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Telomeric plasmid induces human cancer cell dysfunction depending on ATM activity

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Telomeres are essential for chromosome stability and the regulation of the replicative life-span of somatic cells. Many studies showed that exogenous telomeric repeats could activate p53 protein. It is not known how cell dysfunction is induced by telomeric plasmids. A covalent closed circular (ccc) double-stranded plasmid containing $(TTAGGG)_{96}$ repeats (pRST5) was transiently transfected into the human gastric cancer MGC-803 cells. We first confirmed that the cell viabilities decreased by 27%, cell senescence increased by 62% and G2/M cycle arrested in pRST5 plasmid transfected cells. Compared to control groups, cells transfected with telomeric plasmids showed an ATM-dependent increasing of p53, TRF1, and TRF2 expression. Furthermore, telomere dysfunction-induced foci (TIF) were observed. In conclusion, telomeric plasmids can elicit endogenous telomere dysfunction and induce cell senescence by activating ATM-p53 pathway.

KEY WORDS — senescence; telomere; p53; TRF1; TRF2; ATM

INTRODUCTION

Telomeres, the special DNA–protein complexes at the ends of eukaryotic chromosomes, have important functions in maintaining chromosome stability and regulating the replicative life-span of somatic cells.[1] Telomeres protect chromosome ends from recombination, fusion, and being recognized as damaged DNA. The DNA sequence of telomeres typically consists of tandem GT-rich repeats (TTAGGG in human). The 3′ end of each telomere is a single-stranded G-rich overhang which is important to stabilizing the t-loop structure at the chromosome end.[2–4] The length and structure of telomeres are controlled by a variety of proteins.[5,6] Shelterin is a protein core complex which consists of six subunits: TRF1 and TRF2 directly bind duplex TTAGGG repeats; POT1 recognizes single-stranded telomeric DNA; TIN2, TPP1, and Rap1 mediate the interaction of the above three.[7]

TRF1 is the first identified mammalian telomere binding protein that associates with double-stranded TTAGGG repeats as a homodimer.[8] TRF1 negatively controls telomere length by *cis*-inhibiting telomerase activity. Overexpression of wild type TRF1 leads to telomere shortening, whereas inhibition of TRF1 with a dominant-negative mutant allele leads to telomere lengthening.[9] TRF2 is a key component of human telomere protein complex. TRF2 also binds to duplex telomeric repeats as a homodimer.[10] Like TRF1, overexpression of intact TRF2 results in progressive telomere shortening too.[11] *In vitro*, TRF2 facilitates the formation of the t-loop structure and might enhance the invasion of the G-overhang.[3,12] TRF2 deletion leads to telomere dysfunction and TIF formation. TIF is formed by the association of damaged telomeres with DNA damage response factors, such as ATM, 53BP1, γ-H2AX, Rif1, and the Mre11 complex.[13,14]

Many studies showed that telomeric repeats could activate p53 protein. Feeding cells with 3′ overhang oligonucleotides sequence-specifically resulted in p53 activation and cell cycle arrest without causing cellular telomere disruption.[15] It has been reported that MCF-7 cells transfected with ccc plasmids containing $(TTAGGG)_{40}$ activated p53, but control plasmids containing non-TR sequences, such as T_3AG_3, T_2AG_2C, and T_2AG_5 repeats, empty vector plasmids pBluescript and pSX-neo did not cause p53 protein stabilization, indicating that the cell effects are telomeric repeat $(TTAGGG)_n$ specific.[16] However, it is not known how cell dysfunction is induced by telomeric plasmids.

In this study, a plasmid containing $(TTAGGG)_{96}$ repeats was transiently transfected into human gastric cancer MGC-803 cells. We confirmed the effects of telomeric plasmids on

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cell growth inhibition, and studied the telomeric plasmid-mediated DNA damage response and variation of protein expression.

