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Human mitochondrial transcription factor A functions in both nuclei and mitochondria and regulates cancer cell growth

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ABSTRACT

Mitochondrial transcription factor A (mtTFA) is one of the high mobility group protein family and is required for both transcription from and maintenance of mitochondrial genomes. However, the roles of mtTFA have not been extensively studied in cancer cells. Here, we firstly reported the nuclear localization of mtTFA. The proportion of nuclear-localized mtTFA varied among different cancer cells. Some mtTFA binds tightly to the nuclear chromatin. DNA microarray and chromatin immunoprecipitation assays showed that mtTFA can regulate the expression of nuclear genes. Overexpression of mtTFA enhanced the growth of cancer cell lines, whereas downregulation of mtTFA inhibited their growth by regulating mtTFA target genes, such as baculoviral IAP repeat-containing 5 (*BIRC5*; also known as survivin). Knockdown of mtTFA expression induced p21-dependent G1 cell cycle arrest. These results imply that mtTFA functions in both nuclei and mitochondria to promote cell growth.

1. Introduction

Mitochondrial transcription factor A (TFAM; also known as mtTFA) is a member of the high mobility group (HMG) box protein family and activates transcription from the D-loop region of the mitochondrial genome [1–3]. Mitochondria play a critical role in cancer cell metabolism, which is also essential for cell proliferation [4]. It is well known that mitochondrial uncoupling mediates the metabolic shift to aerobic glycolysis in cancer cells [4,5]. Thus, mitochondria control cell survival and growth. In addition, the number of mitochondria correlates with the growth rate of cancer cells [6,7].

mtTFA-null mice show an embryonic lethal phenotype and apoptosis in heart cells [8]. Nuclear HMG box proteins such as HMGB1/B2 are often overexpressed in cancer cells, bind preferentially to p53 and are involved in apoptosis [9,10]. mtTFA protein multimerizes and binds to mitochondrial DNA, indicating that the mtTFA levels might be increased in cancer cells and be related to malignant progression and proliferative activity.

Prostate cancer is the most prevalent form of cancer among men. Microscopic prostate cancers can be detected in 12.4% elderly men in USA, indicating an association of the cancer with aging and oxidative stress [11]. We previously found that mtTFA is overexpressed in cisplatin-resistant human cancer cells [12]. mtTFA may function to protect mitochondrial DNA from oxidative stress. It has been shown that high expression of mtTFA correlated with poor prognosis in endometrial carcinoma [13] and colon cancer [14]. These data are in accordance one of the hallmarks of cancer: the evasion of apoptosis.

Interestingly, we found that mtTFA was detected within the nuclear chromatin of cancer cells and may regulate nuclear gene expression in addition to its role as a transcription factor in mitochondria.

Abbreviations: BIRC5, baculoviral IAP repeat containing 5; ChIP, chromatin immunoprecipitation; HMG, high mobility group; mtDNA, mitochondrial DNA; MTP, mitochondrial targeting peptide; mtTFA/TFAM, mitochondrial transcription factor A; NLS, nuclear localization signal; PRDX3, peroxiredoxin 3; siRNA, small interfering RNA; YBX1, Y box binding protein 1.

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2. Materials and methods

2.1. Cell culture and antibodies

Human prostate cancer cell line PC3 was obtained from the American Type Culture Collection. The human clear cell renal cancer SKR1 and Caki-1 cells were kindly provided by Dr. Seiji Naito (Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan). Antibodies to p21 (sc-187), baculoviral IAP repeat-containing 5 (BIRC5; also known as survivin) (sc-10811) and β-tubulin (sc-5274) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Flag (M2) and anti-β-actin (AC-15) antibodies were purchased from Sigma (St. Louis, MO). Polyclonal antibodies against mtTFA [15], BRG1-associated factor 57 (SMARCE1, also known as BAF57) [16] and HMGB1 [17] have been described previously. Anti-peroxiredoxin 3 (PRDX3) antibody was a kind gift from Hiroki Nanri (Seinan Jogakuin University, Fukuoka, Japan).

