC TTG GTG-3′; *Txnrd2* FW: 5′-ACA ACT GGA AGA CAA TGG CA-3′, *Txnrd2* RV: 5′-AAC TGT GTG CTC ATC CAC AAA-3′. Primers for *Per2, Dbp, Bmal1, Sod1, p16*^{INK4a}, *Xbp1u, Xbp1s* and *18S rRNA* were described previously (Cannavino et al. 2014; Chen et al. 2011; Tsuru et al. 2016; Yamamoto et al. 2004).

Cell growth assays

Cell growth assays were performed as described previously (Khaidizar et al. 2017). To calculate individual population doubling level (iPDL), we used a formula as follows: $n = 3.32\log(N_{post}/N_{pre})$. n = the iPDL number after a 3-day culture, N_{post} = total cell number after a 3-day culture, N_{pre} = the seeding cell number. Cumulative PDL (cPLD) represents the accumulation of each iPDL number. In this study, we determined the onset of cellular senescence when n is less than 0.5.

Cell viability assay

Cells at passage 3 were seeded in triplicates in 96-well plates at 1,500 cells/well. After 24 hr culture, cells were treated with indicated concentrations of thapsigargin (TOCRIS Bioscience), tunicamycin (SIGMA-ALDRICH) or hydrogen peroxide for 3, 3, or 24 hr, washed twice with PBS, cultured for another 21, 21 or 0 hr culture, respectively, and

performed MTT assays (Nacalai tesque) according to the manufacturer's protocol.

SA-β-Gal assay against ER or oxidative stresses

Cells at passage 3 were seeded in triplicates in 35 mm dishes at 4×10^4 cells and cultured for 24 hr. For SA-β-Gal assay against ER stress, MEF cells were treated with indicated concentrations of tunicamycin or thapsigargin for 3 hr, washed twice with PBS, and cultured for another 6 days with a single cell passaging before performing SA-β-Gal assay. For SA-β-Gal assay against acute oxidative stress, MEF cells were treated with 400 μM of hydrogen peroxide for 24 hr, washed twice with PBS, and cultured for another 3 days before SA-β-Gal assay was performed. For SA-β-Gal assay against chronic oxidative stress, MEF cells were cultured with 100 μM of hydrogen peroxide for 6 days with a single passaging followed by SA-β-Gal assay. Medium with hydrogen peroxide was changed every day. All SA-β-Gal assays were performed as described previously (Khaidizar et al. 2017).

Statistics

Values were reported as mean ± SEM. Statistical differences were determined by a Student's *t*-test. Statistical significance was displayed as * ($p < 0.05$) or ** ($p < 0.01$).

Results

Primary MEF cells possess intrinsic circadian clocks

Although *Bmal1*$^{-/-}$ mice have a short lifespan with many accelerated age-related diseases (Kondratov et al. 2006), the molecular mechanisms of how *Bmal1*, a core circadian clock gene, regulates aging events are still largely unknown. Since organismal aging is regulated by complicated systems, we first attempted to investigate whether *Bmal1* regulates cellular senescence using primary MEF cells derived from *Bmal1*$^{-/-}$ embryos. There are a few literature that shows primary wild type MEF cells possess circadian oscillators (Baeza-Raja et al. 2013; Pando et al. 2002), therefore, we first confirmed whether primary MEF cells have functional circadian clocks (Figure 1). Transcripts of clock components *Per2, Dbp*, and *Bmal1* were found to demonstrate circadian oscillations in primary MEF cells derived from wild type embryos (wt–MEF) at embryonic day (E) 14.5 (the day when the vaginal plug was found was designated as E0.5). However, in primary MEF cells derived also from E14.5 *Bmal1*$^{-/-}$ embryos (*Bmal1*$^{-/-}$–MEF), *Per2* and *Dbp* transcripts did not show any oscillations. These results indicate that primary wt–MEF cells established as early as E14.5 already have intrinsic circadian clocks, but *Bmal1*$^{-/-}$–MEF cells do not.