METHODS

Cell culture and transfection

Human gastric cancer MGC-803 cells were cultured in RPMI 1640 medium (Gibco-BRL, Grand Island, New York, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 $\mu g\,mL^{-1}$ streptomycin, and 100 $U\,mL^{-1}$ penicillin, and incubated at 37°C in a humidified atmosphere containing 5% CO_2. In all experiments, same amounts of cells were seeded in each well. 20 μM wortmannin (final concentration) was added to the cell culture as indicated, 4 h before cell transfection, and remained till cell analysis. Experimental growing cells were transfected with exactly equal amounts (final concentration was 1.0 $\mu g\,mL^{-1}$, except indicated specially) of plasmid pRST5 (a kind gift from J. D. Griffith) or empty vector pBlueScript II SK+ using Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol.

MTT and β-galactosidase staining assay

Cell viability was estimated by MTT assay. Briefly, about 5000 cells per well were seeded in 96-well plates and cultured for 24 h. 0.2 μg of pBlueScript and pRST5 plasmids mixture at various ratios (the amounts of total DNA were kept constant in all transfections) was transiently transfected into the human gastric cancer MGC-803 cells. Twenty-four hours later, the cells were incubated with MTT reagent for 4 h. 200 μL dimethyl sulfoxide (DMSO) was added and incubated at 37°C for 30 min. The absorbance at 570 nm was measured. Cell growth is expressed as: $B/A \times 100\%$, where A is the absorbance value from control groups and B is the absorbance value from experimental groups. For cell senescence associated β-galactosidase (SA-β-Gal) staining, cells were washed in PBS, fixed in 2% formaldehyde/0.2% glutaraldehyde solution in PBS for 5 min, washed in PBS and incubated with fresh SA-β-Gal stain solution (1 $mg\,mL^{-1}$ X-gal, 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 150 mM NaCl, and 2 mM $MgCl_2$) at 37°C for 12 h. Stain solution was removed and cells were covered with PBS and visualized with light microscope. Total 200×3 cells were counted independently and senescence was showed as $S/200 \times 100\%$ (S: counts of stained cells).

Flow cytometry

For flow cytometry (FCM) analysis, about 1×10^6 cells were collected, washed twice with cold PBS, and fixed by 70% ethanol overnight. Then the cells were incubated in 1 mL PBS containing 50 $\mu g\,mL^{-1}$ propidium iodide and 250 $\mu g\,mL^{-1}$ RNase at 37°C for 30 min. The cell cycle distribution was determined by the fluorescence of individual cells measured by FCM (FACSS Vantage, BD, Inc., USA). Percentage of cells in the sub-G1 phase was regarded as apoptotic percentage.

RT-PCR and ATM siRNA

Total RNA was extracted with acid guanidinium thiocyanate–phenol–chloroform. The first-strand cDNA was synthesized using M-MLVreverse transcriptase. The primer sequences used in PCR were as follows: 5′-TGG CTG CCG GCT GGA TGC TG-3′, 5′-TTATTA AGG TCT TGT TGC TG-3′ for TRF1, 5′-CGC TGG GTA CTC AGG TTC TA-3′, 5′-CTG GTG CTG GCTGTT TAT CT-3′ for TRF2, 5′-GTG GGG CGC CCC AGG CACCA-3′, 5′-GTC CTTAAT GTC ACG CAC GAT TTC-3′ for β-actin. PCR products (10 μL) were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. The DNA fragments were visualized using ultraviolet light and photographed. The ATM siRNA and non-specific control (NSC) siRNA were both chemically synthesized by GenePharma (Shanghai, China). The siRNA sequence targeting ATM is 5′-AACATACTACTCAAAGACATT-3′.[17] Cells were transfected with same amounts (final concentration 100 nM) of siRNAs by Oligofectamine (Invitrogen) according to the manufacturer's protocol.

