Acta Biochim Biophys Sin 2011, 43: 372–379 | © The Author 2011. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmr020.
Advance Access Publication 29 March 2011

Original Article

hMTERF4 knockdown in HeLa cells results in sub-G1 cell accumulation and cell death

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Mitochondrial activity and cell energy status play important roles in the regulation of cell cycle and cell proliferation. Regulation of mitochondrial gene expression is crucial for mitochondrial activity regulation. The mitochondrial transcription termination factor (MTERF) family is a group of important mitochondrial transcription regulatory factors. It has been demonstrated that MTERF1–3 are involved in the regulation of mitochondrial gene transcription and oxidative phosphorylation. However, the function of the newest member MTERF4 has not been characterized. In this study, human *MTERF4* full-length open reading frame was cloned, and the protein structure prediction revealed that hMTERF4 protein contained leucine-zipper motifs, which is similar to human MTERF1–3. The expressed pMTERF4-green fluorescence fusion protein in HeLa cells localized the mitochondria. (3(4,5)dimethylthiahiazo(zy1)3,5diphenytetrazoliumromide) (MTT) proliferation assay and flow cytometry analysis showed that *hMTERF4* knockdown induced sub-G1 phase cells accumulation, whereas its overexpression promoted cell proliferation. Furthermore, double staining with Annexin V and PI revealed that *hMTERF4* knockdown increased necrosis but not apoptosis. In conclusion, our data suggested that hMTERF4 is an essential factor for cell proliferation, which is probably modulated by mitochondrial transcription to promote cell proliferation.

Keywords mitochondrional transcription termination factor 4; mitochondrion transcriptional regulation; subcellular location; cell proliferation; HeLa cells

Received: September 17, 2010 Accepted: January 5, 2011

Introduction

Due to its ability to provide energy, the mitochondrion is a ubiquitous organelle and the ‘powerhouse’ of all nucleated cells. It generates adenosinetriphosphate through oxidative phosphorylation (OXPHOS), which is a highly coordinated cellular process dependent on expression of gene products from both the nucleus and the mitochondrial genome (mtDNA). Since all mtDNA-encoded proteins serve as the subunits of the OXPHOS complex, the expression of these genes is regulated solely by nuclear gene-encoded proteins. Three groups of proteins, mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor A (TFAM), and mitochondrial transcription factor B1 or B2 (TFB1M or TFB2M) are necessary and sufficient for specific transcription initiation of mtDNA *in vitro* [1]. Although several new regulatory proteins have been identified [2,3], the mechanism of mitochondrial gene regulation *in vivo* is still poorly defined.

Mitochondrial transcription termination factor (MTERF) is a family of proteins that regulate mitochondrial gene expression. There are four members, MTERF1−4, in mammalian cell [4]. MTERF1 is a mitochondrial DNA-binding protein [5] that regulates mitochondrial transcription. It can terminate the transcription at the boundary of 16S rRNA and the $tRNA^{leu}$ genes, which allows an increase of mitochondrial ribosomal RNA expression relating to downstream genes [6–8]. A recent report has demonstrated that MTERF1 binds to mtDNA not only at the terminal region but also at the initiation site, suggesting a loop model for transcription termination [9]. hMTERF2 is a serum-inhibitory factor [10] that binds to the mtDNA promoter, and regulates OXPHOS by modulating mtDNA transcription [11]. The different observations have shown that *in vivo* MTERF2 is relatively abundant, and displays a non-sequence-specific DNA-binding activity with one monomer present per ~265 bp of mtDNA [12]. The mammalian MTERF3 has been proposed to be a negative regulator of mtDNA transcription [13]. A recent report has demonstrated that overexpression of MTERF2 or MTERF3 impairs the completion of mitochondrial DNA replication. These findings indicated that the MTERF family facilitates the orderly passage of replication and transcription machineries, thus contributing to genome stability [14]. So far, the function of the newest member MTERF4 protein has not been characterized.

In this study, we examined the function of hMTERF4 by overexpression and knock-down approaches, and identified its subcellular localization and its essential role in the regulation of mammalian cell growth. The functional identification of this previously uncharacterized mammalian protein may shed light on the understanding of MTERFs and the role of mitochondrial transcription in cell proliferation and cell cycle regulation.

