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Implication of p53-dependent cellular senescence related gene, TARSH in tumor suppression

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ABSTRACT

A novel target of NESH-SH3 (TARSH) was identified as a cellular senescence related gene in mouse embryonic fibroblasts (MEFs) replicative senescence, the expression of which has been suppressed in primary clinical lung cancer specimens. However, the molecular mechanism underlying the regulation of TARSH involved in pulmonary tumorigenesis remains unclear. Here we demonstrate that the reduction of TARSH gene expression by short hairpin RNA (shRNA) system robustly inhibited the MEFs proliferation with increase in senescence-associated β-galactosidase (SA-β-gal) activity. Using $p53^{-/-}$ MEFs, we further suggest that this growth arrest by loss of TARSH is evoked by p53-dependent $p21^{Cip1}$ accumulation. Moreover, we also reveal that TARSH reduction induces multicentrosome in MEFs, which is linked in chromosome instability and tumor development. These results suggest that TARSH plays an important role in proliferation of replicative senescence and may serve as a trigger of tumor development.

Introduction

Normal primary culture cells represent irreversible arrest of proliferation after serial cell divisions. This growth inhibition is defined as cellular senescence, which can be triggered by telomere shortening, oxidative stress, DNA damage accumulation, or activated oncogenes [1,2]. Abundant recent studies have demonstrated that these senescence-related changes may block tumor development, produce apoptosis, and even lead to aging, depending on several tumor suppressor proteins which play a dual role both in cancer and aging, such as $p16^{INK4a}$, $p19^{ARF}$, or p53 [3–7]. In addition, a quite recent report reveals that secreted cytokine proteins play a crucial role in the process of stress-induced premature senescence, which provides new insights into the induction of cellular senescence and the regulation of tumor promotion [8–10].

We have demonstrated novel cellular senescence related genes in MEFs by cDNA subtractive hybridization method [11,12]. Murine TARSH (mTARSH, target of NESH-SH3/Abi3bp) was initially identified as NESH-SH3-binding protein [13] and we characterized it a cellular senescence related gene because of its robust and transient induction in the early phase of MEFs cellular senescence. Although the expression of TARSH gene is also declined in follicular thyroid carcinomas and primary lung cancer [14,15], little is known about its involvement in tumorigenesis yet. Alternatively, how dynamics of TARSH gene expression involved in both stress-induced senescence and preventing cancer development remains unclear.

In this study, to elucidate further physiological function of TARSH in cellular senescence, we carried out the short hairpin RNA (shRNA)-mediated TARSH gene knockdown in MEFs and demonstrated that TARSH-suppressed MEFs were drastically inhibited their proliferation in a p53-dependent manner. We found that TARSH expression was necessary for cell cycle progression because its decline preciously represented the accumulation of G0/G1 population with a concomitant activation of a cyclin-dependent kinase inhibitor (CKI), $p21^{Cip1}$ whose expression was tightly controlled by p53. Intriguingly, we also demonstrated that TARSH depletion frequently represented aberrant number of centrosomes, which probably causes aneuploidy as the hallmark of the cancer. On the basis of these results, we here adduce evidence to show that hitherto veiled function of TARSH in replicative senescence and trigger of tumor suppression.

Abbreviations: PDs, population doublings; PI, propidium iodide; TARSH, target of NESH-SH3/Abi3bp; MEFs, mouse embryonic fibroblasts; BrdU, bromodeoxyuridine; 7-AAD, 7-aminoactinomycin D.

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Materials and methods

Cells culture and conditions. 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA), 2 mM glutamine, and 100 U/ml penicillin and streptomycin. Wild type and $p53^{-/-}$ MEFs derived from C57BL/6 mice were cultured as described above condition except with 0.1 mM nonessential amino acid and 50 μM 2-mercaptoethanol. All cells were incubated at 37 °C in 5% CO_2. For growth curve analysis, the 3T3 protocol was performed. Population doublings (PDs) were determined by the previous formula described [16].

Retrovirus production and infection. For short hairpin TARSH (shTARSH) knockdown construction, the sense shRNA target sequence (5′-GAAATAGTGGTGTTTGCCA-3′) or shRNA that did not target any known sequence on mouse genome (shScramble) [17] were annealed and cloned into pSUPERretro vector (Oligoengine, USA). For centrosome detection and focus formation assay, we used another control shRNA retrovirus which also contained a puromycin resistance gene [18]. Cells cultures, puromycin selections, and generation of retroviral supernatant for each infection were carried out as described previously [19]. Unless otherwise described, for all knockdown experiments, retrovirus-infected MEFs at passage 3 were utilized.

Quantitative real-time RT-PCR. Quantitative real-time RT-PCR analysis was preformed with SYBR Green Realtime PCR Master Mix (TOYOBO). Following PCR primers were used; mouse $p21^{Cip1}$ (sense: 5′-CGAGAACGGTGGAACTTTGAC-3′, antisense: 5′-CAGG GCTCAGGTAGACCTTG-3′). The sequences of both human and mouse TARSH and GAPDH were described previously [12,14]. Any relative TARSH expression level was normalized with GAPDH expression as described previously [11].

SA-β-gal staining. SA-β-gal staining was performed using senescence β-galactosidase staining Kit (CST) according to the manufacturer's instructions.

