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Complete List of Authors:	Feng, Feifei; College of Public Health, Zhengzhou University, Yang, Yongli; College of Public Health, Zhengzhou University, Li, Zhitao; Medical College, Henan Science and Technology University, Song, Jinyan; College of Public Health, Zhengzhou University, Zhu, Hansong; College of Public Health, Zhengzhou University, Wang, Lixia; College of Public Health, Zhengzhou University, Zhao, Yong; College of Public Health, Zhengzhou University, Xu, Dong; College of Public Health, Zhengzhou University, Wu, Yongjun; College of Public Health, Zhengzhou University, Wu, Yiming; College of Public Health, Zhengzhou University, wang, wei; zhengzhou university, colloge of public health

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Feifei Feng[a, #], Yongli Yang [b, #], Zhitao Li[c, #], Jinyan Song[d, #], Hansong Zhu[d], Lixia Wang[d], Yong Zhao[d], Dong Xu[d], Yongjun Wu[a], Yiming Wu[d], Wei Wang[d *]

The affiliations and addresses of the authors:

[a] Department of Toxicology, College of Public Health, Zhengzhou University, Zhenzhou, 450001, China.

[b] Department of Epidemiology and Biostatistics, College of Public Health, Zhengzhou University, Zhenzhou, 450001, China.

[c]Department of Immunology, Medical College, Henan Science and Technology University, Luoyang, 471003, China.

[d] Department of Occupational Health and Occupational Medicine, College of Public Health, Zhengzhou University, Zhenzhou, 450001, China.

# Equal contributors

Key words: Coal tar pitch; Telomere length; Telomerase activity; Telomere-associated proteins

***Corresponding Author:** Wei Wang, PhD, Department of Occupational Health and Occupational Medicine, College of Public Health, Zhengzhou University, Zhenzhou, 450001, China, email: ww375@126.com, phone: 86-0371-67781466, fax: 86-0371-67781868.

Running title: Telomere change in CTP-induced BEAS-2B cells

Abstract

Coal tar pitch is a confirmed human carcinogen and is composed mainly of polycyclic aromatic hydrocarbons. Coal tar pitch extract (CTPE) is toxic to humans and animals due to its ability to induce carcinogenesis, teratogenesis, and mutagenesis. The aim of this study was to explore the effects of CTPE on BEAS-2B cells in vitro and the mechanism responsible for the changes in telomere length and telomerase activity in lung cancer. In this study, BEAS-2B cells were divided into three groups: the Blank, DMSO and CTPE group, we found that the telomere length shortened significantly and telomerase activity increased in cells in CTPE group at passage 20 and 30 compared to cells in CTPE group at passage 10. In addition, the gene and protein expression levels of POT1, TRF1 in BEAS-2B cells in CTPE group at passage 20 and 30 were significantly reduced, and the gene and protein expression levels of TRF2 were increased, compared to those in cells in CTPE group at passage 10. CTPE can shorten telomere DNA and increase telomerase activity in human bronchial epithelial cells, the phenomenon of chromosomal instability may be related with the lower expression of POT1 and TRF1 and the higher expression of TRF2.

Introduction

Coal tar pitch, a confirmed human carcinogen, is the waste residue generated in the process of coal tar distillation[1], and it accounts for approximately 54~56% of the total amount of processed coal tar. The composition of coal tar pitch is complicated, and there are some differences in its physical properties. The main chemical component of coal tar pitch is polycyclic aromatic hydrocarbons (PAHs) such as anthracene, phenanthrene and pyrene[2]. Coal tar pitch (CTP) is toxic to human beings and animals due to its ability to induce carcinogenesis of lung, teratogenesis, and mutagenesis[3-5].

A shortened telomere length is a predictive marker of lung cancer risk[6]. The telomere is a special DNA protein structure that is located at the end of linear chromosomes, and its main function is to prevent DNA degradation and protect chromosomes against instability. Telomeric repeats are subject to progressive shortening with each cell division. This phenomenon is related to the end replication problem and may serve as a mitotic clock to determine the replicative capacity of the cell[7]. In contrast to normal somatic cells, immortal cells that have overcome the cellular senescence blockade acquire the ability to stabilize their telomere length by expressing telomerase[8]. It has been suggested that telomerase activity is essential for cell immortalization and tumor progression, although the mechanism underlying its activation and regulation remain unknown. There is a large negative correlation between

the length of the telomere and telomerase activity.