Materials and Methods

cDNA cloning and sequencing

Total RNA was isolated from HeLa cells using the Column Animal RNAout Kit (Tiandz, Beijing, China), and then 1 μg of total RNA was reverse transcribed into cDNA using a Reverse-Transcription Kit (TIANGEN, Beijing, China) according to the manufacturer's protocol. The full-length open reading frame (ORF) of *hMTERF4* was amplified from the cDNA. The primers were designed based on the known human *MTERF4* cDNA in GenBank (accession no. NM_182501) and their sequences were: 5′-cgtaact gctgccatcttct-3′ (forward) and 5′-ctttcgctctagtccttcca-3′ (reverse). Conditions for PCR amplifications were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 58°C, and 120 s extension at 72°C; and a final extension at 72°C for 10 min. The PCR products were separated by agarose gel electrophoresis and purified with a Gel Extraction Kit (Omega Bio-tek, Norcross, USA). The purified fragment was cloned into the T-vector and the sequence of the insert was confirmed by direct sequencing using a T7 primer (Sangon, Shanghai, China).

Plasmid construction

The *hMTERF4* ORF was subcloned into several expression vectors for application. First, it was subcloned into an expression vector pEGFP-N3 between *Eco*RI and *Bam*HI sites to generate a recombinant plasmid pMTER4-GFP (green fluorescence). For *hMTERF4* gene knockdown, we constructed siRNA expression vector. The mRNA sequence of *hMTERF4* (NM-182501) was placed in Dharmacon siDESIGN Center (http://www.dharmacon.com/sidesign/siRNA) to design siRNAs targeting different sequences. Two independent target siRNAs were selected on the basis of ranking criteria of Reynolds *et al*. [15]. The siRNAs were converted to shRNAs by using the Insert Design Tool for the pSilencer Vector (http://www.ambion.com/techlib/misc/psilencerconverter.html). A single-loop sequence derived from miRNA was chosen for all the shRNAs. The Vienna RNA Secondary Package (http://www.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) was used to predict the secondary structure of the antisense strand of the shRNA.

The shRNA-annealed oligonucleotides were ligated into the siRNA expression vector pSilencer 4.1-cytomegalomavirus (CMV)-neo (Ambion, Astin, USA) between the *Bam*HI and *Hin*dIII sites by T4 DNA ligase (TakaRa, Dalian, China) according to the protocol of manufacture. The recombinant plasmids were designated as pSi1-MTERF4 and pSi2-MTERF4. The pSilencer4.1-CMV-neo was used as a negative control (we designate it as pSi-Negative) encoding a hairpin shRNA with a random sequence that was not found in the mouse, human, or porcine circovirus genome databases. HeLa cells were transfected with pSi1-MTERF4, pSi2-MTERF4, and pSi-Negative, respectively. The total RNA of transfected cells was extracted and semi-quantitative RT–PCR was performed to detect the *hMTERF4* expression level. For *hMTER4* overexpression, *hMTERF4* ORF was subcloned into p3×FLAG-CMV-14 vector, which was kindly provided by Dr Jia Fei (Institute of Veterinary Pharmacology and Toxicology, Vetsuisse Faculty, University of Zurich). The insert was confirmed by direct sequencing with sequencing primers: 5′- aggcgattaagttgggta-3′ (forward) and 5′-cggtaggcgtgtacggtg-3′ (reverse) (Sangon). The recombinant eukaryotic expression plasmid was named pMTERF4-Flag.

Bioinformatics analysis

Alignment of four members of the human MTERF family was performed using ClustalW (http://align.genome.jp/). Mitochondrial localization of hMTERF4 was predicted using TargetP and PSORT prediction programs. Coiled-coils were predicted using COILS program. Protein motifs in hMTERFs were predicted using SMART software.

Cell culture and transfection

HeLa cells were kindly provided by Dr Guoyang Liao (Kunming Biological Institute of Medical Science, Chinese Academy of Medical Sciences), and were maintained in RPMI-1640 medium (Gibco-BRL, Carlsbad, USA) supplemented with 10% fetal bovine serum (complete medium) at 37°C in 5% CO_2. Cells were seeded in six-well plates, cultured to 70% of confluence, and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) with the designed constructs.