Bmal1$^{-/-}$–MEF cells show normal replicative senescence

Primary cells have a limited capacity to proliferate, in which the proliferative phase progressively switches towards a growth stationary phase (Hayflick 1965; Hayflick and Moorhead 1961). In order to assess at which passage cells begin to cease proliferation, individual and cumulative population doubling levels (iPDL and cPDL, respectively) were recorded by subculturing primary MEF cells every 3 days. Here, we defined a cease of cell proliferation (CCP) as the point at which iPDL value dips below 0.5. Littermate wt–MEF cells reached CCP at passage 7 with cPDL of 10.15 (Figure 2A, B), which is consistent with our recent report (Khaidizar et al. 2017). Surprisingly, *Bmal1*$^{-/-}$–MEFs reached CCP at passage 7 with cPDL of 10.37 (Figure 2A, B), similar to CCP and cPDL of wt–MEF cells, suggesting that replicative senescence is not affected by *Bmal1* deficiency.

We further investigated two parameters to corroborate our initial observation; cellular level of senescence associated β-galactosidase (SA-β-Gal) activity (Debacq-Chainiaux et al. 2009) and expression level of senescence marker gene $p16^{INK4a}$ (Krishnamurthy et al. 2004; Stein et al. 1999). Consistent with our recent report (Khaidizar et al. 2017), the number of positive cells for SA-β-Gal activity in wt–MEFs was 0.96% at passage 5 and dramatically increased to 41.35% at passage 6. Similarly, the number of SA-β-Gal positive *Bmal1*$^{-/-}$–MEFs was 1.85% at passage 5 and significantly increased to 33.43% at passage 6 (Figure2C, D). However, there was no statistical difference between genotypes. Consistent with the results of SA-β-Gal assay, expression level of

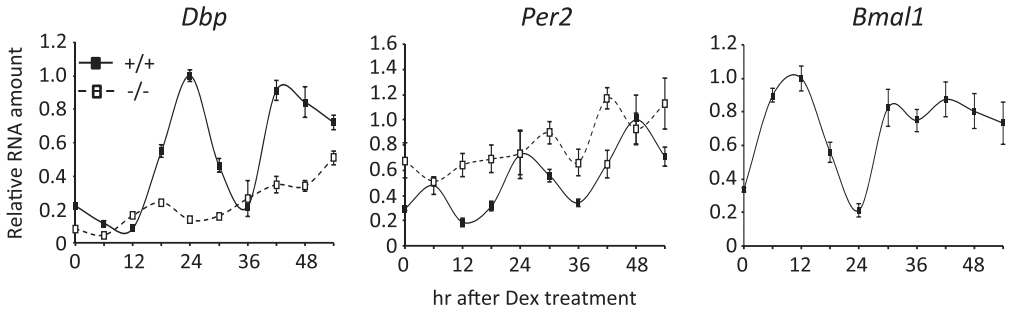

Figure 1. Primary wt- (+/+; black square with solid line) or *bmal1-/–* (-/-; white square with dotted line) MEF cells were stimulated by 1 μM Dexamethasone for 1 hr. Total RNAs were isolated at each time point. Quantitative real-time RT-PCR was performed using *Dbp* (left panel), *Per2* (middle panel), *Bmal1* (right panel) and 18S rRNA primers. mRNA amount at time 24, 42 or 12 hr of *Dbp, Per2* or *Bmal1*, respectively, in wt-MEFs were set to 1. The relative level of each mRNA was normalized to the corresponding 18S rRNA level. Data are means ± SEM of three independent samples.