Telomerase is expressed in most human cancers including lung cancer. Lung cancer cells could avoid the progressive attrition of telomeres by expressing telomerase. Early studies have demonstrated the activity of telomerase in most primary lung cancer samples[9-10]. Several studies have shown that a shortened telomere length and high levels of telomerase activity occur in lung cancer and that telomerase and the telomere complex play a key role in lung tumor progression[11-13]. To explore changes in telomere length and telomerase activity in Coal tar pitch extract (CTPE)-induced BEAS-2B cells and the related mechanism, we examined the telomere length, telomerase activity, and gene and protein expression profiles of three telomere-associated proteins [protection of telomeres 1 (POT1), telomeric repeat-binding factor 1 (TRF1), telomeric repeat-binding factor 2, (TRF2)] in CTPE-induced BEAS-2B cells with malignant transformation[14] at passage 10, 20 and 30. Our findings provide new evidence for the prevention of lung carcinogenesis induced by coal tar pitch in occupational settings.

Materials and methods

Preparation of CTPE solution

Coal tar pitch was obtained from the coking plant at Anyang Iron and Steel Company (Henan, China) and stored at room temperature. It was ground into powder with a diameter of 10~20 μm and then added to a beaker, which was placed in an exhaust hood

with a flat-panel heater. The smoke of the coal tar pitch was generated at 400□ and collected using a dust sampler with a nitrocellulose filter membrane. The flow rate was 20 L/min, and the sampling was performed three times for a duration of 40 min each. The filter membrane was then weighed, cut into pieces and dissolved in 350 mL of dichloromethane in a flask with a plug by supersonic vibration for 40 min. The solution was filtered using a sand core funnel to produce crude extracts, which were further dried in a baking oven at 45□. After it was completely dried, the smoke extracts were dissolved in dimethylsulfoxide (DMSO) to a final concentration of 2.0 mg/mL[15].

Cell culture

BEAS-2B cells are a SV40 hybrid (Ad12 SV40) transformed human bronchial epithelial cell line[15]. These cells were cultured in standard medium (RPMI 1640 containing 10% fetal bovine serum) in at 37□ with 5% CO_2. When the adherent monolayer was 90% confluent in the flask, the medium was discarded with a pipette, and the cells were rinsed with cold 1× PBS and detached with 0.25% trypsin. The cells were inoculated and incubated at a density of 2×10^5 cells/mL, and were sub-cultured every six days.

Established malignant transformation cell model

The cytotoxic effect of CTPE was assessed using the MTT assay according to Mosmann[16]. The immortalized human bronchial epithelial cell line BEAS-2B was treated with 2.0 mg/L of CTPE for 72 hours. The first passage of the BEAS-2B cells was counted as passage 0 and was followed by CTPE treatment. The cells were then

sub-cultured in standard medium (Blank), and DMSO served as the vehicle control.

Fluorescence-based Quantitative PCR (SYBR Green I)

DNA extraction was performed by SDS-phenol-chloroform. According to the detection method of telomere length from literature[17], primers of telomere length and internal reference are as follows: Tel1 5′-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT -3′, Tel2 5′-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3′. 36B4u 5′-CAGCAAGTGGGAAGGTGTAATCC-3′, 36B4d 5′-CCCATTCTATCATCAACGGGTACAA-3′.

The final concentrations of PCR reagents （20ul）consisted of the following: 150 nM ROX and 0.2×Sybr Green I, 15 mM Tris–HCl pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM each dNTP, 5 mM DTT, 1% DMSO and 1.25U GOTaq Gold DNA polymerase. The final telomere primer concentrations were: tel 1, 270 nM; tel 2, 900 nM. The thermal cycling profile for both amplicons began with a 95℃ incubation for 5 min, there followed 18 cycles of 95℃ for 15 s, 54℃ for 2 min. For 36B4 PCR, there followed 30 cycles of 95℃ for 15 s, 58℃ for 1 min.