Subcellular localization of hMTERF4

HeLa cells were transfected with pMTERF4-GFP or the empty pEGFP-N3 vector and grown on glass coverslips in the complete medium for 24 h. The medium was then replaced with pre-warmed (37°C) fresh medium containing 100 mmol/L MitoTracker Red CMXRos (Molecular Probes, Eugene, USA). After incubation for 15–45 min, the medium was replaced with fresh pre-warmed medium.

The coverslips with transfectants were viewed under a confocal fluorescent microscope (LSM510 META; Zeiss, Göttingen, Germany) at 488 nm exciting light and 579 nm emitting light, and images were obtained for the GFP and red fluorescence (mitochondrial tracker) in live cells.

Semi-quantitative RT–PCR

HeLa cells were transfected with plasmids pMTERF4-Flag, pSi1-MTERF4, pSi2-MTERF4, and empty vectors. Forty-eight hours later, total RNAs were extracted from transfected cells and subjected to RT–PCR as described above. Primers for PCR detection of *hMTERF4* mRNA were 5′-actgactacagcctccaatgg-3′ (forward) and 5′-ttcagggcaacagtaaagca-3′ (reverse). The expression levels of β-actin in the same RNA samples were also examined by RT–PCR. The primers for human β-actin were 5′-cgggaaatcgtgcgtgac-3′ (forward) and 5′-caggaaggaaggct ggaa-3′ (reverse). PCR was carried out for 24 cycles for *hMTERF4* and 20 cycles for β-actin. The annealing temperatures used for amplification of *hMTERF4* and β-actin were 58 and 57°C, respectively. The PCR products were analyzed by agarose gel electrophoresis and the DNA band intensities were quantified by the Gel Doc1000 gel scanner (Bio-Rad, Hercules, USA).

MTT cell proliferation assay

HeLa cells were seeded in 24-well plates at the density of 1×10^3 cells per well with the complete medium, and incubated at 37°C in 5% CO_2. When subconfluent, cells were transfected with pMTERF4-Flag, pSi1-MTERF4, pSi2-MTERF4, and vector control, respectively. After 0, 24, and 48 h, 3(4,5)dimethylthiahiazo(zy1)3,5diphenytetrazoliumromide (MTT) reagent (5 mg/ml) was added into the media. And the cells were incubated for another 4 h. DMSO (150 μl) was then added into wells, and the plates were shaken for 10 min to dissolve the formazan crystals inside the cells. The plates were then read in a microplate reader (Bio-Rad) for absorbance at 570 nm. The relative cell number in each well was expressed as the values of A570. The experiment was repeated thrice.

Flow cytometry analysis for cell cycle progression

HeLa cells were transfected with pMTERF4-Flag, pS1i-MTERF4, pSi2-MTERF4, and their respective control plasmids. Forty-eight hours later, cells were trypsinized and harvested by centrifugation at 10,000 *g* for 5 min. Cells were re-suspended in cold phosphate buffered saline (PBS), fixed in 70% ethanol for at least 1 h at 4°C, then washed twice with cold PBS, followed by staining with 500 mg/ml propidium iodide (PI) at 4°C for 30 min for DNA content. The cells were treated with 10 mg/ml RNase A for 30 min at 37°C, and then analyzed for cell cycle progression with a fluorescence activated cell sorter (FACS) (Becton-Dickinson, Franklin Lakes, USA). Approximately 20,000 cells were counted, and data were analyzed with the software provided by the FACS manufacturer. This experiment was repeated twice.

Flow cytometry detection of apoptosis

HeLa cells were transfected with pMTERF4-Flag, pS1i-MTERF4, pSi2-MTERF4, and vector control, respectively. Cells were washed with PBS and collected by trypsinization. The cells were then re-suspended in PBS and stained with Annexin V-FITC Apoptosis Detection Kit (Beyotime, Haimen, China) according to the manufacturer's instructions. Samples were analyzed by FACS under both green and red channels. Cells stained positive for the green fluorescent Annexin V-FITC were apoptotic, whereas the red fluorescent dye PI were necrotic. HeLa cells that were treated for 6 h with 12% ethanol to induce significant apoptosis in most cells were used as apoptosis controls.