2.2. Plasmid preparation

Human mtTFA cDNA without a stop codon was amplified by reverse-transcription PCR from total RNA extracted from HeLa cells with the primer pair: 5′-CGATGGCGTTTCTCCGAAGC-3′ and 5′-ACACTCCTCAGCACCATATTTTCG-3′. The underline indicates the start codon. To obtain the mtTFA-3×Flag expression plasmid (pcDNA3-mtTFA-3×Flag), the mtTFA cDNA fragment containing 3×Flag sequences at the C-terminus was ligated into a pcDNA3.1/hygromycin mammalian expression plasmid (Invitrogen, Carlsbad, CA). To construct BIRC5-luciferase reporter plasmid, putative BIRC5 promoter region (−992 to +110) was amplified by PCR using the human genomic DNA and the following primer pairs: GGGAGGGGTGGGGAGAGGTTGC and AAGCTTCGCGGGACCCGTTGGCAGAGGTG. Underlining indicates the HindIII restriction enzyme cleavage site. PCR product was cloned and ligated into the EcoRV–HindIII site of the pGL4.16 luc2CP/Hygro vector (Promega, Madison, WI).

2.3. Cloning of stable transfectants

The cloning of stable transfectants was described previously [18]. Briefly, PC3 cells were transfected with pcDNA3-mtTFA-3×Flag with Superfect Transfection Reagent (Qiagen, Valencia, CA) and cultured with medium containing 500 μg/ml hygromycin for 20 days. The resulting colonies were isolated and the expression level of mtTFA was investigated by Western blotting with the anti-mtTFA antibody. Two stable transfectants named as mtTFA-3F CL1 and CL9 were established.

2.4. Cell fractionation

Cell pellets were resuspended in hypotonic buffer A, containing 10 mM Hepes–KOH, pH 7.9, 10 mM KCl, 0.1 mM EDTA–NaOH, pH 8.0, 0.1 mM EGTA, 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and incubated for 15 min on ice. After addition of Nonidet-P40 to 0.3% final concentration, cells were gently resuspended and centrifuged at 4200*g* for 5 min. The supernatant was stored for the cytoplasmic fraction (CF). The nuclear pellet was resuspended in high salt buffer C (1/4 volume of buffer A), containing 20 mM Hepes–KOH, pH 7.9, 0.4 M NaCl, 1 mM EDTA–NaOH, 1 mM EGTA, 1 mM DTT and 0.5 mM PMSF. The half volume of buffer C containing nucleus was sonicated for 20 s for nuclear fraction (NF). The remained half volume of nuclei in buffer C was incubated for 30 min on ice and centrifuged at 21,000*g* for 10 min. The supernatant was stored for use as the nuclear extract (NE), i.e., proteins that loosely bind to DNA. The pellet was sonicated in buffer C (the same volume of NE) for 20 s and stored for use as the chromatin fraction (ChF), i.e., proteins that tightly bind to DNA. For preparation of whole cell lysate, the cell pellet was sonicated in buffer A for 20 s.

2.5. Indirect immunofluorescence

PC3 cells were divided into cytoplasm and nuclei as described above. Nuclei were suspended with 15% formalin neutral buffer solution (Wako, Osaka, Japan) for 10 min and mounted on glass coverslips coated with poly-L-lysine (3438-100-01, R&D Systems) and were permeabilized with PBS containing 0.2% Triton X-100 for 10 min at room temperature, and then incubated with normal serum or serum containing anti-mtTFA antibody (1:200 dilution) in PBS including 0.3% BSA for 60 min at room temperature. After washing three times with PBS, the samples were incubated with fluorescein-conjugated goat anti-rabbit IgG (Alexa Fluor 488; Molecular Probes, OR) and 4,6-diamidino-2-phenylindole (DAPI; Sigma) in PBS including 0.3% BSA for 45 min at room temperature. The samples were washed three times with PBS and mounted directly on slides with Gel/Mount (M01, Biomeda). Digital photographs were taken with a Nikon ECLIPSE E600 fluorescence microscope and DS-5M, -L1 (Nikon, Tokyo, Japan). Exposure time was fixed in each picture.

2.6. Western blotting

Western blotting was described previously [18]. For calculating the ratio between fractionations, indicated amounts of volume were subjected by Western blotting. Detection was performed using enhanced chemiluminescence (Amersham, Piscataway, NJ). The protein expression levels were quantitated using a Multi Gauge Version 3.0 (Fujifilm, Tokyo, Japan).