RNA extraction was performed with TRIzol. Reverse transcription was performed using an Omniscript RT-PCR kit. The RNA was quantified using a UV spectrophotometer, and the RNA purity was determined by the A260/A280 ratio. The integrity was assessed by gel electrophoresis. Using ß-actin as an internal reference, primers were designed

using software PP5.0 and synthesized according to the POT1, TRF1, TRF2, and ß-actin mRNA sequences in GeneBank by Shanghai Biological Technology Service Co. Ltd. The primers were then diluted to 50 μmol/L in sterile double-distilled water and stored at -20℃. The sequences of the primers for these four genes were as follows: POT1, forward primer, 5′-TCAGATGTTATCTGTCAATCAGAACCT-3′, reverse primer, 5′-TGTTGACATCTTTCTACCTCGTATAAT-3′ (86bp amplicon); TRF1, forward primer, 5′- GCAACAGCGCAGAGGCTATTAT-3′, reverse primer, 5′-AGGGCTGATTCCAAGGGTGTA-3′ (160bp amplicon); TRF2, forward primer, 5′-ACCAGGGCCTGTGGAAAAG-3′, reverse primer, 5′-GCACCAGACAGAGTCTTGAAAGC-3′ (111bp amplicon); ß-actin, forward primer 5′-ATCATGTTTGAGACCTTCAACA-3′, reverse primer 5′-CATCTCTTGCTCGAAGTCCA-3′ (231bp amplicon).

The PCR reaction (30 μL) for the POT1, TRF1, TRF2, and ß-actin gene amplification consisted of the following: 18.86 μL of DEPC, 6 μL of 5× buffer, 0.5 μL of dNTPs (10 mmol/L), 0.2 μL of GOTaq DNA polymerase, 0.12 μL of sense primer (50 μM), 0.12 μL of antisense primer (50 μM), 0.2 μL of 50× SYBR Green Ⅰ and 4 μL of DNA template (10 ng/μL). The thermal cycling profile consisted of an initial denaturation at 95℃ for 5 min followed by 35 cycles at 95℃ for 15 s and 58℃ for 45 s. Following amplification, a melting curve was created to confirm the specificity of the reaction.

Telomeric Repeat Amplification Protocol (TRAP)

Telomerase activity assay was assessed according to the telomeric repeat amplification protocol (TRAP)[18-19]. Firstly, telomerase extension products were amplified using PCR, telomerase synthesized telomeric repeats onto the nontelomeric oligonucleotide TS (5′-AATCCGTCGAGCAGAGTT-3'), and the reaction (30 μL) for the telomerase amplification included the following: 19.9 μL of DEPC, 6 μL 5× buffer, 0.5 μL of dNTPs (10 mmol/L), 0.5 μL of RNase inhibitor (40 U/μl), 0.1 μL of TS primer (10 μmol), and 3 μL of telomerase template. The elongation parameters were 30℃ for 20 min. And then, such telomerase products are specifically amplified by PCR with the downstream primer CX (5'-CCCTTACCCTTACCCTTACCCTAA-3') and the upstream primer TS (5′- AATCCGTCGAGCAGAGTT-3'), which could measure the level of telomerase (Telomerase activity).

Immunohistochemistry Analysis

The expression of three telomere-associated proteins was determined using immunohistochemistry. Coverslips were placed in 6-well culture plates. BEAS-2B cells at passage 30 in logarithmic growth phase in each group were collected as a single cell suspension, and the concentration of cells was adjusted to 1×10^6 cells/mL. The cells were planted to 6-well plates as 1×10^5 cells/well. The SP method was used to stain the cells on cover slip for immunohistochemistry when the cells grew to 80% confluency. PBS served as the negative control. The images were recorded with a camera, and the

average optical density (AOD) for the protein expression was analyzed using Image-Pro Plus 6.0 software.

Statistical Analysis

The experimental results were processed and analyzed using SPSS12.0 statistical software (SPSS, Chicago, IL.). Normally distributed data are expressed as the mean ± standard deviation ($\bar{x} \pm s$). Differences among groups were examined for statistical significance using one-way analysis of variance, and differences between two groups were examined using the LSD test. All of the statistical tests were two-sided, and the level of statistical significance was set at $\alpha=0.05$.