Results

Cloning and bioinformatics analysis of *hMTERF4*

Total RNA was isolated from HeLa cells and reverse transcribed into cDNA. The full *hMTERF4* ORF of 1146 bp was obtained by PCR and its sequence was confirmed by sequencing. The nucleotide sequence and predicted amino acid sequence matched with the cDNA and protein sequence in the GenBank, respectively (data not shown). The protein sequence was subjected to bioinformatics analysis for the prediction of secondary structure and subcellular localization.

A high probability of mitochondrial localization was predicted by TargetP (75.7%) and PSORT (71%). Leu zipper motifs were also identified in hMTERF4 (**Fig. 1**). ClustalW alignment showed a high sequence homology among four members of the MTERF family in human. hMTERF1 contains two basic domains and three leucine-zipper (Lz) motifs (Lz1a, Lz1b, and Lz2, Lz3) which are necessary for its binding to mtDNA [5,7,8]. hMTERF2 has two basic domains and three Lz motifs (Lz1a, Lz1b, Lz2a, Lz2b, Lz3a, and Lz3b) [10]. Sequence analysis of the hMTERF4 protein revealed multiple Lz motifs (**Fig. 1**). The first Lz motif (Lz1) is located between residues 184–204 (Lz1a), the second one (Lz2) has two peptide fragments localized between residues 213–233 (Lz2a) and 251–271 (Lz2b). The last potential Lz motif resides between residues 284 and 304 (Lz3). The Lz1 in hMTERF4 overlaps with the Lz1b in hMTERF1, and the Lz2a in hMTERF4 overlaps with the Lz2 in hMTERF1, and the Lz2a in hMTERF2. Further, the Lz3 in hMTERF4 overlaps with the Lz3a in hMTERF2, suggesting the functional importance of these Lz motifs of hMTERF4 in