2.7. Knockdown analysis using small interfering RNAs (siRNAs)

Knockdown using siRNAs was performed as described previously [19]. The following 25-bp, double-stranded RNA oligonucleotides were commercially generated (Invitrogen) 5′-UGCACAGCUCUGCUCCAGACCUUCC-3′ and 5′-GGAAGGUCUGGAGCAGAGCUGUGCA-3′ for *mtTFA*-siRNA (siTFAM) #1, 5′-AUACCUGCCACUCCGCCCUAUAAGC-3′ and 5′-GCUUAUAGGGCGGAGU- GGCAGGUAU-3′ for siTFAM #2.

2.8. Cell proliferation assay

Cell proliferation assays were performed as described previously [19].

2.9. Flow cytometry

Flow cytometry assays were performed as described previously [20].

2.10. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay with transient transfection and anti-Flag antibody was described previously [21]. The purified DNA was used for PCR analysis with the following primer pairs: 5′-GGGAGGGGTGGGGAGAGGTTGC-3′ and 5′-CGCTGTCCCGGCGAGTACATCG-3′ for the *BIRC5* promoter, 5′-GCCCGGCACTACGGGCTGCG-3′ and 5′-GTGTGCGCAGGCCGCGGACG-3′ for the Y-box binding protein 1 (*YBX1*) gene. The PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide.

2.11. Microarray analysis

PC3 cells were incubated for 48 h after transfection with control siRNA or siTFAM. The methods for high quality RNA preparation and microarray analysis were described previously [22].

2.12. Reporter assay

Transient transfection and a luciferase assay were performed as described previously [23]. The indicated amounts of BIRC5 reporter plasmid were co-transfected with mtTFA expression plasmid or siRNA using Superfect reagent (Qiagen) or Lipofectamine RNAiMAX (Invitrogen) into PC3 cells, respectively. After 48 h, cells were lysed with reporter lysis buffer (Promega) and luciferase activity was detected by Picagene kit (Toyoinki, Tokyo, Japan), and the light intensity was measured with a luminometer (Luminescencer JNII RAB-2300, ATTO, Japan). The results shown are normalized to protein concentration measured using the Bradford method and are representative of at least three independent experiments.

2.13. Statistical analysis

Pearson's correlation was used for statistical analysis, and significance was set at the 5% level.

3. Results

3.1. Nuclear localization of mtTFA in cancer cells

Nuclear HMG-box proteins possess nuclear localization signal (NLS) in the HMG boxes and are localized both in nuclei and cytoplasm. On the other hand, cellular localization of mtTFA, one of the HMG-box protein family, has not been extensively studied, because mtTFA possess mitochondrial targeting peptide (MTP) in the amino terminal end. To investigate whether mtTFA is found in nuclei, we first searched the NLS in HMG-boxes of mtTFA, and found two putative nuclear localization signals (NLS) in each HMG-box (Fig. 1A). Immunoblots of whole cell lysate, cytoplasmic fraction (CF), nuclear extract (NE) and chromatin fraction (ChF) from PC3 and SKR1 cancer cell lines are shown in Fig. 1B. mtTFA was present not only in the cytoplasmic fraction containing mitochondria but also in the chromatin fraction of the two cancer cell lines. Each fraction was stringently monitored for contamination using markers for nucleus (BAF57 and HMGB1) and mitochondria (PRDX3). The mitochondrial PRDX3 was not detected in both nuclear extract and the chromatin fraction. The majority of both BAF57 and HMGB1 was localized in the nuclear fraction. Then, we performed indirect immunofluorescence with nuclei of PC3 cells to avoid the strong intensity of mtTFA in mitochondria. Signal intensity was observed in nuclei when rabbit serum containing anti-mtTFA antibody was used, but not normal rabbit serum