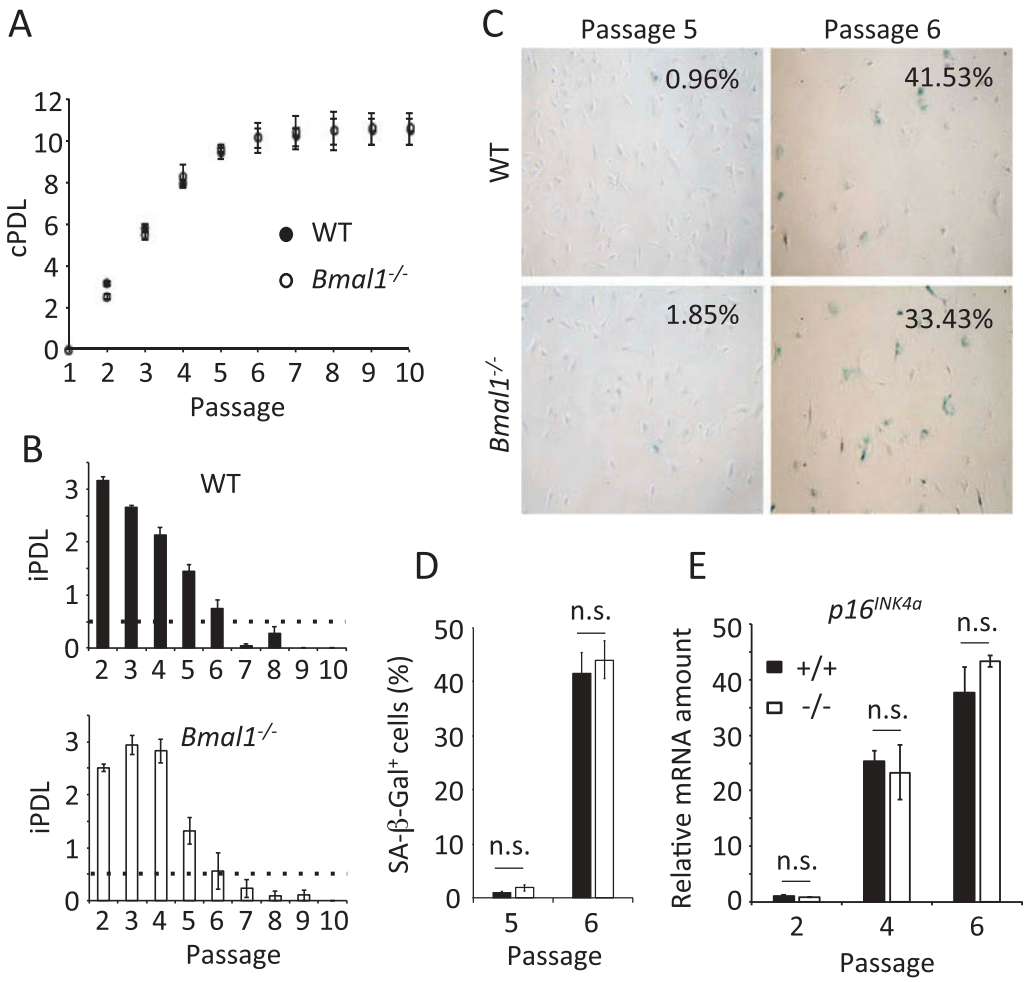

Figure 2. Cumulative (A) and individual (B) PDL of wt- and *Bmal1-/-*-MEF cells were measured. (C) Photos of SA-β-Gal positive cells (green) in primary Wt- and *Bmal1-/-*-MEF cells at passage 5 or 6 were taken. (D) The percentage of SA-β-Gal positive cells were quantified. (E) Expression levels of $p16^{INK4a}$ at indicated passages were quantified by qPCR. $p16^{INK4a}$ mRNA amount in wt-MEFs at passage 2 was set to 1. The relative level of each mRNA was normalized to the corresponding 18S rRNA level. Data are means ± SEM of three independent samples except D.

$p16^{INK4a}$ was increased with passages in both wt– and $Bmal1^{-/-}$–MEF cells, but without significant difference between genotypes (Figure 2E). All the abovementioned results indicate that *Bmal1* deficiency has no effect on the process of replicative senescence *in vitro* under unperturbed conditions.