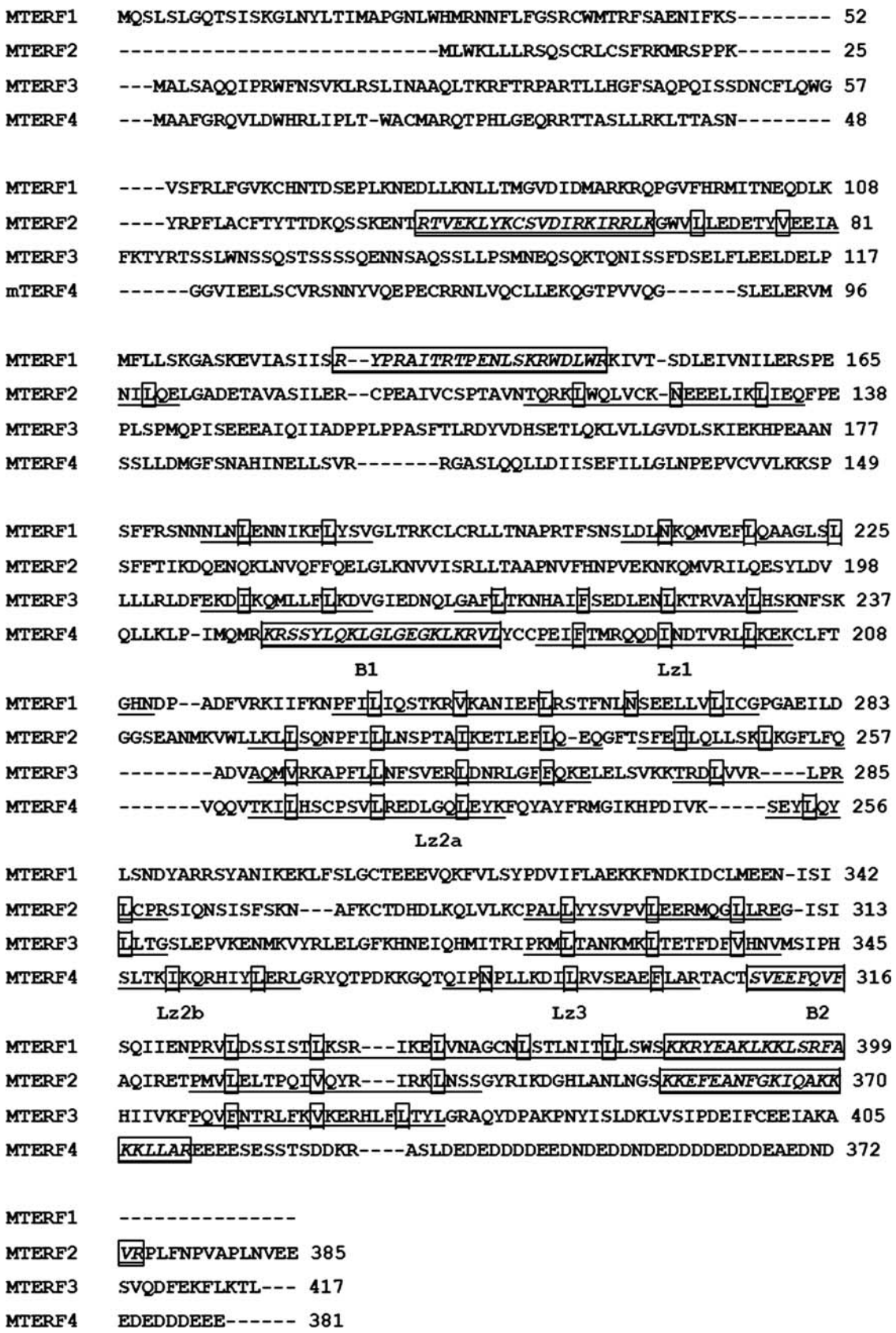

Figure 1 Comparison of the domain structure among members of hMTERF family Alignment of four members of the human MTERF family was performed using ClustalW. Underlined amino acids indicated the Lz motifs with L indicating leucine or similar hydrophobic amino acids. DNA-binding regions were outlined.

mitochondria. In addition, hMTERF4 also contains two basic DNA-binding domains (B1 and B2). The B1 domain is between residues 161 and 180 and the B2 domain is located between residues 309 and 322 near the C-terminus. The C-terminus contains many acidic amino acid residues (D and E), which are different from the C-terminus sequences of other members in this family. These bioinformatics data suggest that hMTERF4 protein is probably a mitochondrial DNA-binding protein containing Lz-like (Leu zipper) motifs. The overall structural similarity and difference between hMTERF4 and other MTERF members imply a concordant, yet divergent, role of hMTERF4 from MTERF1–3 in the regulation of mitochondrial transcription.

hMTERF4 is localized in mitochondria

Full-length *hMTERF4* ORF was subcloned into pEGFP-N3 and the recombinant plasmid pMTERF4-GFP or pEGFP-N3 vector was transiently transfected into HeLa cells. The transfectants were stained with mitochondrial probe MitoTracker Red CMXRos. The green fluorescent signals from fusion protein MTERF4-GFP were co-localized with the mitochondrial tracker (**Fig. 2**, lower panel), whereas GFP expressed from the control plasmid distributed diffusely in cells (**Fig. 2**, upper panel). This observation indicates that MTERF4 is present and functionally active in the mitochondria, similar to other members of its family.

hMTERF4 knockdown suppresses cell growth and induces sub-G1 cells accumulation

HeLa cells were transfected with pMTERF4-Flag, pSi1-MTERF4, pSi2-MTERF4, and vector control, respectively. The cells were collected 48 h after transfection and *hMTERF4* mRNA levels were assessed by RT–PCR. The result of RT–PCR showed that the *hMTERF4* mRNA level decreased to 71% of the control in pSi1-MTERF4 transfected cells, and in pSi2-MTERF4 transfected cells the mRNA level decreased to 27% of the control. In contrast, the *hMTERF4* mRNA level in pMTERF4-Flag transfected cells increased to 180% of the control [**Fig. 3(A)**]. Thus, pSi2-MTERF4 transfection strongly inhibited the *hMTERF4* gene expression, whereas pMTERF4-Flag transfection enhanced its expression.