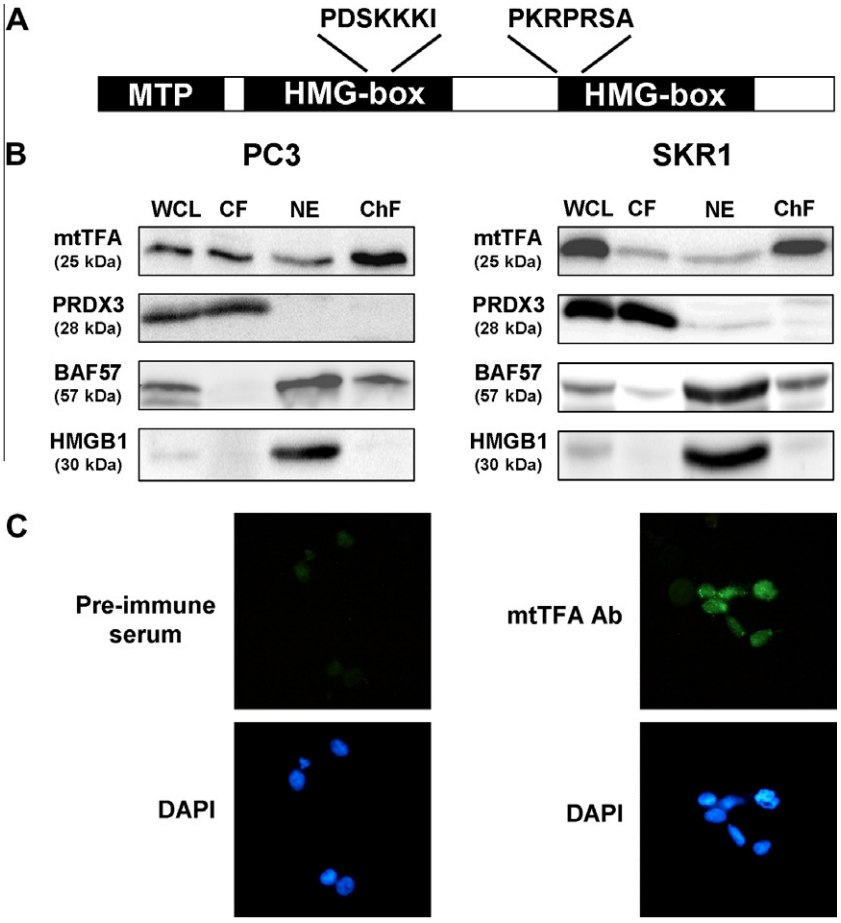

Fig. 1. Cellular localization of mtTFA in cancer cells. (A) Nuclear localization signals (NLS) were represented. MTP indicates mitochondrial targeting peptide. (B) Each 50 μg of whole-cell lysate (WCL), cytoplasmic fraction (CF), nuclear extract (NE) and chromatin fraction (ChF) were prepared from PC3 and SKR1 cells and subjected to SDS–PAGE. Western blotting was performed with the indicated antibodies. (C) Nuclei of PC3 cells were stained with DAPI and serum containing anti-mtTFA antibody or normal rabbit serum. DAPI indicates nuclear marker.