Bmal1$^{-/-}$–MEF cells show normal stress-induced premature senescence

We next investigated whether *Bmal1* deficiency affects the process of stress-induced premature senescence. Since it has been reported that oxidative stress accelerates aging at cellular and organismal levels (Bhatia-Dey et al. 2016; Rodier and Campisi 2011), we investigated whether oxidative stress accelerates senescence in *Bmal1*$^{-/-}$–MEFs. To that end, we first examined whether deficiency of *Bmal1* has any effect on cell viability after an acute (24 hr) hydrogen peroxide (H_2O_2) treatment. Acute H_2O_2 treatments decreased cell viability for both wt– and *Bmal1*$^{-/-}$–MEF cells in a dose-dependent manner (Figure 3A). However, differences between genotypes were not observed at all the doses tested, indicating that sensitivity and/or response against oxidative stress is not influenced by *Bmal1*. To address whether *Bmal1* is associated with oxidative stress-induced premature senescence, we performed SA-β-Gal assay after an acute H_2O_2 treatment. SA-β-Gal assays were performed after 24 hr H_2O_2 (400 μM) treatment followed by a 3-day culture. Although cellular senescence was induced by an acute H_2O_2 treatment, there was no difference between genotypes (Figure 3B). We further performed SA-β-Gal assay after a 6-day continuous H_2O_2 treatment at a sublethal concentration (100 μM). Although percentages of SA-β-Gal positive cells tended to be low in *Bmal1*$^{-/-}$–MEF cells, there were no significant differences between genotypes at both concentrations (0 μM: $p = 0.07$; 100 μM: $p = 0.23$). Finally, we quantified antioxidant gene expressions in MEF cells. *Nrf2*, the master regulator of oxidative stress (Kensler et al. 2007), and other antioxidant genes tested were expressed to the same levels in both primary wt– and *Bmal1*$^{-/-}$–MEF cells (Figure 3D).

Endoplasmic reticulum (ER) stress and its downstream pathways, collectively known as the unfolded protein response (UPR) signaling pathways, have recently been reported to be associated with aging in multiple tissues (Pluquet et al. 2015; Salminen and Kaarniranta 2010). Moreover, genetic or pharmacological manipulations of some components of the

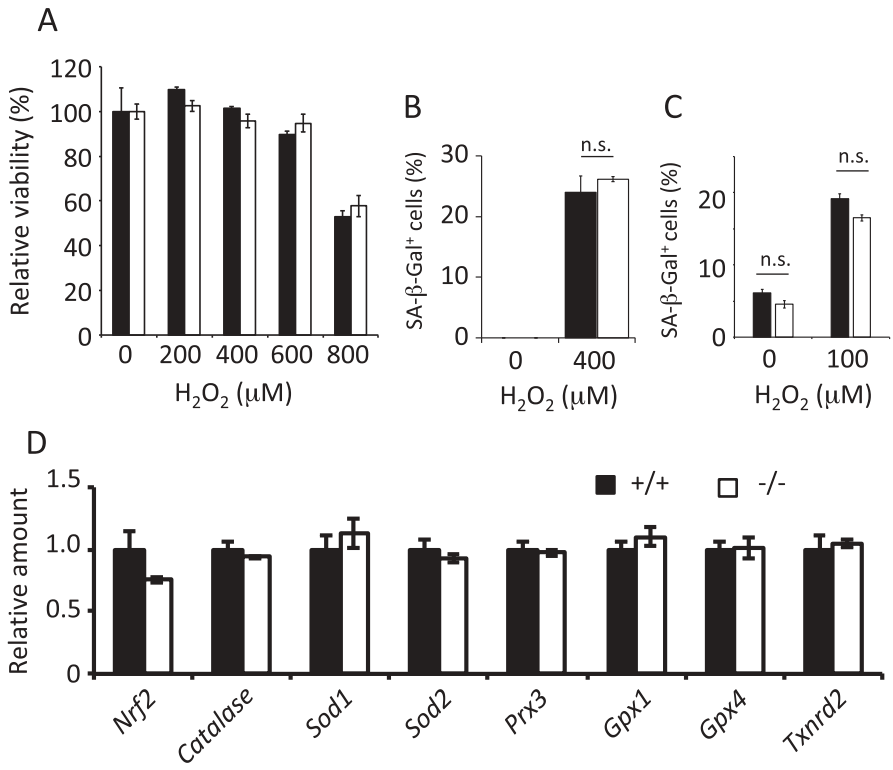

Figure 3. (A) Cell viabilities of wt- (black bar) and *Bmal1-/--* (white bar) MEF cells were measured after 24 hr hydrogen peroxide treatment at indicated concentrations. (B, C) Percentages of SA-β-Gal positive cells in wt- (black bar) or *Bmal1-/--* (white bar) MEFs treated with acute (B) or chronic (C) conditions were quantified. (D) Total RNAs were isolated from wt- (black bar) or *Bmal1-/--* (white bar) MEF cells. qPCR was performed using indicated primer sets. The relative level of each mRNA was normalized to the corresponding 18S rRNA level.

