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A NOVEL P53-DEPENDENT APOPTOSIS FUNCTION OF TARSH IN TUMOR DEVELOPMENT

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ABSTRACT

A target of NESH-SH3/Abi3bp (TARSH) was originally identified as an SH3 domain-binding molecule of the NESH-SH3/Abi3 protein that is involved in Rac-dependent actin polymerization. In recent studies, TARSH gene expression was dramatically induced in mouse embryonic fibroblasts (MEFs) replicative senescence and suppressed in human lung carcinoma specimens and thyroid carcinomas. However, the molecular mechanism underlying the regulation of TARSH in tumorigenesis remains unclear. Here, we address a p53-dependent apoptosis function of the mouse TARSH gene using RNAi-mediated suppression of endogenous TARSH expression. Our results will be useful in the discovery of a novel therapeutic target in lung carcinoma.

Key Words: Cell cycle, Apoptosis, Tumor suppression, TARSH, p53

INTRODUCTION

Human TARSH was initially identified as a NESH-SH3-binding protein by the yeast two-hybridization system.[1)] Recently, Uekawa et al.[2,3)] characterized mouse TARSH as a novel cellular senescence-related gene in MEFs by the cDNA subtractive hybridization method due to its robust induction in MEF cellular senescence. It was also shown that the expression of TARSH was dramatically reduced in human lung cancer cell lines and primary lung carcinomas by quantitative real-time RT-PCR.[3,4)] Although the expression of the TARSH gene was also decreased in follicular thyroid carcinomas,[5)] little is known about its involvement in tumorigenesis.

Cell cycle progression is tightly controlled by cyclin and cyclin-dependent kinase inhibitor (CKI) molecules. Cell cycle surveillance is also carried out at several checkpoints by p53, $p16^{INK4a}$, $p19^{ARF}$ or RB.[6-8)] These molecules also play a role as tumor suppressors, indicating that the cell cycle control system is closely related with tumor development. For example, p53 suppresses tumor initiation and progression by halting the cell cycle and inducing apoptosis.[9)] The activity of the p53 tumor suppressor in induction of apoptosis largely depends on caspase-3 activity.

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In the present study, to further reveal the physiological function of TARSH in tumor progression, we carried out short hairpin RNA (shRNA)-mediated TARSH gene knockdown in MEFs and demonstrated that TARSH-suppressed MEFs drastically induced caspase-3-mediated apoptosis in a p53-dependent manner. These results suggest that TARSH may be a trigger of the p53-dependent apoptosis pathway against tumor development.

MATERIALS AND METHODS

Cell culture

293T and NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 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, with the addition of 0.1 mM nonessential amino acids and 50 μM 2-mercaptoethanol. All cells were incubated at 37°C in 5% CO_2.

Retrovirus production and infection

The short hairpin mouse TARSH (sh-TARSH) sense shRNA target sequence (5'-GAAATAGTG-GTGTTTGCCA-3') or shRNA that did not target any known sequence in the mouse genome (sh-Scramble) [10] was annealed and cloned into the pSUPERretro vector (Oligoengine). Retrovirus production and infection were carried out as described previously.[7]

Quantitative real-time RT-PCR

Total RNA was extracted by TRI-Reagent (Invitrogen) and treated with DNaseI (Invitrogen). cDNA was synthesized using the RevaTra Ace Kit (TOYOBO) with Oligo-dT primer. Quantitative real-time RT-PCR analysis was performed with SYBR Green Realtime PCR Master Mix (TOYOBO). TARSH and GAPDH internal control primer were used as described previously.[3] TARSH expression levels were normalized GAPDH expression.

FACS analysis

To detect DNA contents, 5×10^5 NIH-3T3 cells were seeded into 10 cm dishes for overnight. Cells were then harvested and fixed with 70% ethanol/PBS. Followed by staining with propidium iodide (PI) (BD Pharmingen), cells were analyzed by FACS. To detect apoptotic population in sh-TARSH or sh-Scramble retrovirus infected, wild-type and *p53*-deficient MEFs were stained with FITC-conjugated anti-Annexin V antibody and propidium iodide. The apoptotic cells were analyzed as percentages by FACS calibur.