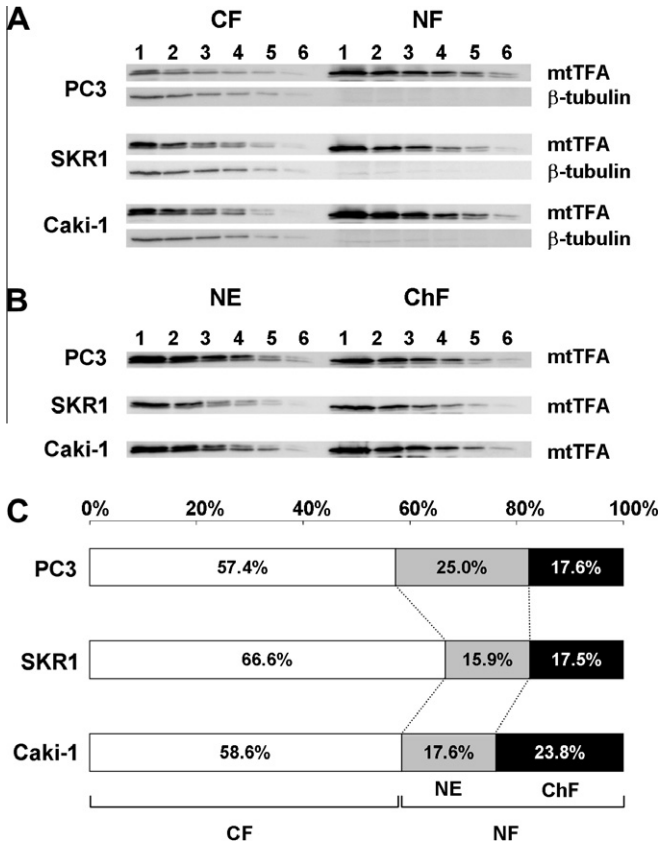

Fig. 2. The ratio of mtTFA content in the cellular fractions. (A) PC3, SKR1 and Caki-1 cells were divided into cytoplasmic fraction (CF) and nuclear fraction (NF) with volume ratio 4 to 1. Each cell lysate was diluted 1/2 sequentially. The same volume was subjected to SDS–PAGE and Western blotting was performed with the indicated antibodies. Maximum amount of protein is under 100 μg. β-Tubulin is a control of CF. (B) Nuclear fraction (NF) were divided into nuclear extract (NE) and chromatin fraction (ChF) with same volume. Each cell lysate was diluted 1/2 sequentially. The same volume was subjected to SDS–PAGE and Western blotting was performed with anti-mtTFA antibody. Maximum amount of protein is under 100 μg. (C) Based on the results of (A) and (B), the ratio of cellular localization was calculated.

(Fig. 1C). Next, we investigated the nuclear contents of mtTFA using three prostate cancer cell lines. The ratio of mtTFA between each cellular fraction varied among cancer cell lines. Approximately 30–40% of mtTFA was localized in the nuclear fraction (Fig. 2A and C), and almost equal amount of mtTFA was localized in the nuclear extract (NE) and the chromatin fraction (ChF) (Fig. 2B and C).

3.2. Identification of mtTFA target genes by DNA microarray analysis

Nuclear HMG-box proteins participate in the process of transcription as coactivators [24]. To elucidate the nuclear function of mtTFA, we performed DNA microarray. DNA microarray analysis showed that 596 genes in PC3 cells were downregulated after transfection with specific siRNA against *mtTFA* (data not shown). To categorize the 596 genes, we employed the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources Program (http://david.abcc.ncifcrf.gov/). Among 596 genes, almost genes were encoded in nuclear genome and 32 genes categorized as concerned with apoptosis were shown in Supplementary Table 1. This result indicated that mtTFA might localize in nucleus and regulate nuclear gene expressions.

3.3. mtTFA directly regulated BIRC5 gene expression

Next, we searched for putative mtTFA binding sites in the promoter region of these genes using DataBase Transcriptional Start Sites (DBTSS) Version 7.0 (http://dbtss.hgc.jp/). The number of mtTFA binding site within 1 kb upstream from the putative transcription start site was also presented in Supplementary Table 1. We found two forward-oriented binding sites are localized in the promoter of *BIRC5* gene. Then, we focused on *BIRC5*, which is involved in both cell growth and apoptosis [25,26]. Consistent with the results of the DNA microarray analysis, knockdown of mtTFA expression downregulated BIRC5 expression (Fig. 3A). Interestingly, we found that downregulation of mtTFA induced expression of the cyclin-dependent kinase inhibitor p21 (CDKN1A) (Fig. 3A). On the other hand, Western blotting showed that BIRC5 expression was markedly increased in mtTFA-overexpressing cells (Fig. 3A). As we found that there are two binding site of mtTFA in the promoter of *BIRC5* gene (Fig. 3B), ChIP assays demonstrated that mtTFA bound to the promoter of the *BIRC5* gene, but not to that of the unrelated *YBX1* gene (Fig. 3C). Furthermore, *BIRC5*-promoter activity was enhanced by cotransfection of mtTFA expression plasmid in dose dependent manner, and was reduced by cotransfection of specific siRNAs against *TFAM*. These data indicate that BRIC5 gene expression is transcriptionally regulated by mtTFA in the nucleus.

3.4. The expression of mtTFA stimulates growth of PC3 cells

To explore the role of mtTFA in PC3 cells, we investigated cell proliferation. Overexpression of mtTFA increased the proliferation rate of PC3 cells (Fig. 4A). Consistent with this result, downregulation of mtTFA significantly reduced the cell growth of both wild-type PC3 cells and mtTFA-overexpressing PC3 cells (Fig. 4B). Then, we performed the cell cycle analysis by flow cytometry. Cell cycle analysis showed that G2/M phase cells were markedly increased in mtTFA-overexpressing cells (Fig. 4C). On the other hand, increase in G1 population of cells and decrease in G2/M phase cells were observed when *mtTFA* expression was depleted by specific siRNAs. In addition, the population of sub-G1 cells was increased approximately 3-fold in siTFAM-treated cells compared with control siRNA-treated cells (Fig. 4D).