Western blotting

The cell pellets were lysed in radioimmuno-precipitation assay buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM dithiothreitol, plus a protease inhibitor cocktail consisting of 1 $\mu g\,mL^{-1}$ aprotinin, 1 $\mu g\,mL^{-1}$ leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Equal amounts of supernatant (about 20 μg of total protein per lane) were electrophoresed through 10% SDS–polyacrylamide gels and blotted onto nitrocellulose membranes. The membranes were blocked in 5% nonfat milk and 0.1% Tween-20 in PBS for 1 h at room temperature, then incubated with primary antibodies: p53, TRF1, TRF2, ATM, Actin (Santa Cruz Biotechnology, Santa Cruz, California, USA), or phosphor-specific (Ser^{15}) p53 (Oncogene) diluted in PBS with 3% milk and 0.1% Tween-20. Then blots were washed three times and incubated with horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h, washed, and detected using the enhanced chemiluminescence kit (Amersham Life Science, Amersham, UK).

Immunofluorescence

Cells were grown on polylysine-coated coverslips, rinsed in phosphate-buffered saline, fixed in cold methanol for 20 min, permeabilized for 10 min in 0.5% Triton X-100 on ice and blocked in 5% BSA for 30 min at room temperature. The coverslips were incubated with mouse monoclonal anti-TRF2 (BD Biosciences Pharmingen) and rabbit monoclonal anti-phospho-Histone-H2AX (Cell signaling) primary antibodies for 1 h at 25°C. The coverslips

were washed and incubated with fluorescein conjugated goat anti-mouse (1:1000) and rhodamine conjugated goat anti-rabbit (1:1000) secondary antibodies. DNA was stained with 0.1 $\mu g\,mL^{-1}$ DAPI. Fluorescence images were captured using confocal microscope Olympus FV500.

Statistical analysis

The data are expressed as means + standard deviations, and statistical significance was assessed by the Student's *t*-test. All experiments were performed independently at least three times.

RESULTS

Telomeric plasmid transfection induced cell senescence and S-G2/M cycle arrest

Cells were transfected with telomeric plasmid pRST5. Twenty-four hours later, the cell growth was obviously inhibited (Figure 1A). 0.5 $\mu g\,mL^{-1}$ pRST5 plasmid transfection decreased cell viability by 27% and increased cell senescence by 62% ($p < 0.05$). However, empty vector pBlueScript caused no obvious cell growth inhibition.

Cell cycle analysis (Figure 1B) showed that $10.5 \pm 2.4\%$ of the cells were in the sub-G1 phase 24 h later after plasmid pRST5 trasfection, while $1.4 \pm 0.9\%$ of untreated cells and $1.3 \pm 0.3\%$ of plasmid pBlueScript transfected cells were in the sub-G1 phase. Cells transfected with pRST5 plasmids also displayed increase in S phase (untreated cells: $27.5 \pm 1.2\%$; empty vector transfected cells: $24.4 \pm 0.9\%$; plasmids pRST5 transfected cells: $36.8 \pm 1.5\%$) and G2/M phase (untreated cells: $17.5 \pm 1.8\%$; pBlueScript transfected cells: $17.5 \pm 1.3\%$; pRST5 transfected cells: $27.9 \pm 1.1\%$). It revealed that the transfection of plasmids pRST5 resulted in cell apoptosis and S and G2/M cycle arrest.

Transfection with the telomeric plasmid increased p53, TRF1, and TRF2 expression and was dependent on ATM

Compared to control groups, cells transfected with telomeric plasmids showed an obvious increase of p53 protein level in a dose dependent manner. And the phosphorylation at the 15th serine residue of p53 protein also increased (Figure 2A). In addition, cells transfected with plasmid pRST5 displayed increase of TRF1 and TRF2 mRNA and protein level (Figure 2B). Wortmannin is a specific inhibitor of protein kinases such as ATM and PI3Ks. At a low concentration (<100 μM), it effects on ATM rather than PI3Ks. As shown in Figure 2A, 20 μM wortmannin effectively inhibited the phosphorylation of ATM and the increase of p53, TRF1, and TRF2, suggesting that the upregulation of these proteins might be correlated with ATM activity.