Western blotting

Preparation of whole cell lysates and Western blotting were performed as described.[1] The following primary antibodies were used: anti-cleaved caspase-3 (Asp175, Cell Signaling Technology), anti-β-tubulin (D66, Sigma). For secondary antibodies, HRP-conjugated anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories) were used. Proteins were visualized by enhanced chemiluminescence (ECL) (GE Healthcare), captured with a CCD camera, and measured by densitometric analysis (Gel-Pro analyzer, Media Cybernetics).

RESULTS

TARSH gene expression reduced in cell cycle progression

To investigate the molecular function of TARSH in cell cycle progression, we synchronized NIH-3T3 cells in G1 phase by contact inhibition. After serum starvation for 16 h, cells were released and TARSH mRNA expression was measured every 6 h by quantitative real-time RT-PCR. The TARSH mRNA level at each time point was normalized with GAPDH internal control, and the relative abundance of TARSH mRNA expression is shown in Fig. 1A. As a result, we found that the TARSH gene was reduced during cell cycle reentry. We next designed shRNAs recognizing the sequence at the 3' UTR of the mTARSH gene (sh-TARSH). Endogenous TARSH mRNA expression was detected by quantitative real-time RT-PCR (Fig. 1B). TARSH mRNA expression in shTARSH-infected NIH-3T3 cells was decreased to less than 2% compared to sh-Scramble infected cells. Then, we synchronized sh-TARSH- or sh-Scramble-infected NIH-3T3 cells to detect the function of TARSH in cell cycle reentry (Fig. 1C). We did not find any significant difference between sh-Scramble- and sh-TARSH-infected cells. These results suggest that mTARSH is not involved in cell cycle progression. On the other hand, we found

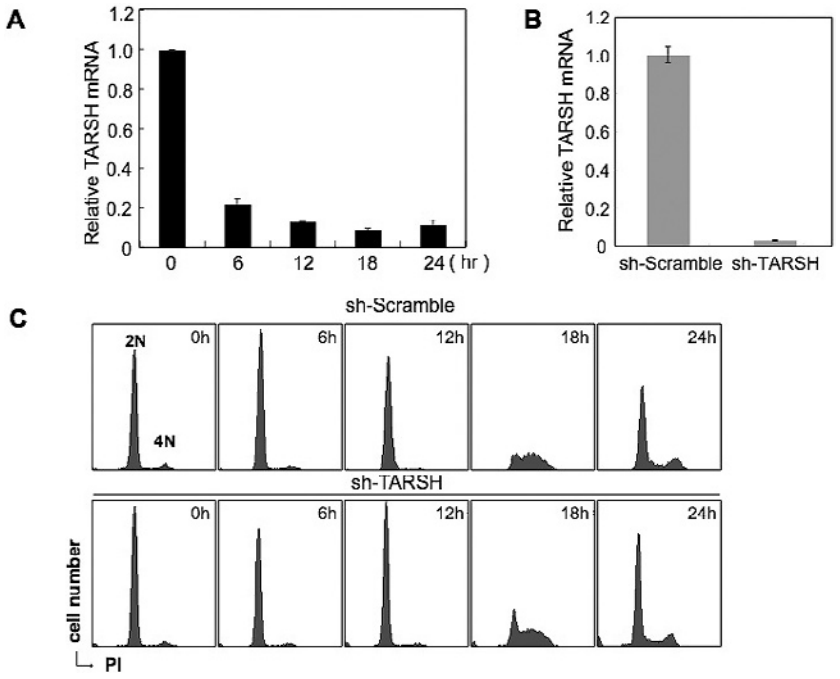
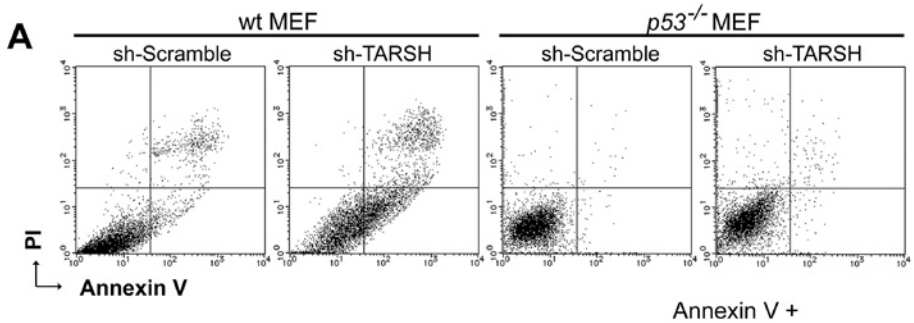