4. Discussion

mtTFA is the primary transcription factor in mitochondria [1]. In addition to its role as a transcription factor, mtTFA has also been implicated as a primary architectural protein of the mitochondrial genome by packing the mitochondrial DNA (mtDNA) [27]. Our demonstration of mtTFA localization in the nucleus is not without precedent: unexpected subcellular localization of proteins has been previously reported. Nuclear transcription factors such as signal transducer and activator of transcription 3 (STAT3) have been found in mitochondria [28]. In addition, an alternatively spliced form of mtRNA polymerase has been localized to nucleus, where it supports nuclear gene expression [29]. Here, we firstly demonstrated that mtTFA was found in the nuclei and bound tightly to the chromatin. Because mtTFA is one of the HMG box proteins, the nuclear localization of mtTFA may be due to the nuclear localization signal (NLS) in the HMG box (Fig. 1A). Mitochondrial DNA transcription requires two transcription factors, mtTFB1 and mtTFB2 [1]. It is unknown whether nuclear components corresponding to these transcription factors are present. In view of the mtDNA packaging function of mtTFA, it is possible that chromatin-bound mtTFA may function not only as a transcription factor but also as an architectural component of nuclear chromatin.

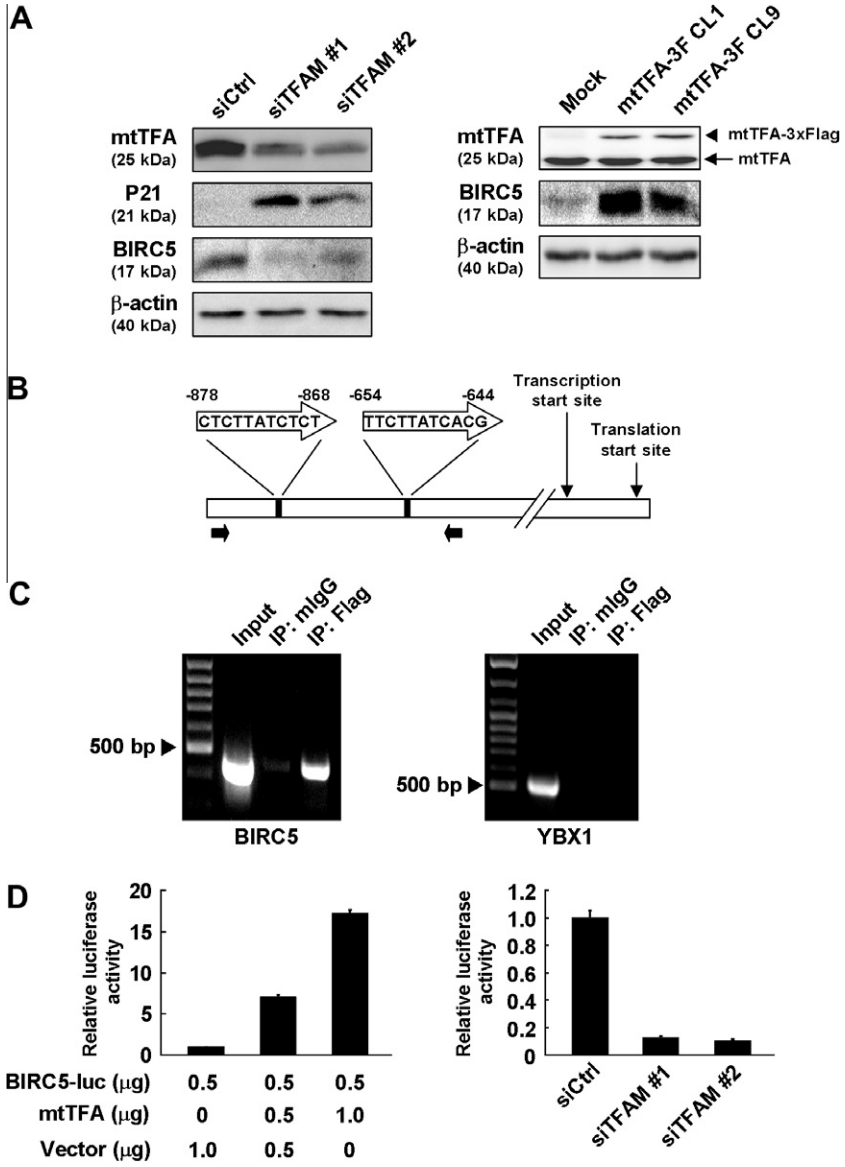

Fig. 3. The target genes of mtTFA. (A) Whole cell lysates (100 μg) of PC3 cells transfected with control siRNA (siCtrl) or *mtTFA*-siRNA (siTFAM) #1 or #2 (left panel) and mtTFA-overexpressing PC3 cell lines (mtTFA-3F CL1 and CL9) and a control cell line (Mock) (right panel) were subjected to SDS–PAGE, and Western blotting was performed with the indicated antibodies. (B) Two putative mtTFA binding sites are presented with the binding sequences. Two closed arrows present the position of the primers for ChIP analysis. (C) Chromatin immunoprecipitation assay of the mtTFA-overexpressing PC3 cell line (mtTFA-3F CL9). Immunoprecipitated DNAs were amplified by PCR using specific primer pairs for *BIRC5* and *YBX1* (as negative control). (D) BIRC5-luciferase reporter plasmid (0.5 μg) was co-transfected into 1×10^4 PC3 cells with indicated amounts of pcDNA3 vector or pcDNA3-mtTFA-3F (left panel) and 50 pmol of control siRNA (siCtrl) or *mtTFA*-siRNA (siTFAM) #1 or #2 (right panel). After 48 h, the luciferase activity was detected using a Picagene kit and the light intensity was measured using a luminometer. The results shown are normalized for protein concentration measured using the Bradford method, and are representative of at least three independent experiments.