To further confirm this, we knocked down the ATM expression with RNA interference 24 h before the telomeric plasmid pRST5 or control plasmid pBlueScript transfection. As shown in Figure 2B, the ATM siRNA effectively reduced the ATM protein level, and inhibited the increase of p53, TRF1, and TRF2 protein level induced by pRST5 transfection, while mock siRNA displayed no effects. At the same time, abrogating the ATM protein by wortmannin or RNA interference rescued cell viability and reduced the β-gal positive cells after the transfection (Figure 1A). The

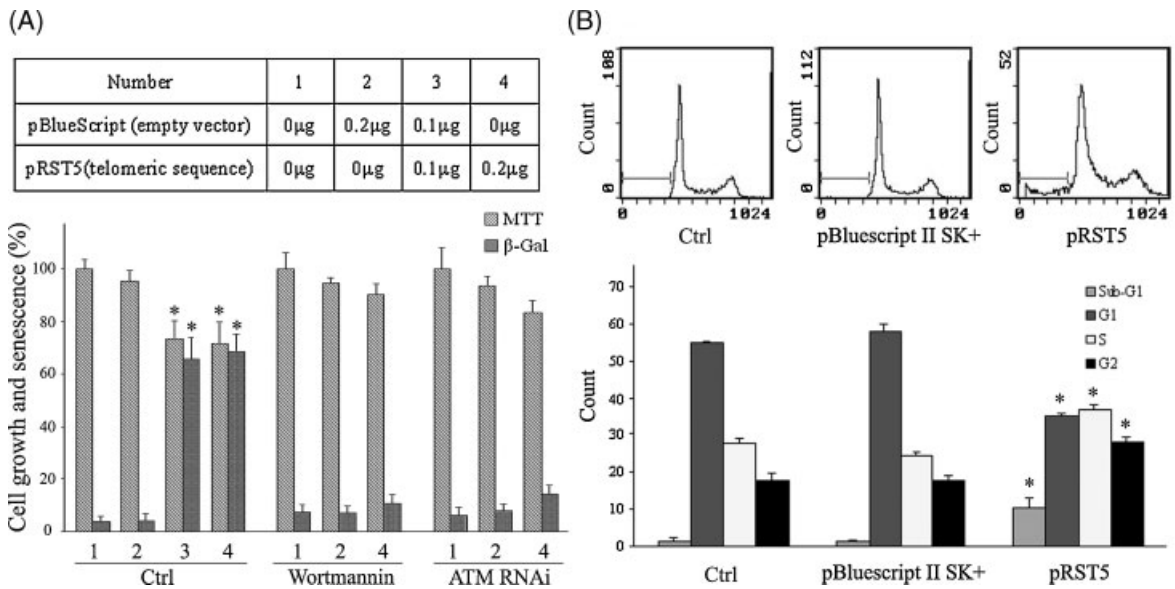

Figure 1. Cell senescence and cycle arrest induced by telomeric plasmids. (A) Treated MGC-803 cells were transfected with equal amounts of plasmids as indicated in the table. Twenty-four hours later, cell growth inhibition was estimated by MTT assay, and cell senescence was determined by SA-β-Gal staining. (B) FCM analysis of cells, 24 h later after transfection with the indicated plasmids. DNA was stained with propidium iodide. Data are presented as mean values + SD from three independent experiments. A significant difference ($p < 0.05$) to Ctrl group was denoted by an asterisk

