Biochemical and Biophysical Research Communications xxx (2018) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Loss of circadian protein TIMELESS accelerates the progression of cellular senescence

Xiaomeng Shen, Mingzhe Li, Zebin Mao**, Wenhua Yu*

Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing, 100191, People's Republic of China

ARTICLE INFO

Article history:
Received 28 July 2018
Accepted 5 August 2018
Available online xxx

Keywords:
TIMELESS
E2F1
Senescence

ABSTRACT

TIMELESS protein is known to be essential for normal circadian rhythms. Aging is a deleterious process which affects all the physiological functions of complex organisms including the circadian rhythms. The circadian aging may produce disorganization among the circadian rhythms, arrhythmicity and even, disconnection from the environment, resulting in a detrimental situation to the organism. However, the role of circadian genes on the aging process is poorly understood. In present study, we found TIMELESS was down-regulated in cellular senescence, and further research indicated E2F1 bound to the promotor of TIMELESS and regulated its expression in cellular senescence. Knockdown of TIMELESS accelerated cellular senescence induced by ectopic expression of RasV12, and overexpression of TIMELESS delayed this kind onset of senescence. Meanwhile, micrococcal nuclease assays proved depletion of TIMELESS exacerbated genomic instability at the onset of senescence. Together, our data reveal that TIMELESS plays a role in OIS, which is associated with genome stability changing.

1. Introduction

The Timeless (Tim) gene is originally identified in *Drosophila melanogaster* and subsequently in mammals, involving in the molecular clockwork that drives 24 h periodicity in physiology and behavior. Although the influence of Tim on rhythmicity has been demonstrated [1,2], it appears to play additional functions in embryonic development, cell cycle progression, DNA replication, and the DNA damage response (DDR) [3]. Human TIMELESS is involved in maintaining replication fork stability during normal DNA replication [4], activating cell S-checkpoints [5], promoting proper sister chromatid binding [4], and participating in DNA damage repair [6], which requires its association with TIPIN. Timeless is thus a multifaceted factor implicated in the maintenance of many cellular processes, tissue functions, and ultimately homeostasis of various organisms, from insects to humans. Aging process would affect circadian system at all its structures and all levels [7]. The circadian aging may produce a disorganization among the circadian rhythms, arrhythmicity and even, disconnection from the environment, resulting in a detrimental situation to the organism. However, the role of circadian genes on aging process is less known.

Cellular senescence is a permanent cell cycle arrest which is triggered by replicative exhaustion, oxidative stresses, oncogenic activation, DNA damage, and other detrimental growth conditions [8,9]. Normal fibroblasts with ectopic expression of RAS induced cellular senescence [10] which is telomere-independent [11], different from replicative senescence, termed as oncogene-induced senescence(OIS) [10]. Research proved initiation and maintenance of OIS was dependent on DDR pathway, which was activated by collapse of replication fork induced by hyper-replication stress [12]. DNA repair deficiencies during OIS might increase the genomic instability [13]. OIS serves as a barrier that normal cells have to overcome in order to transform into cancer cells [14]. Analysis of several types of premalignant tumors, most notably benign skin nevi, has revealed the existence of senescent pretumorigenic cells [14,15]. The functional relevance of spontaneous senescence induction in preventing tumor initiation and progression has been demonstrated by several recent mouse studies [16–18]. Therefore, to identify novel mechanisms of OIS would not only provide novel

* Corresponding author. Department of Biochemistry and Molecular Biology, Peking University Health Science Center, 38 Xue Yuan Road, Beijing, 100191, People's Republic of China.
** Corresponding author. Department of Biochemistry and Molecular Biology, Peking University Health Science Center, 38 Xue Yuan Road, Beijing, 100191, People's Republic of China.
E-mail addresses: zbmao@bjmu.edu.cn (Z. Mao), yuwenhua@hsc.pku.edu.cn (W. Yu).

https://doi.org/10.1016/j.bbrc.2018.08.040
0006-291X/

insights into senescence regulation, but could also facilitate development of novel prosenescence therapy strategies.

We reported here that TIMELESS could function as a regulator during the process of OIS. We showed that TIMELESS is down-regulated in cellular senescence. Our investigation demonstrated the expression of TIMELESS is regulated by E2F1; depletion of TIMELESS accelerated Ras^{G12V} induced senescence and over-expression of TIMELESS delayed the onset of senescence; furthermore, TIMELESS depletion exacerbates genomic instability at the onset of senescence. Taken together, our data revealed that loss of clock protein TIMELESS could facilitate the cellular senescence accompanied by increased genome instability, which may suggest a potential application of TIMELESS inhibitor in cancer prevention.

2. Materials and methods

2.1. Cell culture and reagent

HEK293T cells and Human diploid fibroblasts 2BS cells were obtained from the National Institute of Biological Products, Beijing, China. These cells were cultured in complete Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS), streptomycin (100 mg/ml), penicillin (100 U/ml), and maintained in a humidified atmosphere of 5% CO2 at 37 °C. H_2O_2 (H1009; Sigma) was diluted with culture medium to 150 μM and 2BS cells were treated with H_2O_2 for 2 h.