UPR signaling pathways have been shown to impact cellular senescence (Pluquet et al. 2015). As ER stressors have recently been demonstrated to trigger stress-induced premature cellular senescence (Liu et al. 2014; Liu et al. 2015), we sought to find out whether *Bmal1* regulates ER stress-induced premature senescence. Since splicing of *Xbp1* mRNA is one of the indicators and triggers of ER stress responses (Yoshida et al. 2001), we first evaluated *Xbp1* mRNA amount with or without ER stressor. Semi-quantitative PCR showed that the amount of unspliced *Xbp1* mRNA (*Xbp1u*) in *Bmal1*$^{-/-}$–MEFs without ER stressor was comparable to that in wt–MEF cells (Figure 4A, B upper panels). Furthermore, the amounts of spliced form of *Xbp1* (*Xbp1s*) in response to ER stressors, tunicamycin or thapsigargin, were almost similar between genotypes (Figure 4A, B upper panels). To quantify the ratio of *Xbp1s/Xbp1u*, quantitative PCR was performed and we confirmed that the ratios of *Xbp1s/Xbp1u* between genotypes had no statistical difference under both steady-state and stress-induced conditions (Figure 3A, B bottom panels). These results suggest that the regulatory mechanism upstream of *Xbp1* splicing pathway is independent of *Bmal1*. Next, to investigate downstream of Xbp1 splicing pathway, we investigated cell viability and cellular senescence against ER stressors. After a 3-hr treatment of ER stressor followed by a 21-hr culture, cell viability decreased in a dose-dependent manner in both wt– and *Bmal1*$^{-/-}$–MEF cells, however, we could not observe any differences between genotypes (Figure 4C, D). Percentages of SA-β-Gal positive cells, after a 3-hr tunicamycin or thapsigargin treatment followed by a 6-day culture, were increased in both genotypes of MEF cells which was similar to a previous report of cellular senescence induction by ER stressor in proximal tubular epithelial cells (Liu et al. 2014; Liu et al. 2015). However, percentages of SA-β-Gal positive cells in *Bmal1*$^{-/-}$–MEFs were comparable to those in wt–MEFs (Figure 4E). Taken together, we conclude that neither oxidative- nor ER-stress induced premature senescence is controlled by *Bmal1*.

Discussion

In this study, we established primary MEF cells from *Bmal1*$^{-/-}$ and wild type littermate embryos at embryonic day (E) 14.5 and confirmed that primary wt–MEF cells possess intrinsic circadian oscillators, but *Bmal1*$^{-/-}$ cells do not. Contrary to our hypothesis that *Bmal1*$^{-/-}$ mice are vulnerable and prone to senesce, primary *Bmal1*$^{-/-}$–MEF cells showed an acceleration in neither replicative nor stress-induced senescence, indicating that *in vitro* cellular senescence is not controlled by *Bmal1*.

Kondratov et al. reported that no difference was detected with regards to *in vitro* replicative senescence between primary fibroblast cells derived from adult lung in wt and *Bmal1*$^{-/-}$ mice (Khapre et al. 2011), which is consistent with our findings in this study. However, they also reported that *Bmal1*$^{-/-}$ fibroblast cells derived from adult lung are sensitive to oxidative stress (Kondratov et al. 2009), while our cells were not. Moreover, our result demonstrated no difference in transcript levels of *Nrf2* and all the antioxidant genes tested between wt– and *Bmal1*$^{-/-}$–MEF cells *in vitro*, although *Nrf2* is reported to be directly regulated by CLOCK:BMAL1 (Pekovic-Vaughan et al. 2014). Discrepancy between these results might be due to the environment in which fibroblast cells were isolated. For example, cells in adult tissues are exposed to a variety of stress, such as oxidative or nutritional stresses, compared to cells in embryo. Also, cells in *Bmal1*$^{-/-}$ adult mice may be exposed to excess stress due to the disruption of circadian clock. These stresses may affect the sensitivity against hydrogen peroxide in cells isolated from *Bmal1*$^{-/-}$ adult mice. Actually, Lee et al. reported that pancreatic β cells derived from *Bmal1*$^{-/-}$ adult mice expressed lower levels of some antioxidant genes (Lee et al. 2013). Furthermore, MEF cells cultured in this study were exposed to oxidative stress compared to *in vivo* condition; in contrast to 3 ~ 5% physiological *in vivo* oxygen concentrations, oxygen concentration of our culture condition was at atmospheric level (21%), suggesting that *Nrf2* gene expression in *Bmal1*$^{-/-}$–MEF cells has been fully activated by CLOCK:BMAL1-independent manner and is comparable to wt–MEF cells.