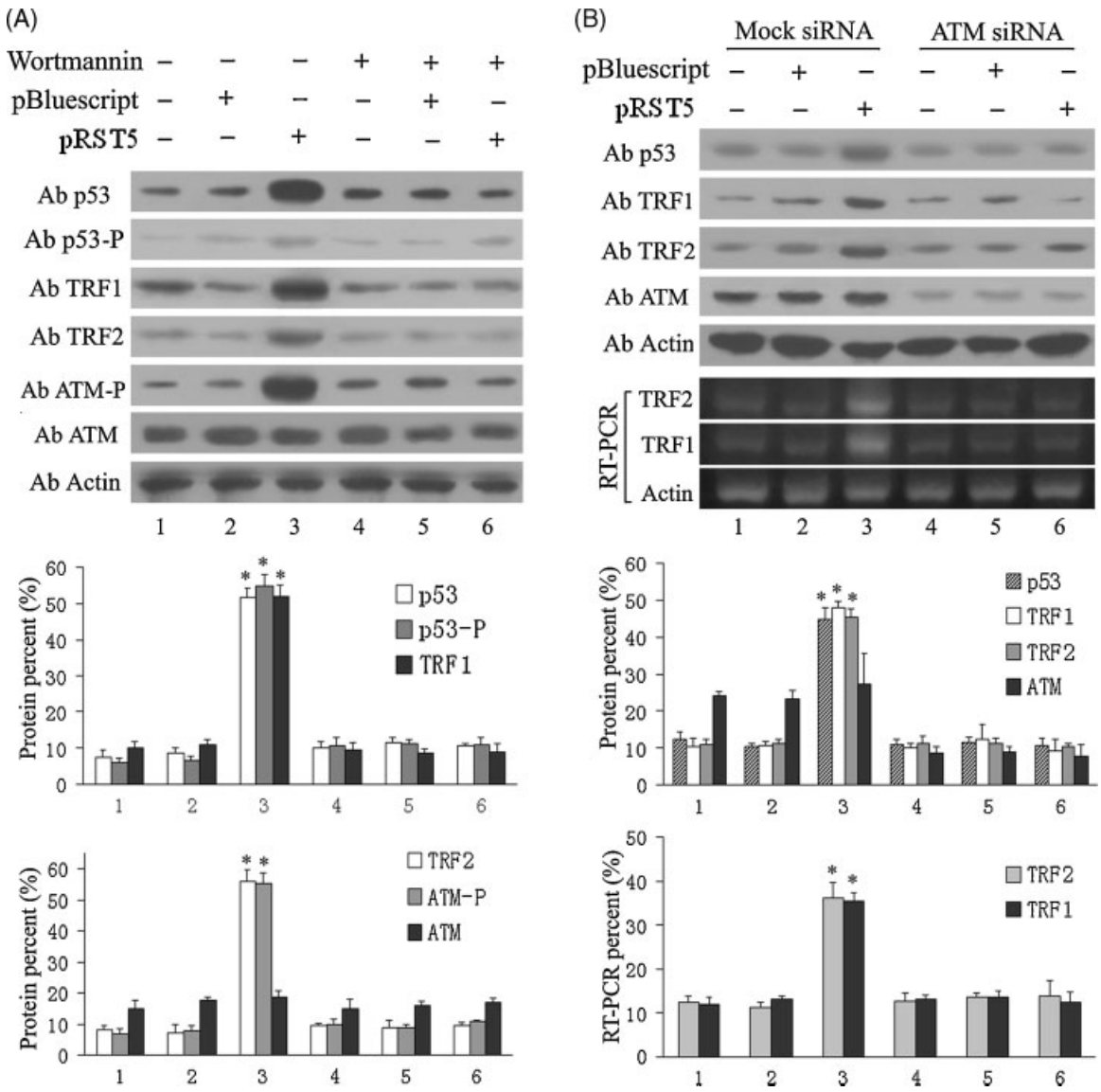

Figure 2. p53, TRF1, and TRF2 upregulation depending on ATM activity. (A) MGC-803 cells were pretreated with or without 20 μM wortmannin 4 h before same amounts of plasmids pRST5 or pBlueScript were transfected. Twenty-four hours later, protein levels were estimated by Western Blotting. (B) ATM was knocked down with siRNA interference 24 h before same amounts of plasmids pRST5 or pBlueScript transfection. Protein levels were estimated by Western Blotting 24 h later and TRF1/2 mRNA levels were determined by RT-PCR. One typical experiment out of three is shown. Amounts of proteins and mRNA were determined by densitometry of the blots and normalized to Actins. Mean values from three experiments and standard deviations were shown. Significant differences ($p < 0.01$) to mocking treated samples were indicated with asterisks

above results suggested that the protein expression increase induced by telomeric plasmid transfection was dependent on ATM activity.