The effect of *hMTERF4* on cell proliferation was examined at 0, 24, and 48 h post-transfection by MTT assay. Compared with the control, the number of viable cells transfected with pMTERF4-Flag increased at 24 h post-transfection. By contrast, the number of viable cells transfected with pSi1-MTERF4 and pSi2-MTERF4 were markedly reduced at 24 h and remained at a low level at 48 h [**Fig. 3(B)**]. These data indicated that *hMTERF4* might be important for cell proliferation, because knockdown of this gene inhibited cell growth while

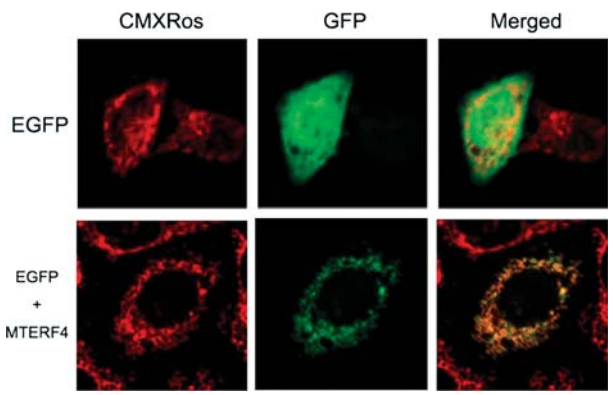

Figure 2 Subcellular localization of hMTERF4 in HeLa cells HeLa cells were transfected with pEGFP-N3 vector (upper panel) or pMTREF4-GFP recombinant plasmid (lower panel), and stained with the mitochondrial tracker CMXRos. The green signals showed the GFP or MTERF4-GFP fusion proteins and the red signals showed the mitochondria. Yellow signals were merged signals from both CMXRos and GFP, indicating the localization of GFP-containing proteins in the mitochondria. Magnification, × 500.

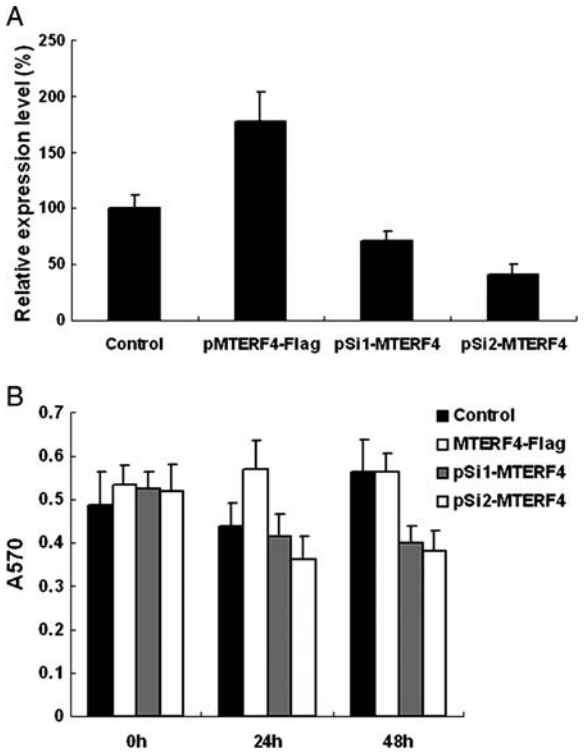

Figure 3 Results of RT–PCR and MTT assay Hela cells were transfected with pMTERF4-Flag, pSi1-MTERF4, pSi2-MTERF4, and vector control, respectively Cells were harvested at 48 h after transfection, the total RNA was extracted and subjected to RT–PCR analysis. (A) Relative level of mRNA from above four groups of cells. Data were presented as mean ± SD from three experiments. (B) Cells were also treated with MTT for 4 h at 0, 24, and 48 h post-transfection. MTT assay was carried out in triplicate and the mean values were graphed. Error bars represented the standard deviation.

