Transforming growth factor-β1-induced epithelial to mesenchymal transition increases mitochondrial content in the A549 non-small cell lung cancer cell line

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Abstract. The mitochondrial genome DNA copy number is critical for the functional maintenance of the mitochondria and energy acquisition for cell metabolism. Epithelial to mesenchymal transition (EMT) is an important process during embryonic development and has also been hypothesized to exhibit a significant role in cancer cell invasion and metastasis. In the present study, EMT was induced in the A549 non-small cell lung cancer (NSCLC) cell line, using transforming growth factor-β1 (TGF-β1) and changes in mitochondrial content, mitochondrial DNA (mtDNA) copy number and protein cytochrome *c* (Cyt *c*) were determined by reverse transcritpion-quantitative polymerase chain reaction and western blot analysis, respectively. mtDNA copy number and Cyt *c* protein levels were observed to increase following the induction of EMT in NSCLC cells. Results of the current study indicate that energy metabolism is adapted to facilitate EMT in NSCLC cells.

Introduction

At present, lung cancer is the most commonly diagnosed cancer and is the most common cause of cancer-related mortality, with an increasing number of diagnoses every day worldwide (1). Among the subtypes of lung cancer, non-small cell lung cancer (NSCLC) is the most common type, accounting for 85% of newly diagnosed cases (2). The majority of patients with NSCLC exhibit metastases in local lymph nodes or distant sites (3).

Epithelial to mesenchymal transition (EMT) has been hypothesized as a key step in determining the metastatic potential of cancer cells (4). During embryonic development, epithelial cells undergo EMT, in which epithelial cells transform into mesenchymal cells. During this transition, epithelial cells lose their epithelial characteristics, including cell polarity and specialized cell-to-cell contacts, and acquire mesenchymal characteristics. These characteristics include change from a cobblestone to elongated morphology and individual growth (5-7). At the molecular level, several specific markers of EMT have been identified with significantly altered levels of expression during EMT progression (8). For example, loss or gain of the cell adhesion molecules, E-cadherin, N-cadherin and vimentin, is considered to be the most important molecular marker of EMT. Several other EMT markers, including the transcription factors Snail (Snai1), Slug (Snai2), ZEB1 and ZEB2/Sip1 have been demonstrated to inhibit E-cadherin expression (9,10).

In *in vitro* studies, EMT is induced in cancer cells by transforming growth factor-β1 (TGF-β1). Induced cells exhibit altered expression of EMT markers, undergo morphological transitions and acquire characteristics of stem cells (11) and drug resistance (12,13).

Previous studies have revealed that EMT is a common cell behavior in NSCLC (14-17). When EMT occurs, multiple changes at the molecular and cellular levels occur to alter transcription profiling, the cell cycle or cell energy metabolism. Mitochondria are known as the energy producers of the cell and are able to adapt to conditions in various cellular contexts (18-20). The aim of the present study was to determine whether mitochondria number and mitochondrial DNA (mtDNA) copy number is modified during EMT in NSCLC cells.

Materials and methods

Cell culture. The A549 NSCLC cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco-BRL), 100 IU/ml penicillin (Sigma-Aldrich, St Louis, MO, USA), 100 μg/ml streptomycin (Sigma-Aldrich), 2 mM glutamine (Gibco-BRL) and 1 mM sodium pyruvate (Gibco-BRL) in a humidified incubator at 37˚C and 5% CO_2 atmosphere in 100-mm culture dishes.