Results

Changes in telomere length and telomerase activity in BEAS-2B cells induced by CTPE

As shown in **Figure 1**, there were no significant differences in telomere length and telomerase activity in BEAS-2B cells among the Blank, DMSO and CTPE group at passage 10; however, at passages 20 and 30, the telomere length of CTPE-induced cells was significantly shorter than that in Blank or DMSO group; In contrast, the telomerase activity of BEAS-2B cells with CTPE stimulation was increased compared with that of the cells in Blank or DMSO group.

The telomere length and telomerase activity of BEAS-2B cells at passages 10, 20 and

30 (**Figure 2**) were not significantly different in the Blank or DMSO group. However, in CTPE group, the telomere length decreased and the levels of telomerase activity increased in BEAS-2B cells at passages 20 and 30 in comparison to passage 10. Furthermore, the telomere length in CTPE-induced cells at passage 30 was shorter than that at passage 20.

mRNA levels of POT1, TRF1 and TRF2 in BEAS-2B cells in different groups at passages 10, 20 and 30

Figure 3 shows that the levels of POT1 and TRF1 mRNA in CTPE-induced BEAS-2B cells decreased significantly, while the levels of TRF2 mRNA in cells stimulated with CTPE increased, as compared with those of cells in the Blank or DMSO group.

A comparison of the mRNA levels of POT1, TRF1 and TRF2 at passages 10, 20 and 30 (**Figure 4**) revealed decreased mRNA levels of POT1 and TRF1 and increased mRNA levels of TRF2 in CTPE-induced cells at passages 20 and 30, as compared with passage 10. In addition, there were significant differences in the levels of TRF1 and TRF2 mRNA in CTPE-induced cells at passage 30 compared with passage 20. However, there were no significant differences in the levels of POT1 mRNA in CTPE-induced cells between passages 20 and 30.

Protein levels of POT1, TRF1 and TRF2 in BEAS-2B cells in different groups at passages 10, 20 and 30

The protein expression of POT1, TRF1 and TRF2 in BEAS-2B cells at passage 30 in the different groups was determined using cell immunohistochemistry. When we chose cell immunohistochemistry analysis to detect the protein levels of POT1, TRF1 and TRF2 in BEAS-2B cells, we wanted to not only detect the protein levels, but also the position of these three proteins in cells. The images in **Figure 5** are the representatives of POT1, TRF1 and TRF2 protein expression in BEAS-2B cells at passage 30. From these pictures, we can see that the yellow-brown color of POT1 and TRF1 protein in cytoplasm of CTPE-induced cells was lighter than those of BEAS-2B cells with blank or DMSO, but the yellow-brown color of TRF2 in cytoplasm of BEAS-2B cells was darker than that in blank or DMSO group at passage 30. The average optical density (AOD) for the protein expression of POT1, TRF1 and TRF2 in BEAS-2B cells for the different groups and different passages is illustrated in **Figure 6** and **Figure 7**.

The protein expression levels of POT1, TRF1 and TRF2 in BEAS-2B cells were consistent with their mRNA expression levels. As shown in **Figure 6**, at passage 20 and 30, the protein levels of POT1 and TRF1 in CTPE-induced BEAS-2B cells decreased significantly, while those of TRF2 in BEAS-2B cells stimulated with CTPE increased, as compared with the cells in the Blank or DMSO group.

Figure 7 shows decreased protein levels of POT1 and TRF1 and increased protein levels of TRF2 in CTPE-induced cells at passages 20 and 30 compared with CTPE-treated BEAS-2B cells at passage 10. Furthermore, there were significant

differences in the protein levels of TRF1 and TRF2 in CTPE-induced BEAS-2B cells at passage 30 compared with passage 20. However, there were no significant differences in the levels of POT1 protein in CTPE-induced cells between passages 20 and 30.