Kondratov group has further reported that chronic oral administration of rapamycin, the inhibitor of mammalian Target of Rapamycin Complex 1 (mTORC1), partially mitigates the shortening of lifespan observed in *Bmal1*$^{-/-}$ mice (Khapre et al. 2014). They also demonstrated in that study that activity of mTORC1 is increased in tissues and primary fibroblast cells isolated from *Bmal1*$^{-/-}$ mice. An increased mTOR signaling is associated with accelerated aging,

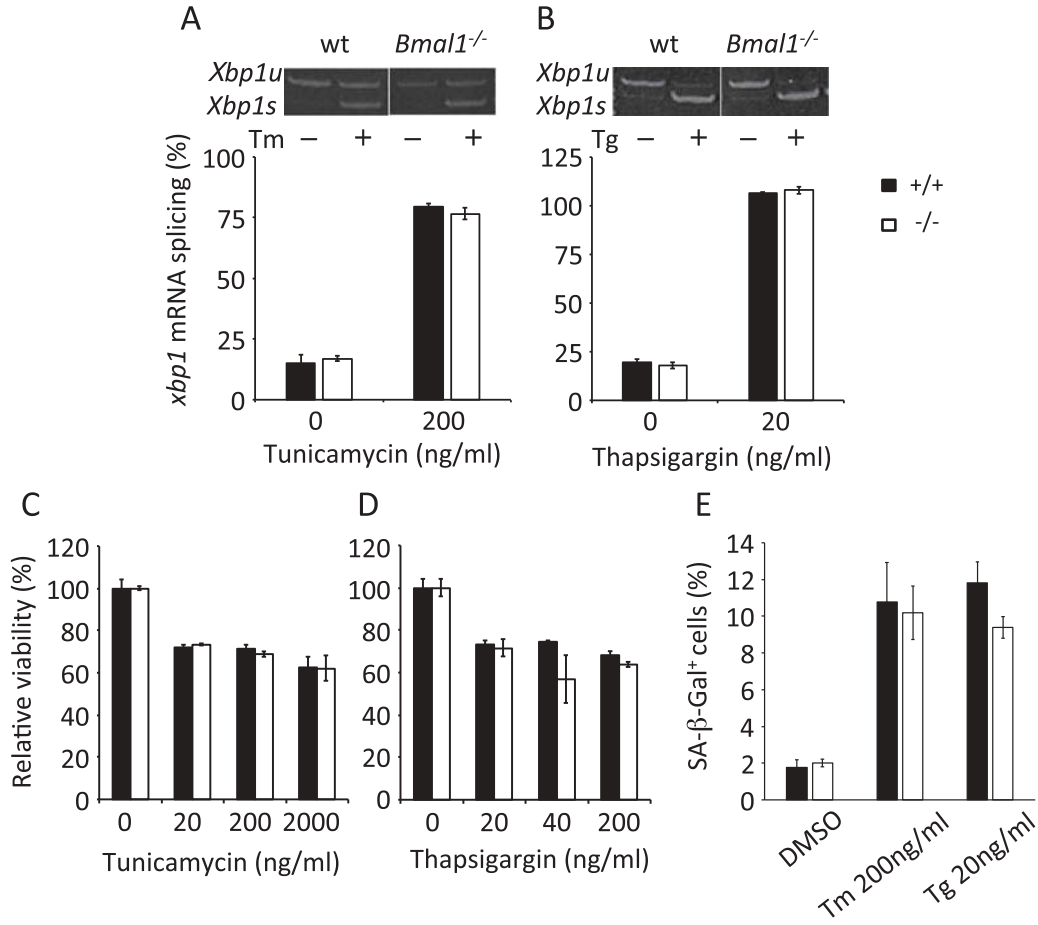

Figure 4. (A, B; upper panels) Results of semi-quantitative PCR treated with (+) or without (-) 200 ng/ml tunicamycin (Tm) or 20 ng/ml thapsigargin (Tg). Upper bands indicate unspliced form of *Xbp1 (Xbp1u)* and lower bands spliced form of *Xbp1 (Xbp1s)*. (A, B; bottom panels) Total RNAs were isolated from wt- and *Bmal1-/-*-MEF cells treated with or without 200 ng/ml Tm or 20 ng/ml Tg. Ratio of *Xbp1s/Xbp1u* were quantified by qPCR. (C, D) Cell viabilities of wt- (black bar) and *Bmal1-/-* (white bar) MEF cells were measured after 24 hr treatment with tunicamycin or thapsigargin at indicated concentrations. (E) The percentage of SA-β-Gal positive cells in DMSO (negative control), 200 ng/ml Tm or 20 ng/ml Tg treated wt- or *Bmal1-/-*-MEF cells were quantified.

hence their results suggest that primary *Bmal1*$^{-/-}$ fibroblast cells undergo accelerated cellular senescence. Investigations on whether *Bmal1* is associated with mTOR signaling pathway-dependent *in vitro* cellular senescence will be performed in future.

We cannot deny the possibility that *Bmal1*$^{-/-}$–MEF cells undergo accelerated *in vitro* cellular senescence under certain stress/culture conditions, or that senescent cells in *Bmal1*$^{-/-}$tissues are the tissue-specific cells, not fibroblast cells. However, results from our and other groups suggest that the premature *in vivo* cellular senescence in *Bmal1*$^{-/-}$ mice is due to dysregulations of inter- and/or intra-organ communication network and is not cell-autonomous. Yang et al. recently generated conditional *Bmal1*$^{-/-}$ (iKO) mice that lacked the BMAL1 protein from the age of 