Fig. 1 TARSH was down regulated in cell cycle progression.
(A) NIH-3T3 cells were synchronized in quiescence phase by contact inhibition. After serum starvation for 16 hours, cells were released to reentry into the cell cycle. Endogenous TARSH mRNA expression was measured every 6 hours after the point of release by quantitative real-time PCR. Data were normalized with GAPDH internal control (n=3). (B) NIH-3T3 cells were infected with retrovirus carrying sh-TARSH short-hairpin RNA. After puromycin selection for 2 days, endogenous TARSH mRNA expression was measured by quantitative real-time PCR. Data were normalized with GAPDH internal control (n=3). (C) Cells were harvested every 6 hours after release and DNA content was stained with PI and analyzed by FACS.

that binucleated cells were drastically increased in sh-TARSH infected cells, although they did not accumulate 4N or 8N DNA content, implying that binucleated cells were eliminated rapidly (unpublished data).

Suppression of TARSH enhanced p53-dependent apoptosis

It is well known that p53 tumor suppressor-mediated apoptosis pathway via caspase-3 can be induced by DNA damage and mitotic catastrophe.[12-14] To determine whether or not the p53-mediated apoptosis pathway was activated in TARSH-infected cells, we next measured the apoptotic population by Annexin V and PI staining. Because primary MEFs are well known to analyze p53-dependent biological function, we used primary MEFs in this experiment. As shown in Fig. 2A and 2B, we found a significant increase in both pre-apoptosis (Annexin V^+ PI^-) and apoptosis (Annexin V^+ PI^+) populations compared with the sh-Scramble control. In addition, we noted that an elevated Annexin V-positive apoptosis population, via the suppression of mTARSH, was not obvious in the *p53*-deleted MEFs. To gain insight into the molecular events underlying the apoptosis caused by mTARSH loss, we examined the cleaved caspase-3 protein levels of sh-TARSH-infected MEFs by Western blotting. As shown in Fig. 2C, we detected a distinct



B

		Living Cells	Annexin V + PI -	Annexin V + PI +
wt MEF	sh-Scramble	85.8%	5.2%	8.3%
	sh-TARSH	53.6%	29.2%	16.7%
p53$^{-/-}$ MEF	sh-Scramble	94.2%	1.0%	0.4%
	sh-TARSH	91.5%	1.4%	1.6%

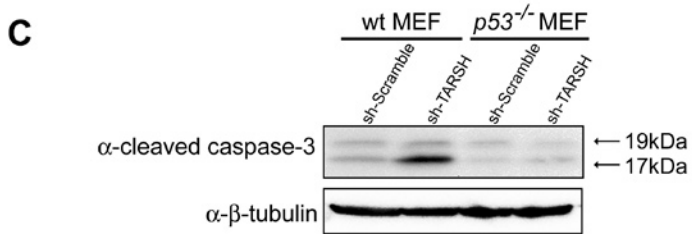


Fig. 2 TARSH reduction activated p53-dependent apoptosis in MEFs.
(A, B) Wild-type or *p53*$^{-/-}$ MEFs infected with indicated retrovirus were stained with anti-Annexin V and PI and analyzed by FACS. The representative data of three independent experiments are shown. (C) Western blotting of cleaved caspase-3 protein and β-tubulin levels in sh-TARSH- or sh-Scramble-infected MEFs.