Discussion

Professional who work in the CTP production process are exposed to CTP, and long-term exposure causes respiratory damage. Epidemiological studies of aluminum smelter workers have demonstrated an association between exposure to CTP and the risk of lung cancer [20]. At present, a few studies have investigated CTP occupational exposure as a cause of lung cancer[21-23]. Many studies have shown that telomeres and telomerase play an important role in the development of lung cancer[24-26]. Knockdown of telomerase activity results in accelerated telomere shortening and has a notable impact on lung structure and cellular compartments[27]. Telomerase plays a pivotal role in telomere protection in both normal and cancer cells. Protected telomeres ensure normal chromosomal segregation during mitosis while concomitantly endowing genetically abnormal cancer cells with immortality. The current hypothesis regarding telomere involvement in cancer states that proliferative preneoplastic cells suffer from persistent telomere shortening that leads to massive senescence in all but a few positively selected cells, which are then able to bypass senescence by altering their DNA damage via mutation or silencing of related proteins[28]. These cells have an extended life span and

continue to lose telomere fragments until their telomeres become dysfunctional, causing genomic instability and subsequent apoptosis. Most somatic cells do not have detectable telomerase activity, mainly due to a lack of telomerase expression. However, stem and embryonic cells express telomerase to prevent telomere attrition[29]. Because of its importance for cell fate, the length of the telomere is finely regulated. Telomerase activity is the main mechanism responsible for telomere maintenance, and thus, telomerase activity itself is also carefully controlled.

Telomerase plays a key role in the maintenance of telomere length and chromosome integrity[12]. Many malignant tumors exhibit telomerase activity and thereby telomere lengthening capacity[13]. Several studies have been published regarding the expression of telomerase in preneoplastic lesions in lung cancer and other tumors. In general, these reports show an increase in telomerase expression in late dysplastic lesions[12, 30]. The mechanisms underlying telomerase activation in lung cancer cells are not yet known. The expression of other telomere-associated proteins has also been reported to be altered in lung cancer, and studies utilizing quantitative polymerase chain reaction studies have shown that TRF1 expression is reduced in non-small cell lung cancer (NSCLC) tissues when compared with the adjacent normal tissues[31]. One study indicated that a shorter telomere length is associated with chronic PAHs exposure[6]. The shortened, dysfunctional telomere might activate a DNA damage response pathway and lead to cell cycle arrest and a senescent phenotype[32]. We used an in vitro experiment to

simulate the environment of occupational workers who were exposed to coal tar pitch. The results demonstrated that at passages 20 and 30, the telomere length of CTPE-induced cells was significantly shorter and the telomerase activity of BEAS-2B cells with CTPE stimulation was increased compared with those of the cells in Blank or DMSO group. (**Fig. 1**). This phenomenon suggests that shortening telomeres are associated with elevated telomerase activity, and thus, telomeres and telomerase play key roles in chromosome instability due to end-to-end fusion, recombination, and degradation; that is to say, telomeres are critical for the maintenance of genome integrity [33].

Increased telomerase activity will repair telomere shortening caused by cell division. TRF1, TRF2 and POT1 are telomere-associated proteins that are part of the telomere structure and play essential roles in controlling the length of the telomere[11]. One study assessed the expression of three telomere-associated proteins in normal and tumor tissues of lung cancer patients and demonstrated a significant down-regulation of TRF1 in tumor samples and no significant differences in the expression of TRF2 and POT1 between tumor and normal tissues[31]. The long-term overexpression of TRF1 in a telomerase-positive tumor cell line resulted in gradual and progressive telomere shortening, whereas telomere elongation was induced by the expression of a dominant-negative form of TRF1. TRF2 is also a negative regulator of telomere length, and overexpression of TRF2 results in a progressive shortening of telomere length.

More importantly, TRF2 protects human telomeres against end-to-end fusions, and thus, TRF2 plays a key role in maintaining telomere integrity[34].