Telomeric plasmid induced endogenous telomere damages

Figure 3 showed that telomere dysfunction-induced foci (TIF)were formed in cells transfected with telomeric plasmid pRST5. The phosphorylated γH2AX foci that colocalized with TRF2 increased by 41% ($p < 0.01$) in cells transfected with pRST5 plasmid. However, cells transfected with empty vector pBluescript showed almost no detectable phosphorylated γH2AX foci, indicating that the telomeric plasmid transfection induced cellular telomere damage.

DISCUSSION

Previous studies showed that telomeric plasmids can induce p53 protein stabilization in MCF-7 and HTC116 cells and cause growth suppression in a p53-dependent manner.[16] However, the mechanism was not clear. To confirm the effect

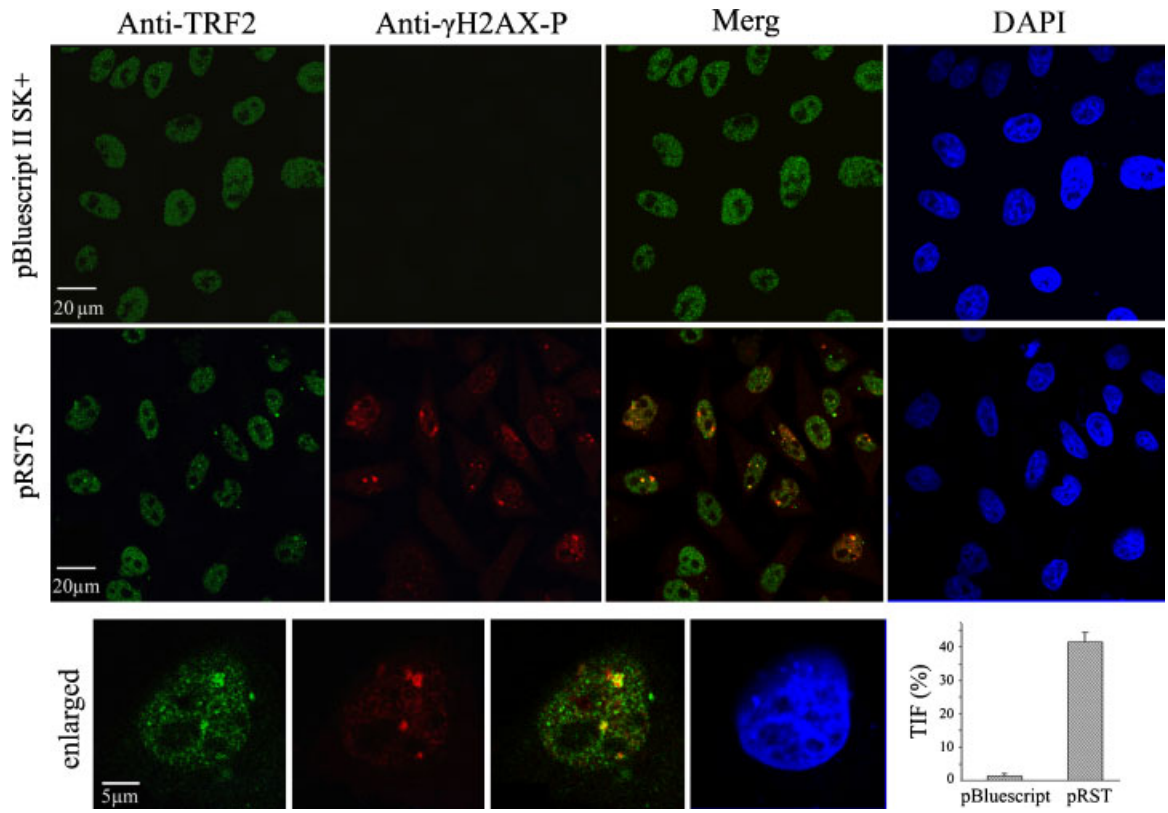

Figure 3. Telomeric plasmid induced TIF. MGC-803 cells were transfected with indicated plasmids. Twenty-four hours later, cells were stained with antibodies to TRF2 and phosphorylated γH2AX. DNA was stained with 0.1 μg mL^{-1} DAPI. Cells containing more than one TIF were counted in three independent experiments(total 200 × 3 cells)

of telomeric plasmid on cell senescence, we transiently transfected human gastric cancer MGC-803 cells with a mixture of pBlueScript and pRST5 plasmids at various ratios (total plasmid amounts were constant). pRST5 plasmid led to a decrease of cell viabilities by 27% and an increase of cell senescence by 62%, while empty vector pBlueScript plasmid caused no obvious effects. It was of interest to ascertain by what means the telomeric plasmid resulted in these cell effects.