90-day, and reported that lifespan and most of age-related diseases of iKO mice were comparable to wild type mice (Yang et al. 2016). The authors further stressed that many phenotypes in conventional *Bmal1*$^{-/-}$ mice reflect the loss of properties of BMAL1 that are independent of its role in circadian clock. In contrast, some reports suggest that BMAL1 as a core circadian component may function before the age of 90-day. One example is that *Bmal1*$^{-/-}$ mice were demonstrated to develop aberrant adult neurogenesis (Malik et al. 2015). This phenomenon was not only observed in *Bmal1*$^{-/-}$ mice, but also in other circadian clock-deficient mice such as *Per2*$^{-/-}$ (Borgs et al. 2009; Bouchard-Cannon et al. 2013), *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ (Malik et al. 2015) and *Rev-erbα*$^{-/-}$ (Schnell et al. 2014) mice, indicating that circadian clock is essential for adult neurogenesis. These findings raise the possibility that the neurogenesis during

embryogenesis/early postnatal period is also affected by circadian clock, leading to the establishment of aberrant neural networks in clock-deficient mice. If this were the case for *Bmal1* iKO mice, inter- and/or intra-organ communication network including neural network established during embryogenesis/early postnatal period might be critical for healthy lifespan. Additionally, tissue-specific *Bmal1*$^{-/-}$ mouse could be a powerful tool to reveal whether intraorgan communication failures caused by the dysfunction of circadian clock would affect lifespan. While many tissue-specific *Bmal1*$^{-/-}$ mice have been produced, to our knowledge, only cardiac-specific *Bmal1*$^{-/-}$ mice have had their lifespan monitored, in which the mice die in approximately 40 weeks, similar to that of conventional *Bmal1*$^{-/-}$ mice (Kohsaka et al. 2014). Further investigation will therefore be conducted to understand systemic regulations of aging process by circadian clock.

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Declaration of Interest Statement

The authors report no conflicts of interest.

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