We observed decreased mRNA and protein levels of POT1 and TRF1 (**Fig. 4A, 4B; Fig. 7A, 7B**) and increased levels of TRF2 (**Fig. 4C; Fig. 7C**) in CTPE-induced cells at passage 20, and in particular, at passage 30. The change in TRF1, TRF2, and POT1 expression was consistent with the telomere shortening. Some studies have suggested that TRF1 and TRF2 are down-regulated in tumor tissues[35], whereas others have shown that TRF1 or TRF2 was up-regulated[36]. The decrease in POT1 mRNA and protein expression levels indicates reduced telomere protection but, concomitantly, up-regulated telomerase activity. One study showed that the stage of the tumor and the length of the telomere might also influence POT1 expression in cancer[37]. Another study indicated that in stage I/II gastric cancer, POT1 is mostly down-regulated, and in stage III/IV gastric cancer, POT1 is frequently up-regulated. In addition, the expression of POT1 decreases as the telomere shortens[37]. The decrease in TRF1 mRNA and protein expression in the CTPE group may result in the activation or up-regulation of telomerase activity to lengthen shortened telomeres. TRF2 may have a protective role in lung cancer progression[32]. The increase in TRF2 mRNA and protein expression indicates that TRF2 may negatively regulate the telomere length and maintain its structure. As shown in **Fig.3, Fig. 5 and Fig.6**, the gene and protein expression of POT1 and TRF1 were reduced, and the expression of TRF2 was elevated in the CTPE group

compared to the Blank and DMSO groups. The changes in the expression levels of POT1, TRF1, and TRF2 have a concerted influence on the dynamic balance between the structure and function of telomeres, which play an important role in the continuous contraction of telomeres during the transformation of BEAS-2B cells produced by CTPE. However, the changes in the three proteins may lead to an increase in telomerase activity during the process of cell, thus continuous telomere shortening was observed that triggers chromosome end-to-end fusion and breakage-fusion-bridge cycles. These processes result in chromosome instability and malignant changes in cells.

In the present study, POT1 and TRF1 expression levels in BEAS-2B cells were down-regulated and TRF2 was up-regulated in response to CTPE exposure. However, the mechanism responsible for these phenomena is not clear. Therefore, further studies will be conducted in which these proteins will be blocked or stimulated to observe their relationship with telomere length and telomerase activity, with the goal of determining whether they are initiator factors for changes in telomere length and telomerase activity induced by CTPE.

Conclusion

CTPE can shorten telomere DNA and increase telomerase activity in human bronchial epithelial cells, the phenomenon of chromosomal instability may be related with the lower expression of POT1 and TRF1 and the higher expression of TRF2.

There is no any potential conflict.

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Figure legends

Fig. 1 The change of telomere length (A-a, b, c) and telomerase activity (B-a, b, c) of BEAS-2B cells in different groups at passage 10, 20 and 30.

(**A-a and B-a**): There was no significant difference on telomere length and telomerase activity of BEAS-2B cells among blank, DMSO and CTPE group at passage 10. (**A-b and A-c**): The telomere length of CTPE-induced cells was significantly lower than that of BEAS-2B cells with blank or DMSO at passage 20 and passage 30. (**B-b and B-c**): The telomerase activity of BEAS-2B cells was increased compared with that of cells in blank or DMSO group at passage 20 and passage 30. (n=3, *: vs Blank, $P<0.05$; #: vs DMSO, $P<0.05$). Phenotypes were duplicated.

Fig. 2 The change of telomere length (A-a, b, c) and telomerase activity (B-a, b, c) of BEAS-2B cells among different passages in blank, DMSO and CTPE groups.

(**A-a, b** and **B-a, b**): The levels of telomere length and telomerase activity were no significant difference among different passages in Blank or DMSO group. (**A-c and B-c**): The levels of telomere length and telomerase activity in CTPE-induced cells were significant changed at passage 20 and passage 30 comparing with those in CTPE-treated BEAS-2B cells at passage 10. (n=3, Δ: vs passage 10, $P<0.05$; ◆: vs passage 20, $P<0.05$) . Phenotypes were duplicated.

Fig. 3 The mRNA expression of POT1, TRF1 and TRF2 of BEAS-2B cells in different groups at passage 10, 20 and 30.

(**A-a, B-a and C-a**): There was no significant difference on the mRNA levels of POT1, TRF1 and TRF2 of BEAS-2B cells among blank, DMSO and CTPE group at passage 10. (**A-b**, **A-c and B-b, B-c**): The levels POT1 mRNA and TRF1 mRNA of CTPE-induced cells were significantly lower than those of BEAS-2B cells with blank or DMSO at passage 20 and passage 30. (**C-b** and **C-c**):The mRNA levels of TRF2 of BEAS-2B cells was increased compared with that of cells in blank or DMSO group at passage 20 and passage 30. (n=3, *: vs Blank, $P<0.05$; #: vs DMSO, $P<0.05$). Phenotypes were duplicated.