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Induction of EMT using TGF-β1 in culture cells. EMT was induced by TGF-β1 as described previously (21). Briefly, at 70-80% confluence, A549 cells were trypsinized and seeded into 6-well plates in duplicate ($4x10^5$ cells/well). At 24 h, cells were cultured in EMT-induction medium [serum free, 10 ng/ml TGF-β1 and 100 ng/ml epithelial growth factor (EGF)] in a humidified incubator for an additional 48-72 h. Cells were monitored for morphological changes, including loss of cell-to-cell contact and transition from a cobblestone to elongated morphology, using a CKX31 microscope (Olympus Corporation, Tokyo, Japan).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Following induction of EMT, total RNA was extracted using TRIzol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. RNA concentration and quality were determined using a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A reverse transcription kit (Toyobo Co., Ltd., Osaka Japan) was used to generate cDNA from total RNA. qPCR was performed using a StepOne instrument (Applied Biosystems, Bedford, MA, USA) in a final volume of 25 μl (1 μl cDNA, 10.5 μl SYBR green PCR Master mix, 3 μl forward and reverse primers mix, 0.4 μl ROX dye and 10.1 μl distilled deionized water) using a Premix Ex Taq™ PCR kit (Perfect Real Time) (Takara Biotechnology, Co., Ltd., Dalian, China). The PCR conditions were set as follows: 95˚C for 5 min, and 40 cycles of 95˚C for 5 sec and 60˚C for 1 min. A melting curve analysis was then performed, with the temperature increasing from 60-90˚C, in increments of 0.3˚C. GAPDH was used as internal control. Primer sequences were as follows: Forward: 5'-ACCCAGAAGACTGTGGATGG-3' and reverse: 5'-TCTAGACGGCAGGTCAGGTC-3' for GAPDH; forward: 5'-TGCCCAGAAAATGAAAAAGG-3' and reverse 5'-GTGTATGTGGCAATGCGTTC-3' for E-cadherin; forward: 5'-ACAGTGGCCACCTACAAAGG-3' and reverse: 5'-CCGAGATGGGGTTGATAATGN-3' for N-cadherin; forward: 5'-CAGTGGGAGACCTCGAGAAG-3' and reverse: 5'-TCCCTCGGAACATCAGAAAC-3' for fibronectin; forward 5'-GAGAACTTTGCCGTTGAAGC-3' and reverse 5'-GCTTCCTGTAGGTGGCAATC-3' for vimentin; forward: 5'-CCTCCCTGTCAGATGAGGAC-3' and reverse 5'-CCAGGCTGAGGTATTCCTTG-3' for Snail; forward: 5'-GGAGTCCGCAGTCTTACGAG-3' and reverse: 5'-TCTGGAGGACCTGGTAGAGG-3' for Twist; and forward: 5'-GGGGAGAAGCCTTTTTCTTG-3' and reverse: 5'-TCCTCATGTTTGTGCAGGAG-3' for Slug (11).

Western blot analysis. EMT-induced A549 cells were washed twice in ice-cold PBS and lysed on ice using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM Na_3VO_4, 1 mM EDTA, 1 mM EDTA and protease inhibitor PMSF; Beyotime Institute of Biotechnology, Jiangsu, China). Protein concentration of lysates was determined using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology) and then several marker proteins of EMT were detected using SDS-PAGE electrophoresis. Antibodies against N-cadherin, E-cadherin, vimentin (11) and cytochrome *c* (Cyt *c*) were purchased from Abcam (Cambridge, UK) and Cell Signaling Technologies, Inc. (Danvers, MA, USA). Finally, protein bands were detected using the chemiluminescence detection kit (Beyotime Institute of Biotechnology).

Mitochondrial density of A549 EMT cells determined by MitoTracker green staining. A549 cells were seeded in 24-well plates (Corning Incorporated, Corning, NY, USA) and treated with EMT induction medium. Following induction of EMT, cells were stained with a MitoTracker Green kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions to determine mitochondrial density (22). Briefly, cells were washed twice in PBS and incubated at 37˚C with 50 nM MitoTracker Green probe for 30 min. Next, the staining buffer was removed and changed for fresh complete medium. Fluorescence was detected under a fluorescence microscope (Olympus, Tokyo, Japan).

Genome DNA extraction and qPCR analysis of mtDNA. mtDNA copy number in EMT-induced A549 cells was determined as described previously (23). Total genomic DNA was isolated using the PureGene kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. mtDNA content was determined in cells by qPCR using an SYBR green assay. The method for detection of mtDNA copy number was based on qPCR and utilized a 107 bp amplicon of mtDNA $tRNA^{Leu\ (UUR)}$ (forward: 5'-CACCCAAGAACAGGGTTTGT-3' and reverse: 5'-TGGCCATGGGTATGTTGTTA-3'). An 86 bp amplicon of β2-microglobulin (forward: 5'-TGCTGTCTCCATGTTTGATGTATCT-3' and reverse: 5'-TCTCTGCTCCCCACCTCTAAGT-3') was used to determine nuclear DNA (nDNA) as an internal control (23). qPCR was performed as follows: 1 cycle of 95˚C for 10 min; followed by 40 cycles of 95˚C 15 sec and 62˚C 30 sec; and melting curve acquisition at 50-95˚C and measuring points at 0.5˚C intervals and performed using a StepOne system (Applied Biosystems). qPCR analysis was performed in triplicate for each DNA sample. The expression of mtDNA copy number relative to nDNA was determined using the formula: $2x2^{\Delta CT}$ with ΔCT representing the difference in CT values between the β2-microglobulin gene and $tRNA^{Leu\ (UUR)}$.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical significance of differences was evaluated using an unpaired, non-parametric Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Induction of EMT in A549 NSCLC cells by TGF-β1. A number of previous studies have reported that exposure of the A549 NSCLC cell line to appropriate concentrations of TGF-β1 induces transition from an epithelial to mesenchymal phenotype (17,24,25). In the present study, A549 cells were cultured in serum free medium containing 10 ng/ml TGF-β1 and 100 ng/ml EGF. Following 48-72 h treatment, morphological changes of A549 cells were observed, including loss of epithelial morphology and gain of mesenchymal phenotype, i.e., elongated and individual appearance (Fig. 1A and B).