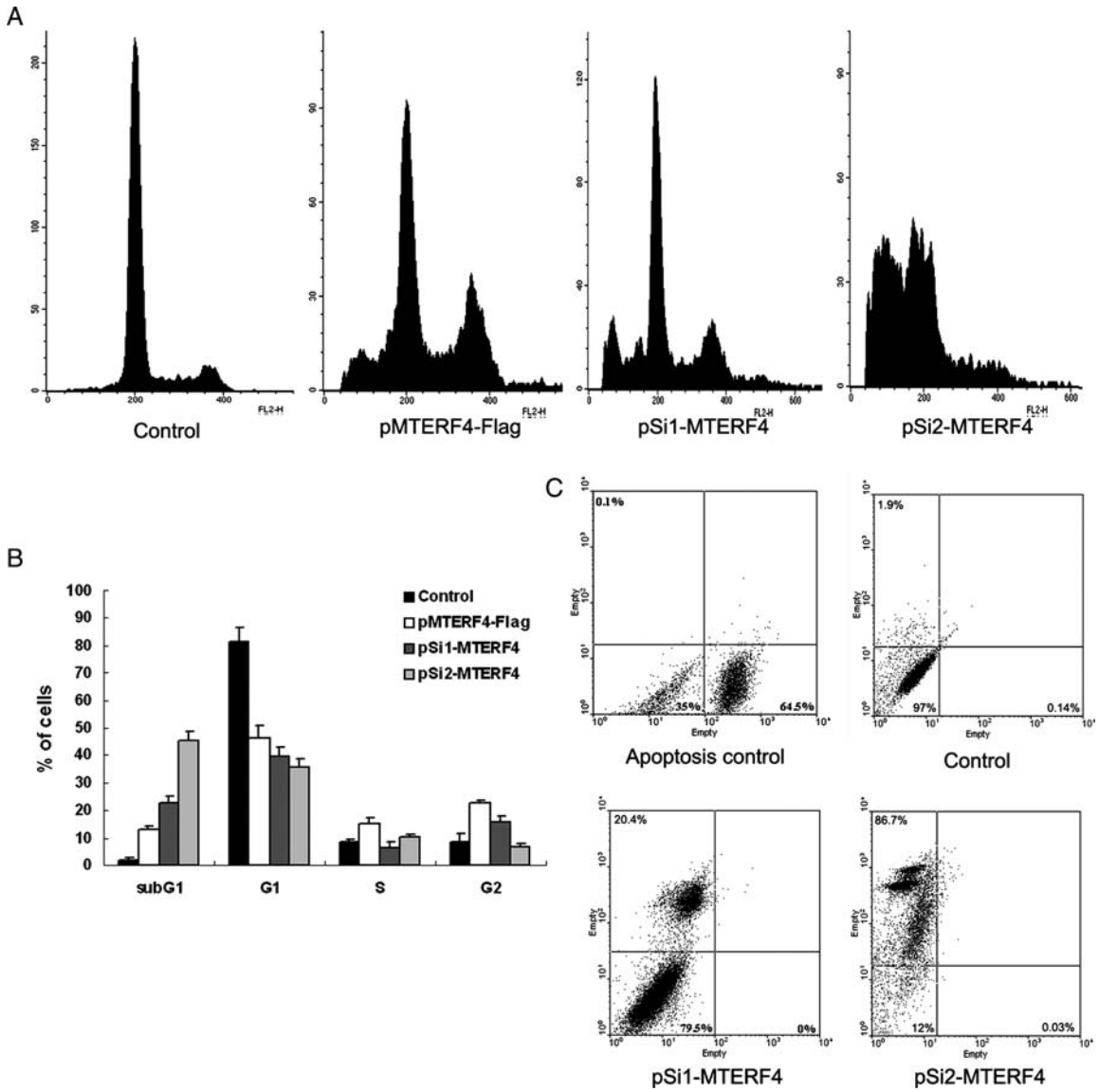

Figure 4 Knockdown of MTERF4 leads to sub-G1 phage cells accumulation and cell death (A) Representative histograms of flow cytometry analysis showing the cell cycle profiles of HeLa cells transfected with pMTERF4-Flag, pSi1-MTERF4, pSi2-MTERF4, and vector control. (B) Distribution in different cell cycle phases among cells transfected with pMTERF4-Flag, pSi1-MTERF4, pSi2-MTERF4, or the control vectors. Data were presented as mean values of three independent experiments and bars indicate standard error of the mean. (C) The FACS scatter plot obtained from HeLa cells double stained with Annexin V and PI. Top left corner showed the late apoptotic or necrotic cells ($FITC^+/PI^+$), bottom left corner showed healthy live cells ($FITC^-/PI^-$), and bottom right corner showed early apoptotic cells ($FITC^+/PI^-$).