Using DNA microarray analysis, we found that the expression of a subset of nuclear genes was regulated by mtTFA, especially apoptosis related genes (Supplementary Table 1). We also survey the mtTFA binding sites in the promoter region of these genes and found two putative mtTFA binding sites in the promoter region of BIRC5 gene. As reported previously, the role of BIRC5 in cancer pathogenesis is not limited to apoptosis inhibition but also involves regulation of the mitotic spindle checkpoints and the promotion of angiogenesis and chemoresistance [30]. Transfection of *BIRC5*-siRNA significantly inhibited the growth of gastric carcinoma cells [26]. We showed that the expression of BIRC5 is regulated by mtTFA expression. As shown in Fig. 4, mtTFA-overexpressing cells grew more rapidly than control cells, and down-regulation of mtTFA reduced the growth of cancer cells. The induction of G2/M phase is one of the functions of BIRC5 in the cell cycle [31,32]. Here, we showed that the population of G2/M phase cells was also increased in mtTFA-overexpressing cells. Taken together, the growth of cancer cells may be partly regulated by the mtTFA/BIRC5 pathway. Interestingly, knockdown of mtTFA expression induced p21-dependent G1 cell cycle arrest in PC3 cells which is null for p53 [33], indicating that mtTFA may be a promising target for cancer therapeutics, regardless of p53 status.

We have recently reported that mtTFA expression may be useful for predicting the clinical outcome of metastatic colorectal cancer patients treated with FOLFOX (chemotherapy protocol consisting of oxaliplatin, 5-fluorouracil and folinic acid) [14]. We also reported the relationship between mtTFA expression and clinicopathological variables in 276 endometrial carcinomas [13]. In summary, mtTFA may regulate nuclear gene expression and/or chromatin structure in addition to its mitochondrial role. Furthermore, the data presented here support the notion that mtTFA expression is involved in tumor progression, cancer cell growth