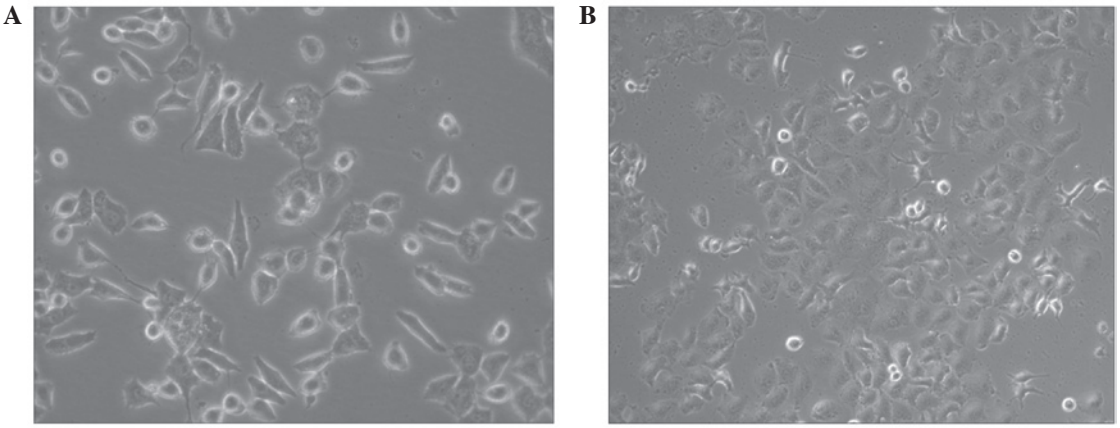

Figure 1. Morphological changes of A549 cells incubated with TGF-β1 (normal light, magnification x100). (A) A549 cells were treated with TGF-β1 and EGF for 72 or 96 h; (B) Control A549 cells were cultured in RPMI-1640 medium only. Cells incubated with TGF-β1 and EGF revealed mesenchymal characteristics, including elongated morphology and independent growth. TGF-β1, transforming growth factor-β1; EGF, epidermal growth factor.

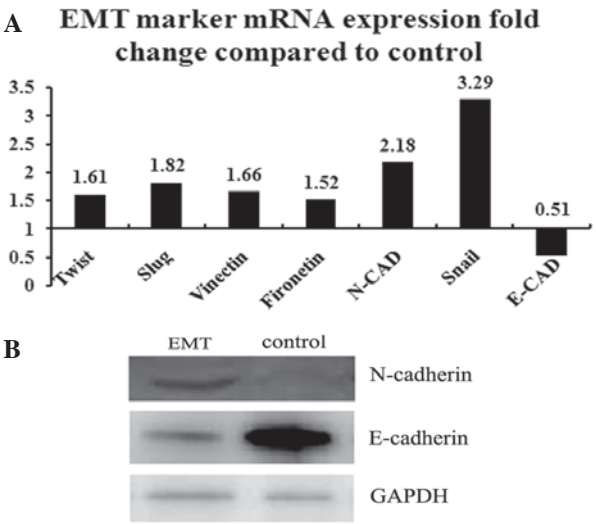

Figure 2. Expression of EMT markers. (A) Reverse transcription-quantitative polymerase chain reaction analysis of Twist, Slug, vimentin, fibronectin, N-CAD, Snail and E-CAD expression in EMT-induced A549 cells. (B) Western blot analysis of N-CAD and E-CAD protein expression. E-CAD decreased and N-CAD increased following induction of EMT. EMT, epithelial to mesenchymal transition; E-CAD, E-cadherin; N-CAD, N-cadherin.