2.2. Western blot and antibodies

Western blot was performed by standard method. The primary antibodies used were for TIMELESS (ab72458, Abcam), Actin (PM053, MB), p21 (ab109520, Abcam), p16 (sc-468, Santa Cruz), p53 (sc-126, Santa Cruz), RORα (10616-1-AP, Proteintech), DEC1 (17895-1-AP, Proteintech), DEC2 (12688-1-AP, Proteintech); CLOCK (YT0978, Immunoway); Per1 (13463-1-AP, Proteintech); Per2 (ab180655, abcam) ; Per3 (12550-1-AP, Proteintech); Bmal2 (ab198293, abcam); γ-H2A.X (2577S, Cell Signaling Technology); E2F1 (ab179445, abcam); E7(ab100967, abcam); GAPDH (AG04191532, Bioss).

2.3. SA-β-gal and EdU incorporation assay

SA-β-gal activity was performed as described previously [19]. EdU incorporation assay was performed with EdU incorporation assay kit according to the manufacturer's instructions. All cells were examined using fluorescence microscopy with the appropriate filters. At least 500 cells were counted in randomly chosen fields from each culture well.

2.4. Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 15 min and were permeabilized with phosphate-buffered saline containing 0.25% Triton X-100 for 10 min. Cells were incubated with 5% bovine serum albumin for 1 h at 37 °C and followed by incubation with antibodies specific for γ-H2AX (1:200, CST) overnight at 4 °C. Cells were then washed with phosphate-buffered saline and incubated with a fluorophore-conjugated secondary antibody. Finally, cells were counterstained with 4′,6-diamidino-2-phenylindole to visualize the nuclei. Cells were imaged on a Zeiss LSM410 confocal laser scanning microscope with × 60 magnification.

2.5. Luciferase activity assay

For the TIMELESS promoter activity assay, the luciferase reporters were all constructed in the pGL3 basic vector. 2 days after infection with the indicated vectors, 2BS cells were seeded and transfected with TIMELESS reporter plasmid together with control. After 48 h, the cells were harvested and luciferase activity was measured with a dual luciferase kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Firefly luciferase activity was normalized to the Renilla luciferase activity for each transfected well. The quantitative results were presented as mean ± SD for triplicated experiments.

2.6. RNA interference

The following shRNA plasmids were constructed in the pLVX-shRNA vector (Clontech). sh-TIM-1:5′-GAGCUAAGAAGCCUAGGGGT T; sh-TIM-2: 5′-GCACCCAAGAAACGACAATTG; sh-TIM-3:5′-GTAGCT TAGTCCTTTCAAATT. Lentiviral gene transduction was carried out as previously described using 293T packaging cells with the Lenti-X HTX Packaging System (Clontech). Medium containing virus was collected, supplemented with 8 μg/ml of polybrene (Sigma), and incubated with target 2BS cells at 37 °C for 12 h. Infected 2BS cells were purified by drug selection (3 μg/ml puromycin).

2.7. Chromatin immunoprecipitation (ChIP)

ChIP assay was performed as previously described by Carey et. Antibodies used for ChIP were anti-E2F1(ab179445, abcam). TIMELESS primer: 5′- TAAATGAACGGGTGGTAG and 5′-GATGAA AGGACGGATGAC; Cyclin A primer: 5′-TCTGCGTCTTCGTTGAGC and 5′-CTTAGCGTCGTTGCCTTC.

2.8. Statistical analysis

Data were presented as mean ± SD from at least three independent experiments. Cell experiments were repeated at least three times and measured by the Student's t-test to evaluate the significance. Spearman's correlation was employed to analyze the correlation. All statistical analyses were measured using SPSS 20 (IBM), and a P-value o0.05 was considered significant.

3. Result

3.1. TIMELESS expression is reduced in senescent cells

It has been previously demonstrated that upon introducing mutant RasG12V in human diploid fibroblasts (HDF) in the absence of cooperating lesions, cells enter premature senescence around day 5–6 [20]. In our study, the cellular OIS model was validated by senescence-associated beta-galactosidase (SA-β-gal) staining and EdU incorporation analysis. The percentage of senescent cells identified by SA-β-gal staining is significantly increased in response to Ras^{G12V} in 2BS human diploid fibroblasts (Fig. 1A) and the percentage of cells with EdU incorporated is decreased (Fig. 1A). According to previous reports, oncogenic stress could induce OIS through p53-p21 and p16-pRB pathways [9]. The cellular senescence model was further confirmed by Western Blot analysis of increased expression of p53, p21 and p16 (Fig. 1B). To our surprise, the expression of circadian protein TIMELESS was down-regulated during the onset of OIS (Fig. 1B), while other members of circadian clock showed no significant changes in their expression (Fig. 1C). The downregulation of TIMELESS was also confirmed in replicative senescence (Fig. 1D) and H_2O_2 induced senescence (Fig. 1E), suggesting TIMELESS downregulation might be a common event happened during cellular senescence.

Please cite this article in press as: X. Shen, et al., Loss of circadian protein TIMELESS accelerates the progression of cellular senescence, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.08.040