Fig. 4 The mRNA expression of POT1, TRF1 and TRF2 of BEAS-2B cells among different passages in blank, DMSO and CTPE groups.

(**A-a, A-b**, **B-a, B-b, C-a and C-b**): The levels of POT1, TRF1 and TRF2 mRNA were no significant difference among different passages in Blank or DMSO group. (**A-c, B-c and C-c**): The mRNA levels of POT1, TRF1 and TRF2 in CTPE-induced cells were significant changed at passage 20 and passage 30 comparing with those in CTPE-treated BEAS-2B cells at passage 10. (n=3, Δ: vs passage 10, $P<0.05$; ◆: vs passage 20, $P<0.05$) . Phenotypes were duplicated.

Fig. 5 Representatives of POT1, TRF1 and TRF2 protein expression in BEAS-2B cells at passage 30 in Blank, DMSO and CTPE groups (×400)**.** (A-C): POT1 protein expression in BEAS-2B cells at passage 30 in Blank, DMSO and CTPE groups; (D-F): TRF1 protein expression in BEAS-2B cells at passage 30 in Blank, DMSO and CTPE groups; (G-I): TRF2 protein expression in BEAS-2B cells at

passage 30 in Blank, DMSO and CTPE groups.

Fig. 6 The protein expression of POT1, TRF1 and TRF2 of BEAS-2B cells in different groups at passage 10, 20 and 30.

(**A-a, B-a and C-a**): There was no significant difference on the protein levels of POT1, TRF1 and TRF2 of BEAS-2B cells among blank, DMSO and CTPE group at passage 10. (**A-b**, **A-c and B-b, B-c**): The levels POT1 and TRF1 protein of CTPE-induced cells were significantly lower than those of BEAS-2B cells with blank or DMSO at passage 20 and passage 30. (**C-b** and **C-c**):The protein levels of TRF2 of BEAS-2B cells was increased compared with that of cells in blank or DMSO group at passage 20 and passage 30. (n=3, *: vs Blank, $P<0.05$; #: vs DMSO, $P<0.05$). Phenotypes were duplicated.

Fig. 7 The protein expression of POT1, TRF1 and TRF2 of BEAS-2B cells among different passages in blank, DMSO and CTPE groups.

(**A-a, A-b**, **B-a, B-b, C-a and C-b**): The levels of POT1, TRF1 and TRF2 protein were no significant difference among different passages in Blank or DMSO group. (**A-c, B-c and C-c**): The protein levels of POT1, TRF1 and TRF2 in CTPE-induced cells were significant changed at passage 20 and passage 30 comparing with those in CTPE-treated BEAS-2B cells at passage 10. (n=3, Δ: vs passage 10, $P<0.05$; ◆: vs passage 20, $P<0.05$) . Phenotypes were duplicated.