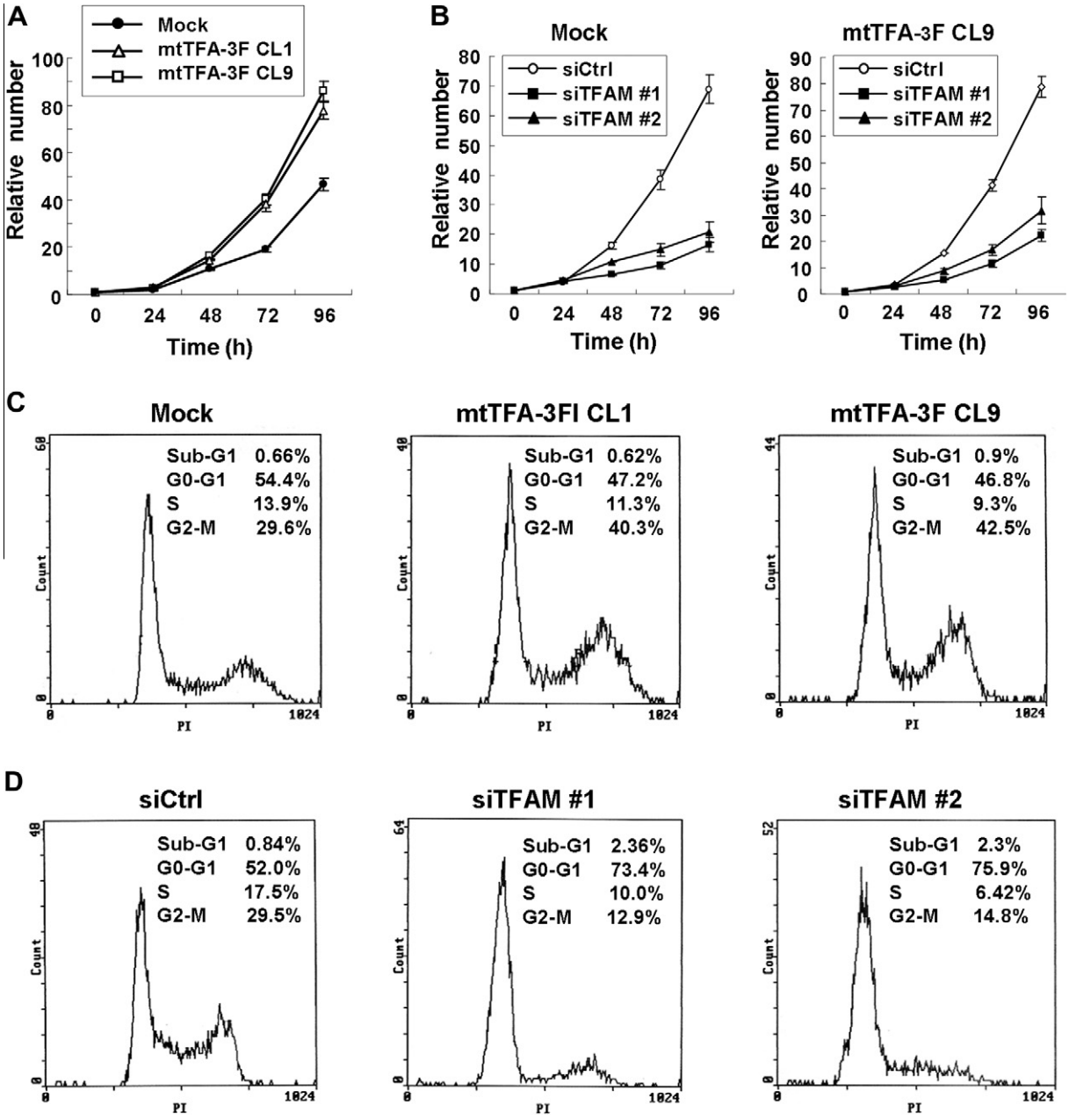

Fig. 4. Cancer cell growth in mtTFA-overexpressing and mtTFA knockdown cells. (A) 2×10^4 mtTFA-3F CL1, CL9 or mock cells were seeded onto 12-well plates and were counted every 24 h. Twenty-four hours after seeding was set as time zero. The data represent the mean ± SD of three independent experiments. (B) 2×10^4 mtTFA-3F CL9 or mock cells transfected with indicated siRNAs were seeded onto 24-well plates and counted every 24 h. The data represent the mean ± SD of three independent experiments. (C) mtTFA-3F CL1, CL9 and mock cells were stained with propidium iodide and analyzed by fluorescence-activated cell sorting (FACS). The cell cycle fraction is shown at the top right of each graph. (D) PC3 cells were transfected with indicated siRNAs. After 48 h, cells were stained with propidium iodide and analyzed by fluorescence-activated cell sorting (FACS). The cell cycle fraction is shown at the top right of each graph.

and chemoresistance. mtTFA is involved in these cellular functions by regulating target genes such as BIRC5.

Disclosure of potential conflicts of interest

There are no potential conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.03.114.

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