Cell cycle analysis. The shScramble or shTARSH-infected MEFs (1×10^6 cells) were metabolically labeled for 30 min with 10 μM BrdU. After washing twice with PBS, the cells were fixed and stained with FITC-conjugated anti-BrdU antibody and 7-AAD in accordance with the manufacture's instruction (BD Pharmingen).

Western blotting. Preparation of whole cell lysates and Western blotting were performed as described elsewhere [11]. Following primary antibodies were used: anti-$p21^{Cip1}$ (F-5), anti-$p16^{INK4a}$ (M-156), anti-$p19^{ARF}$ (5C3-1, Santa Curz Biotechnology), anti-p53 (505, Novocastra, UK), anti-β-tubulin (D66, Sigma), and anti-phospho-p53 (Ser6, Ser15, Ser20, Ser392, CST).

Immunofluorescence. Cells on coverslips were fixed with 4% paraformaldehyde in PBS for 10 min and stained with anti-γ-tubulin antibody (GTU-88, Sigma) for 1 h at room temperature. PBS-washed coverslips were incubated with sheep FITC-conjugated anti-mouse IgG secondary antibody (GE Healthcare). A total of 192 control and 377 shTARSH-infected MEFs in three 3.5 cm plates were counted and indicated as percentages of the cells with more than 3 centrosomes.

Focus formation assay. *p53*-deficient MEFs were infected with control or shTARSH-encoding retrovirus. 1×10^4 of infected cells were mixed with 3×10^5 of uninfected MEFs and seeded in 10 cm dishes. Medium was changed every 3 days up to 18 days post-infection, the cells were stained with crystal violet solution as described previously [20].

Result

Effect of TARSH deficiency in cell cycle

To gain further insights into the effect of TARSH loss in promotion or maintenance of replicative senescence in MEFs, we designed shRNAs recognizing the sequence at 3′ UTR of mTARSH gene (shTARSH). Primary MEFs from wild type C57BL/6 mice were infected with retrovirus encoding shTARSH. We first addressed whether shTARSH construct suppressed endogenous mTARSH mRNA expression by quantitative real-time RT-PCR. As shown in Fig. 1A, mTARSH gene expression was decreased to less than 2% of shRNA containing randomized nonspecific sequences (shScramble)-infected MEFs.

Having reported that TARSH expression is altered in replicative senescence in MEFs, we next assessed whether the suppression of endogenous TARSH expression could either delay or promote senescence by infecting retrovirus-carrying shTARSH into MEFs. After puromycin selection, the proliferation potential of infected MEFs was drawn in growth curves (Fig. 1B). Surprisingly, depletion of TARSH with shRNA allowed their proliferation to reduce less than half compared with shScramble control.

We next examined their cell cycle profiles by BrdU incorporation and 7-AAD staining (Fig. 1C). We noted the distinct decreased S population in TARSH-suppressed MEFs (6.8%) compared with shScramble-infected cells (16.8%) in Fig. 1D. We also realized that the G0/G1 population was accumulated in shTARSH-infected cells (67.1%) compared with control cells (57.4%). These results suggest that reduced TARSH expression led to the dysfunction of G1/S transition in cell cycle, which may have partially caused the growth inhibition in replicative senescence.

TARSH is engaged in cellular senescence via p53-dependent $p21^{Cip1}$ pathway

To complement the result with shTARSH in MEFs, we then confirmed whether this impaired proliferation would be associated with replicative cellular senescence by SA-β-gal activity. As shown in Fig. 2A, upper panels, the proportion of SA-β-gal staining positive MEFs was elevated to 11% in cultures infected shTARSH, although it stayed 2% in shScramble control. To elucidate this TARSH-mediated senescent feature involved in p53, we further measured the SA-β-gal activity in TARSH-reduced MEFs with *p53*-depleted background. Consequently, we did not find any substantial differences between shTARSH and shScramble-infected MEFs under no p53 expression as we expected (Fig. 2A, lower panels). Thus, these results strongly support the idea that TARSH is involved in cellular senescence in a p53-dependent manner.

We further searched for the cell cycle regulators associated with a loss of TARSH-mediated growth arrest of MEFs. As shown in Fig. 2B, TARSH suppression hardly resulted in any activation of the CKIs besides p53 other than $p21^{Cip1}$, a known p53-dependent CKI. Given $p21^{Cip1}$ is well-known as the major target of p53 [21,22], it is worth to demonstrate the further evidence that the downregulation of TARSH evokes the p53-mediated $p21^{Cip1}$ activation following to cellular senescence in MEFs. Next, we analyzed the $p21^{Cip1}$ gene expression with or without TARSH depletion by quantitative real-time RT-PCR (Fig. 2C). Consistent with the observations above, quantitative real-time RT-PCR analysis showed that the measurable induction of $p21^{Cip1}$ mRNA accompanied by the TARSH suppression was confirmed in the presence of p53, whereas such induction was abrogated in the absence of p53. These results collectively indicate that increasing $p21^{Cip1}$ gene expression in the condition of TARSH loss is dependent on p53 specific transcriptional activity. Therefore, TARSH is involved in p53-dependent cellular senescence in MEFs. We also examined shTARSH-mediated p53 activation by its phosphorylation. In spite of the prominent induction of p53 protein, we could not identify any phosphorylation on p53 with various anti-phospho-p53 antibodies we used (Fig. 2D).