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TIS21$^{/BTG2/PC3}$ accelerates the repair of DNA double strand breaks by enhancing Mre11 methylation and blocking damage signal transfer to the Chk2^{T68}–p53^{S20} pathway

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ABSTRACT

DNA double strand breaks (DSBs) occur more frequently in TIS21$^{-/-}$ mouse embryo fibroblasts than that in wild type MEFs (wt-MEFs). Therefore, the role TIS21 plays in the DNA damage response was investigated. Adenoviral transduction of Huh7 tumor cells with the TIS21 gene accelerated the repair of DSBs induced by etoposide treatment as evaluated by clearance of γH2AX foci and the Comet assay. TIS21 increased methylation of Mre11 and protein arginine methyltransferase 1 (PRMT1) activity, leading to Mre11 activation *in vitro* and *in vivo*, as determined by immunoprecipitation and radiolabeling analyses. When downstream DNA damage response mediators were evaluated in various human cancer cells lines, TIS21 was found to strongly inhibit Chk2^{T68} and p53^{S20} phosphorylation by p-ATM^{S1981} but not p53^{S15}. The loss of Chk2 activation after etoposide treatment reduced apoptosis in the cells by downregulating the expression of E2F1 and Bax. These data suggest that TIS21 regulates DSB repair and apoptosis. Expression of TIS21 promoted the repair of DSBs and reduced apoptosis by blocking the damage signal from p-ATM^{S1981} to Chk2^{T68}–p53^{S20} *via* the activation of Mre11 and PRMT1.

1. Introduction

TIS21$^{/BTG2/PC3}$ (12-*O*-tetradecanoyl phorbol-13-acetate-inducible sequence 21/B-cell translocation gene 2/pheochromocytoma) is a member of the antiproliferative gene family (APRO), which was originally isolated from SW3T3 mouse fibroblasts [1], rat PC3 cells [2], and a cDNA library of human lymphoblastoid cells [3]. TIS21 has been suggested to be a potential tumor suppressor in thymic carcinoma development in transgenic mice expressing the SV40 large T antigen [4], and an anti-carcinogenic effect has been reported in the prostate [5], in clear cell renal cell carcinoma [6] and in hepatomas grown in TIS21 knockout mice [7]. The expression of TIS21 is induced by various cellular stresses, such as ultraviolet radiation (UV), ionic radiation (IR), reactive oxygen species (ROS) and other DNA damaging agents. Despite the transient and significant induction of TIS21$^{/BTG2/PC3}$ gene expression after DNA damage, the role of TIS21$^{/BTG2/PC3}$ in the DNA repair process still remains to be elucidated.

Expression of TIS21 has been found to be p53-dependent [3] or -independent [8], and TIS21 is rapidly degraded in a ubiquitin-dependent manner by the proteasome [9]. However, forced expression of TIS21 induces growth arrest at the G1/S phase in cells by inhibiting the expression of cyclin D1 in the presence of function pRB [10] or the expression of cyclin E in the absence of functional pRB [11]. Furthermore, the induction of TIS21 strongly induces growth arrest at the G2/M phase in U937 and Huh7 hepatoma cells [7,8]. Therefore, TIS21 is considered to be a pan-cell cycle inhibitor [12].

To date, a few proteins have been identified as binding partners of TIS21 in response to DNA damage. CAF1 can interact with TIS21, suppresses RAD52 mutation by binding to double stranded breaks of DNA (DSBs) and regulates end-to-end assembly [13,14]. TIS21 also interacts with protein-arginine methyltransferase 1 (PRMT1) and regulates its activity [15]. Methylation of arginine residues frequently occurs at a glycine and arginine-rich region (-GRG-) of target proteins [16] by protein methylase I, which was initially isolated from calf thymus by Paik and Kim [17]. Recently, it has been reported that PRMT1 methylates nuclear proteins, including DNA

Abbreviations: TIS21, TPA inducible sequence 21; DSBs, DNA double strand breaks; TIS21$^{-/-}$MEF, TIS21 knockout mouse embryo fibroblast; wt-MEF, wild type mouse embryo fibroblast; PRMT1, protein arginine methyltransferase 1; Mre11, meiotic recombination 11; Chk2, check point kinase 2; ATM, ataxia telangiectasia mutant; MRN complex, Mre11; Rad50, Nbs1 complex.

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damage sensors such as Mre11 and 53BP1 [18,19]. PRMT1-deficient cells are hypersensitive to DNA damaging agents and exhibit a defect in homologous recombination in response to DNA damage [20]. These results imply that PRMT1 is required for genomic integrity and cell proliferation [21]. Therefore, we hypothesized that TIS21 might be involved in DNA repair signaling *via* the regulation of PRMT1.

The ataxia telangiectasia mutated (ATM) and MRN (Mre11, Rad50, Nbs1) complexes have been reported to be activated in response to DNA damage [22–24]. DSBs induce ATM phosphorylation at the Ser^{1981} residue and recruit MRN complexes to the damage site [23,25,26]. Activated ATM phosphorylates Ser^{15} of p53 and Thr^{68} of Chk2, and p-$Chk2^{T68}$ can directly phosphorylate p53 on Ser^{20}, thereby increasing p53 stabilization by preventing MDM2-mediated ubiquitination after DNA damage [27,28]. Therefore, the p-ATM^{S1981}–$Chk2^{T68}$–$p53^{S20}$ pathway can be activated in response to DNA damage [29–31]. If damaged DNA remains unrepaired, it would threaten not only the integrity of the genome but also the survival of the organism. During this complex signal transduction process, however, a potential role of TIS21 in damage repair has not been fully clarified.