induction of cleaved caspase-3 protein as a result of TARSH loss in wild-type MEFs (Fig. 2C, left 2 lanes). In *p53*-deficient MEFs, we did not find any consistent activation of cleaved caspase-3 protein in the absence of mTARSH (Fig. 2C, right 2 lanes). Taken together with the finding that p53 induces the apoptosis pathway via caspase-3, these results strongly suggest that TARSH negatively regulates cell death through the p53-dependent caspase-3 apoptosis pathway.

DISCUSSION

Human TARSH was initially identified as a novel NESH-binding protein is involved in tumor cell mobility and migration.[15] It is possible that TARSH plays a role in tumor cell development via interaction with NESH. In fact, TARSH mRNA expression was dramatically reduced in human lung carcinoma cell lines and clinical samples. Although the molecular mechanism underlying the tumor development is not clear, its development is closely associated with the cell cycle. For example, tumor suppressor proteins such as p53, RB or $p19^{ARF}$ have a critical function in the cell cycle.[6-8] Here, we first detected mTARSH expression in cell cycle progression by quantitative real-time RT-PCR (Fig. 1A). TARSH mRNA level was drastically reduced in cell cycle reentry NIH-3T3 cells. However, mTARSH expression was accumulated in G1 phase. These results imply that mTARSH may be tightly regulated in cell cycle progression. To investigate the detailed biological function of TARSH in cell cycle progression, we silenced endogenous TARSH mRNA expression using a retrovirus-derived shRNA system (Fig. 1B). In TARSH-absent NIH-3T3 cells, we did not find any evidence to suggest that TARSH is involved in the cell cycle (Fig. 1C). However, it is still possible that the reduction of mTARSH gene expression is regulated by cell cycle progression. Interestingly, we found that binucleated cells were increased in sh-TARSH retrovirus-infected cells (unpublished data). This result indicated that mTARSH might be involved in mitosis or cytokinesis. However, we could not find any significant change in cell cycle profiles between sh-Scramble- and sh-TARSH-infected MEFs.

Apoptosis is a critical pathway to eliminate aneuploid or DNA-damaged cells by p53 activity. We measured the apoptotic population of sh-Scramble-and sh-TARSH-infected MEFs (Fig. 2), and found that loss of TARSH induces the apoptosis pathway in a p53-dependent manner presumably through caspase-3 activity. These data strongly suggest that TARSH plays a role in tumor development via the p53-dependent apoptosis pathway. In Fig. 3, we propose a putative function of TARSH in tumor development. In the p53-intact condition, a p53-dependent apoptosis

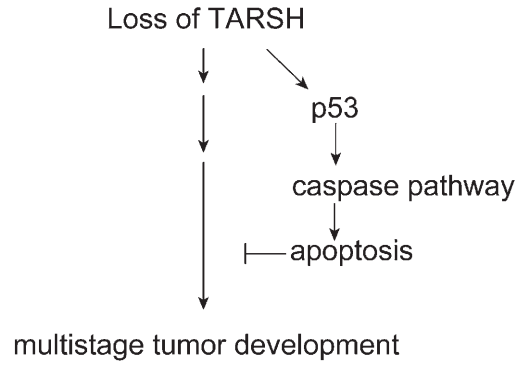


Fig. 3 A putative molecular function of TARSH in multistage tumor development.

pathway suppresses tumor cell development. In contrast, tumor cell accumulation may progress in the p53-absent condition. Although how TARSH and p53 cooperate in tumorigenesis must be clarified in further detail, our study reveals that TARSH may be one initial trigger of multistage tumor cell development.

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