overexpression promoted cell growth. At the same time, we also investigated whether the *hMTERF4* knockdown-induced cell growth inhibition was due to the dysregulation of cell cycle. Flow cytometry analysis showed that compared with the control, the cells transfected with the pSi1-MTERF4 displayed an increase in the percentage of cells at the sub-G1 phase (22.8 $\pm$ 2.3%), and a decrease in the G1 phase (39.9 $\pm$ 2.9%) and an increase in the G2/M phase (15.7 $\pm$ 2.7%). Those cells transfected with pSi2-MTERF4 displayed an obvious increase in percentage of cells at sub-G1 phase (45.5 $\pm$ 3.1%), and a decrease at G1 phase (35.9 $\pm$ 2.8%) and G2/M phase (6.6 $\pm$ 1.3%), and most were in sub-G1 and G1 phases [**Fig. 4(A,B)**]. In contrast, in cells transfected with pMTERF4-Flag, the percentage of G1 cells was lower, whereas the percentage of G2/M cells was higher than that in control cells. These results suggested that overexpression of *hMTERF4* promotes cell proliferation, whereas down-regulation of *hMTERF4* leads to cell accumulation in sub-G1 phase and cell growth inhibition.

Knockdown of hMTERF4 causes cell death

In order to determine whether the *hMTERF4* knockdown would induce the sub-G1 phase cells to apoptotic or necrotic cell death, cells were stained with AnnexinV-FITC and PI. As shown by the scatter plot of green and red fluorescence

[**Fig. 4(C)**], in the apoptosis control cell, 64.5% were $FITC^+/PI^-$ early apoptotic cells, whereas about 35% were $FITC^-/PI^-$ healthy live cells. In the pSi1-MTERF4-transfected cell population, 20.4% were $FITC^+/PI^+$ late apoptotic or necrotic cells, whereas about 79.5% were $FITC^-/PI^-$ healthy live cells, no $FITC^+/PI^-$ early apoptotic cells were detected. In the pSi2-MTERF4-transfected cell population, 86.7% were $FITC^+/PI^+$ late apoptotic or necrotic cells, whereas about 12% were $FITC^-/PI^-$ healthy live cells and 0.03% $FITC^+/PI^-$ early apoptotic cells. In the control group transfected with pSi-Negative plasmid, most of the cells were alive (97%), only about 1.9% cells were necrotic, and 0.14% cells were early apoptotic. These results indicated that *hMTERF4* down-regulation significantly induced necrosis but not apoptosis.

Discussion

Cell growth is regulated by many factors, including growth factors, amino acids, and energy status to ensure that cell growth is appropriate to environmental conditions. Insulin and growth factors regulate cell growth by positively activating target of rapamycin proteins signaling [16,17], amino acids provide nutrients and protein synthesis material to cell growth. The mitochondrion is the cell's powerhouse responsible for energy production. Its gene expression regulation is an important aspect to mitochondrial activity regulation [18,19]. For example, overexpression of a yeast homolog of mammalian TFAM shortens the G1 phase of the cell cycle [20]. *MTERF2* also can regulate OXPHOS by modulating mtDNA transcription [11], and its overexpression inhibits cell growth [10]. In mice, *MTERF3* gene knockout caused embryonic lethality from impaired mitochondrial gene transcription [13]. Therefore, mitochondrial transcription factors can significantly affect cell proliferation.

The function of MTERF4 has not been characterized among the MTERF family. Here, we cloned the *hMTERF4* cDNA from HeLa cells and constructed the overexpression vector or gene silencing vector. When expressed as a GFP-fusion protein, MTERF4 was localized in mitochondria. Using an siRNA approach, we showed that down-regulation of *hMTERF4* inhibited cell proliferation, resulting in cell cycle arrest at sub-G1 phase, and cell death. Bioinformatics analysis revealed the high conservation among four MTERF members and predicted potential Lz motifs and DNA-binding domains in hMTERF4.

Although the precise role of hMTERF4 in mitochondrial transcription regulation remains to be defined, the protein structural features suggest that hMTERF4 may share similar, but not all, functions with other hMTERF family members as a regulator of mitochondrial transcription and mammalian cell cycle.

Funding

This work was supported by grants from the National Natural Science Foundation of China (30760057) and the Key Science and Technology Project of Yunnan Province, China (2008CD069, 2008CC003, and 09Z0005).

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