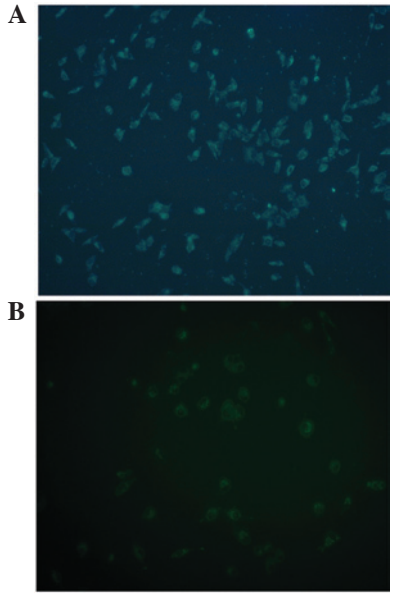

Figure 3. Mitochondria-specific MitoTracker Green staining. (A) EMT-induced A549 cells; (B) control cells. Cells were stained using a green probe and images were captured under a fluorescence microscope (magnification, x100). EMT-induced A549 cells reveal more positive staining than control cells. EMT, epithelial to mesenchymal transition.

TGF-β1-induced A549 cells exhibit altered expression of EMT markers at the mRNA and protein level. In addition to morphological changes, epithelial cells undergoing EMT lose expression of epithelial markers, including E-cadherin and gain expression of mesenchymal markers, such as N-cadherin, fibronectin and vimentin. Simultaneously, transitioned epithelial cells lose their polarity and become more fibroblast-like (26). Confirmation of A549 cell EMT was established at the molecular level. RT-qPCR and western blot analysis were performed to identify the changes in EMT markers. TGF-β1 induced expression of Snail, Slug and Twist transcription factors, as well as a decrease in E-cadherin expression and upregulation of vimentin and N-cadherin through direct suppression or indirect regulation (Fig. 2A). Results of western blot analysis were consistent with these observations (Fig. 2B). These results indicate that EMT was successfully induced in A549 cells.

MitoTracker Green staining of EMT A549 cells. A549 cells were incubated with TGF-β1 for EMT induction and then stained with MitoTracker Green, a specific mitochondrial dye. Fluorescence microscopy was used to observe and capture images of mitochondria in EMT and control A549 cells (Fig. 3). EMT cells were observed to exhibit increased fluorescence intensity compared with control. MitoTracker Green probe stains all mitochondria regardless of competency (27). Image analysis revealed that mitochondrial number increased during EMT in A549 cells.

Mitochondrial content determination: DNA copy number and Cyt c protein. Mitochondrial disorders are a reflection of complicated heterogeneous diseases, which may be caused by molecular or cellular defects. It is clear that a constant number of mtDNA copies is essential for homeostasis in cells. In the

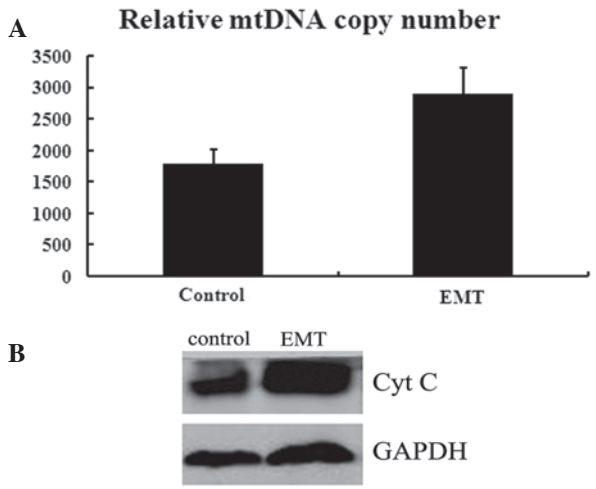

Figure 4. Mitochondrial content following induction of EMT in A549 cells. (A) mtDNA copy number was determined by quantitative polymerase chain reaction. EMT A549 cells revealed significantly increased mtDNA copy number compared with control. (B) Cyt *c* protein levels were measured by western blot analysis. GAPDH was used as a control. Relative Cyt *c* protein levels in EMT cells were increased compared with control cells. EMT, epithelial to mesenchymal transition; mtDNA, mitochondrial DNA; Cyt *c*, cytochrome *c*.

present study, changes in the copy number of mtDNA during EMT were determined. Total genomic DNA was extracted from cells and the relative ratio of mtDNA/nDNA was investigated by qPCR. Using nDNA as an internal control, the relative mtDNA copy number was identified as significantly increased from 1,700 to 2,800 compared with control A549 cells ($P<0.01$; Fig. 4A). To further determine variations in mitochondrial components, protein expression of the mitochondrial protein, Cyt *c*, was analyzed by SDS-PAGE. As demonstrated in Fig. 4B,Cyt *c* levels were augmented following induction of EMT, consistent with observations of mtDNA copy number. These results quantitatively demonstrate that mitochondrial components (mtDNA copy number and Cyt *c* protein) increase during EMT in the A549 NSCLC cell line.