Based on our recent observation of a higher frequency of DSBs in $TIS21^{-/-}$ mouse embryo fibroblasts (MEFs) than in wild type MEFs, in the present study, we investigated the role of TIS21 in damage signal pathways in response to etoposide treatment. Transduction of TIS21 accelerated the repair of etoposide-induced DNA damage, as opposed to inhibiting apoptosis by downregulating $Chk2^{T68}$ phosphorylation. Furthermore, TIS21 was found to regulate the damage repair process by increasing *in vivo* and *in vitro* Mre11 methylation by increasing PRMT1 activity.

2. Materials and methods

2.1. Adenovirus preparation

cDNA encoding TIS21 was inserted into replication-defective E1- and E3-adenoviral vectors containing a CMV enhancer and the chicken β-actin promoter. Adenovirus of TIS21 [Ad-TIS21(HA)] was then amplified in 293 human kidney epithelial cells [7], and the viral particles were purified by filtration (0.45 μm). Similarly, an adenovirus of bacterial β-galactosidase (Ad-LacZ) was also prepared for control experiments.

2.2. Preparation of cell lines

Huh7, Chang liver cells, HeLa, U2OS, A549 and H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Bethesda, MD) with 10% fetal bovine serum (FBS, Gibco BRL). Wild type MEFs and $TIS21^{-/-}$ MEFs were prepared in our laboratory with 13.5 day old embryos isolated from wild type and $TIS21^{-/-}$ mice [7] and were then cultured in DMEM with 10% FBS.

2.3. RT-PCR

Total cellular RNAs were isolated from cells of human cancer lines (Huh7, Chang, and A549). First strand cDNA was synthesized using oligo-dT primers from 1 μg of total cellular RNA by reverse transcription reaction in 10 μl of reaction volume. The primers used were as follows: BTG2, sense 5′-cctgggcagagagtgaaaag-3′ and antisense 5′-cctgggcagagagtgaaaag-3′; human glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense 5′-ggtgctgagtatgtcgtgga-3′ and antisense 5′-gccatgccagtgagcttccc-3′. Amplification was carried out as follows: denaturation at 94 °C for 30 s, annealing at 56°C for 40 s, and extension at 72°C for 40 s. The numbers of total reaction cycle were 32 and 34 for Huh7, Chang liver cells and A549 cells, respectively. PCR products were resolved on 2% agarose gels.

2.4. Immunocytochemistry

TIS21 wild type MEFs, $TIS21^{-/-}$ MEFs and adenovirus-infected Huh7 cells were cultured on a cover glass, fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C for 20 min, and then washed 3 times with PBS containing 0.1% Tween 20 (PBST). The cells were incubated with 1.5% horse serum in PBST for 1 h at room temperature, and anti-γH2AX (Millipore, Billerica, MA), anti-p-ATM^{S1981} (Epitomics, Burlingame, CA) and anti-Mre11 (Novus, Littleton, CO) antibodies were then applied overnight at 4 °C. The secondary antibody (1:500) was applied for 1 h, and the cells were observed using a fluorescence microscope (Carl Zeiss, Axio Imager M1, Germany).

2.5. FACS analysis

For analysis of apoptotic cells, we used FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, Franklin Lakes, NJ). Briefly, Huh7 cells were infected by either Ad-LacZ or Ad-TIS21 (100 moi) for 2 days and treated with 50 μm etoposide for 24 h. Huh7 cells were washed twice with cold PBS and then resuspended in 1 × binding buffer at a concentration of 1×10^5 cells/100 μl. The cells were added to 5 μl each of FITC annexin V and propidium iodide, and then incubated for 15 min at room temperature in the dark. The cells were added to 300 μl of 1 × binding buffer in each tube and analyzed by flow cytometry (BD FACScan II, BD Biosciences, San Jose, CA) within 1 h.

2.6. Comet assay

Huh7 cells were infected with Ad-LacZ or Ad-TIS21 at 100 moi for 2 days and treated with 50 μm etoposide for 1 h. The cells were then harvested and suspended at 1×10^5 cells/ml. Low melting point agarose (LMA) was prepared and mixed with counted cells at 37 °C. A total of 1×10^3 cells were placed on the Comet slide (R&D systems, Minneapolis, MN). The slides were placed flat on the gel at 4 °C in the dark for 10 min and were then immersed in prechilled lysis solution (R&D systems) for 30 min. After discarding the lysis solution, the slides were immersed in fresh alkaline solution (0.3 N NaOH, 10 mM EDTA, pH 10) for 40 min. For electrophoresis, the slides were washed twice with Tris-boric acid electrophoresis buffer (TBE), placed onto a gel tray, and aligned equidistant from the electrodes. The slides were immersed in TBE buffer, and a current of 30 V was applied for 10 min. After electrophoresis, the slides were washed twice with distilled water, immersed in 70% ethanol for 5 min, and then left to dry. Fifty μl of diluted SYBR green I was added to the gels. Every slide was confirmed by fluorescence microscopy.

2.7. Immunoblot analysis

Cell lysates were resolved on 6–13% SDS-polyacrylamide gel electrophoresis, and the gels were then transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) before incubation with various antibodies at room temperature for 1 h or at 4 °C overnight. The antibody against p-ATM^{S1981} was obtained from Epitomics, p-$Chk2^{T68}$ antibody for human form was from Cell Signaling (Danvers, MA) and mouse and rat form from Abcam (Cambridge, UK), p-$Chk2^{S19}$, p-$Chk2^{S33/35}$, p-$Chk1^{S345}$, p-$p53^{S15}$, p-$p53^{S20}$ and Bax antibodies from Cell Signaling, p53, Noxa, $p21^{waf1}$, hemagglutinin (HA) and E2F1 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), Chk2 and γH2AX antibodies from