As is well known, p53 is one of the key molecules in apoptosis and senescence pathways. Previous studies showed that p53 can be activated in a sequence-independent manner by linear ss/dsDNA, circular DNA with a large gap or single-stranded circular phagemid.[18] Telomeric oligonucleotides could induce a sequence-specific cell senescence via p53 activation.[15,19] Telomeric plasmid can also induce p53 protein stabilization in MCF-7, HTC116 cells and cause growth suppression depending on wild-type p53.[16] The MGC-803 cells express wild-type p53 mainly in nuclei. Our results showed that compared to control groups, p53 protein level and phosphorylation at the 15th serine residue of p53 protein increased in cells transfected with telomeric plasmids, and these effects were effectively inhibited by wortmannin or ATM knockdown. These results revealed that p53 protein was stabilized by phosphorylation at the 15th serine residue depending on ATM activation. Previous data have clarified that p53 protein does not bind to double-stranded telomeric DNA sequence specially *in vitro* or *in vivo*, although p53 protein does bind to t-loop junction discovered by EM and AFM *in vitro*.[20,21] Thus it is not possible that the telomeric repeats concealed in the ccc plasmids activated p53 directly. The reliable explanation for p53 activation is that the exogenous telomeric tracts buried in the ccc plasmids interfere with endogenous telomere structure, leading to cellular telomere dysfunction. Then the dysfunctional telomeres activate ATM and downstream p53 phosphorylation and stabilization.

TIF consists of activated DNA damage response factors such as γH2AX and 53BP1 and is a typical mark of telomere dysfunction.[13] We found, in 41% cells transfected with plasmid pRST5, more than one TIF (i.e., phosphorylated γH2AX foci colocalized with TRF2 which mainly localized to telomeres *in vivo*) occurred; however, cells transfected with empty vector pBluescript showed almost no detectable phosphorylated γH2AX foci, indicating that the telomeric plasmid did induce cellular telomere dysfunction.

In addition, the mRNA and protein levels of TRF1 and TRF2 were found increased 24 h later after telomeric plasmid transfection, quite similar to our former results.[20] The regulation of TRF1 and TRF2 expression has not been

clearly elaborated until now. Our results showed that the upregulation of TRF1 and TRF2 was correlated with the ATM activation. As an important upstream DNA damage response factor, ATM is activated by many kinds of DNA damage, especially double strand breaks (DSB) caused by ionizing radiation, through intermolecular autophosphorylation and dimer dissociation.[22] Then activated ATM phosphorylates and activates some downstream signal molecules such as p53, p21, H2AX, and so on. Besides, ATM was supposed to be recruited to telomeres for protection and end processing.[23] It has also been reported that TRF2 was rapidly phosphorylated and relocated to DSB positions prior to ATM and other DNA damage response factors.[24,25] ATM phosphorylated the overexpressed TRF1 at the 219th serine residue and led to G2/M cycle arrest.[26] The above data indicate that ATM performs important roles in telomere protection through interactions with TRF1 and TRF2. On the one hand, the increased TRF1 and TRF2 proteins might be a stress response for cells transfected with telomeric plasmid to enhance the telomere protection in compensation for exogenous competition; on the other hand, excessive TRF1 and TRF2 proteins might restrain the replication of telomeric DNA, which led to G2/M cycle arrest and apoptosis.[27]

In conclusion, telomeric plasmids can elicit endogenous telomere dysfunction, which induces the cell senescence by activating ATM-p53 pathway.

CONFLICT OF INTEREST

None known.

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