Discussion

EMT induced by TGF-β1 is essential for stem cell differentiation and tissue and organ generation during normal mammalian development (6,7). In the tumor microenvironment, TGF-β1 may function as an autocrine or paracrine factor. An increasing number of studies have reported that EMT is involved in the mobility, invasion and migratory ability of cancer cells, providing these cells with enhanced metastatic properties (5,28). When the condition or state of cancer cells is altered, changes in the energy metabolism of these cells occur to adapt to the new status (29,30). Mitochondria are directly involved in cellular energy metabolism and mtDNA copy number and other components affect mitochondrial function (31,32).

In the present study, the A549 NSCLC cell line was induced to undergo EMT by exposure to TGF-β1 and EGF, which was accompanied with increased expression of mesenchymal specific protein markers and decreased expression of epithelial specific protein markers. Compared with control cells, A549 cells treated with TGF-β1 and EGF were successfully induced to undergo EMT, as determined by RT-qPCR and western blot analysis to identify the expression of EMT-specific markers.

Mitochondria are pivotal in cellular and subcellular mechanisms as they function as energy-generating factories. Human mtDNA has a circular double-stranded structure of ~16.6 kbp and codes for 13 proteins of the mammalian mitochondrial respiratory chain (Co I-III, Cytb, ND1-6, 4L, ATP6 and ATP8), 22 tRNAs and 2 rRNAs (23,33). A somatic mammalian cell contains 1,000-10,000 copies of mtDNA. Depletion or deficiency of mtDNA is known to cause several genetic diseases, including deficiencies in SUCLG1 in encephalomyopathy and thymidine phosphorylase in mitochondrial neurogastrointestinal encephalomyopathy and mutations in POLG, DGUOK, MPV17 and TWINKLE in the hepatocerebral form of mtDNA depletion syndrome (23).

In the current study, mitochondrial copy number and the number of mitochrondria were increased following induction of EMT in A549 cells. To the best of our knowledge, no studies have been performed on EMT and the mitochondria. When the cell phenotype is altered, coordinated internal mechanisms also occur in specific systems, including energy metabolism. mtDNA copy number is not random and is specific to the developmental stage of cells, particularly cancer cells (34). To date, the mechanism by which mtDNA copy number is regulated at various stages of the cell life cycle has remained unclear. In the current study, EMT was hypothesized to be accompanied by changes in energy metabolism. Therefore, copy number and Cyt *c* protein expression in A549 cells undergoing EMT were determined. Copy number decreased by ~50% compared with control A549 cells and mitochondrial content in EMT cells increased and Cyt *c* protein was observed to increase significantly.

Variations in mtDNA copy number are associated with a number of diseases (35). Xing *et al* (36) investigated the mtDNA content of patients with renal cell carcinoma and concluded that mtDNA content appeared to exhibit heritability and low mtDNA content was associated with increased risk of renal cell carcinoma. Blokhin *et al* (37) analyzed pathology-related variations in mtDNA copy number in the brains of patients with multiple sclerosis and found significantly higher mtDNA copy number values in neurons of normal-appearing gray matter than in cells of other multiple brain regions. In addition, numerous diseases are associated with a decreased mtDNA copy number, including liver diseases (38), biliary atresia (39), type 2 diabetes (40), cardiomyopathy (41) and breast cancer (41).

In the present study, EMT-induced NSCLC cells were observed to exhibit increases in mtDNA copy number and other mitochondrial contents. These events may represent energy preparation for EMT, a process hypothesized to be important for cancer cell migration, invasion and metastasis (12). In this study, mitochondrial content was altered, particularly the mtDNA copy number. However, the mechanisms involved in this change in mitochondrial content during EMT remain unclear and further studies are required. Although several models of the mechanism by which mtDNA copy number is regulated have been hypothesized, understanding of this process remains extremely limited (34). Results of the present study indicate that increased content of mitochondria may contribute to the increasing energy requirements of cancer cells undergoing EMT and that EMT-mediated metastasis of

malignant cells demands increased energy availability which would affect the subsequent cell behavior.

In the current study, the A549 NSCLC cell line, was induced to undergo EMT and mitochondrial content was found to increase significantly. These observations indicate that epithelial cells undergoing transition to mesenchymal-like cells undergo changes in the energy metabolism system, in additional to the well-known morphological changes. The mechanisms by which the energy system and mitochondria is altered during EMT requires further investigation and novel therapeutic targets for cancer may be identified.

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