Figure 1

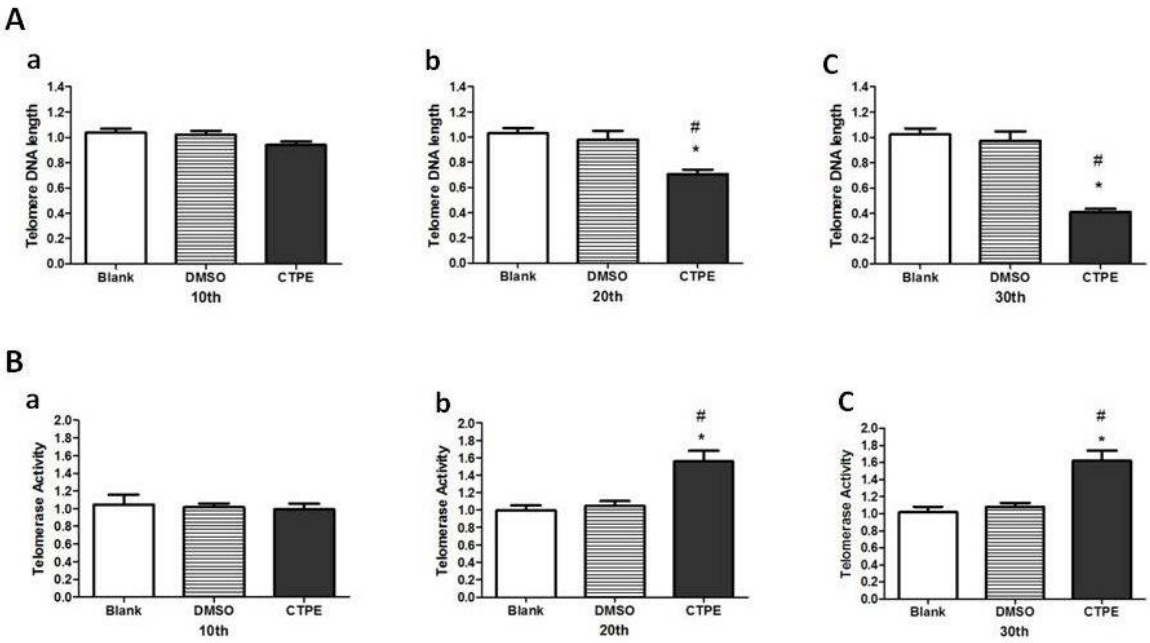

A
a
b
C
Telomere DNA length
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Blank
DMSO
CTPE
10th
20th
30th
#
*
B
a
b
C
Telomerase Activity
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0

Figure 2

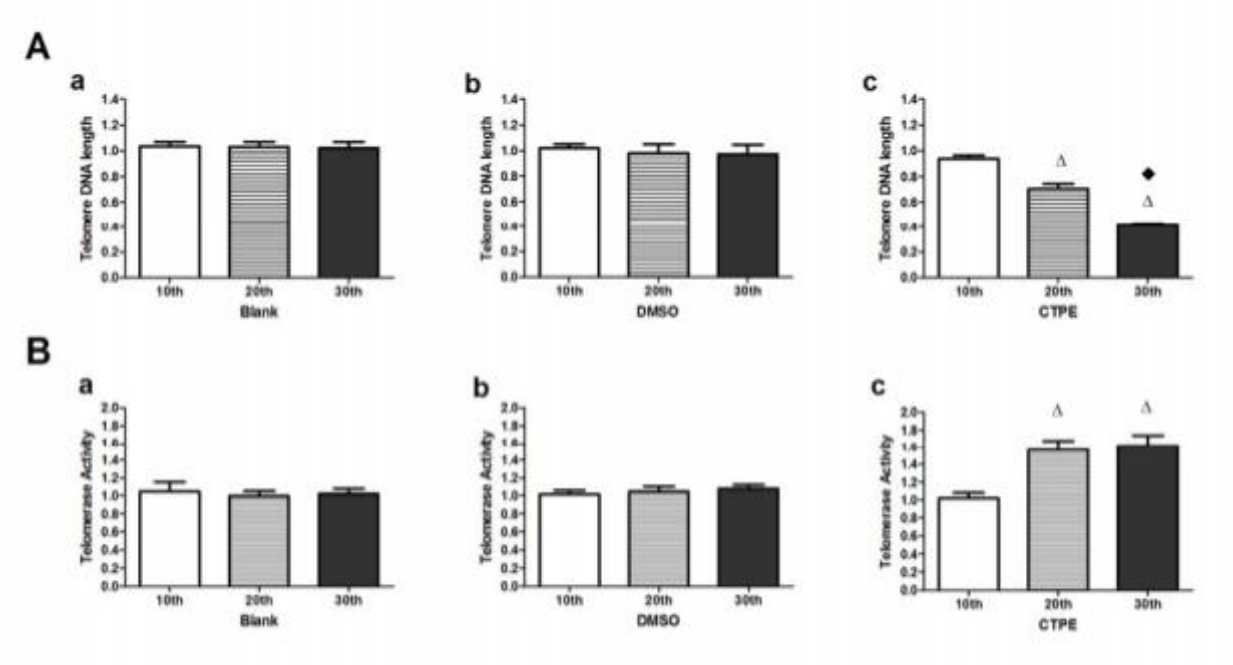

A
a
b
c
Telomere DNA length
10th
20th
30th
Blank
DMSO
CTPE
B
Telomerase Activity

Figure 3

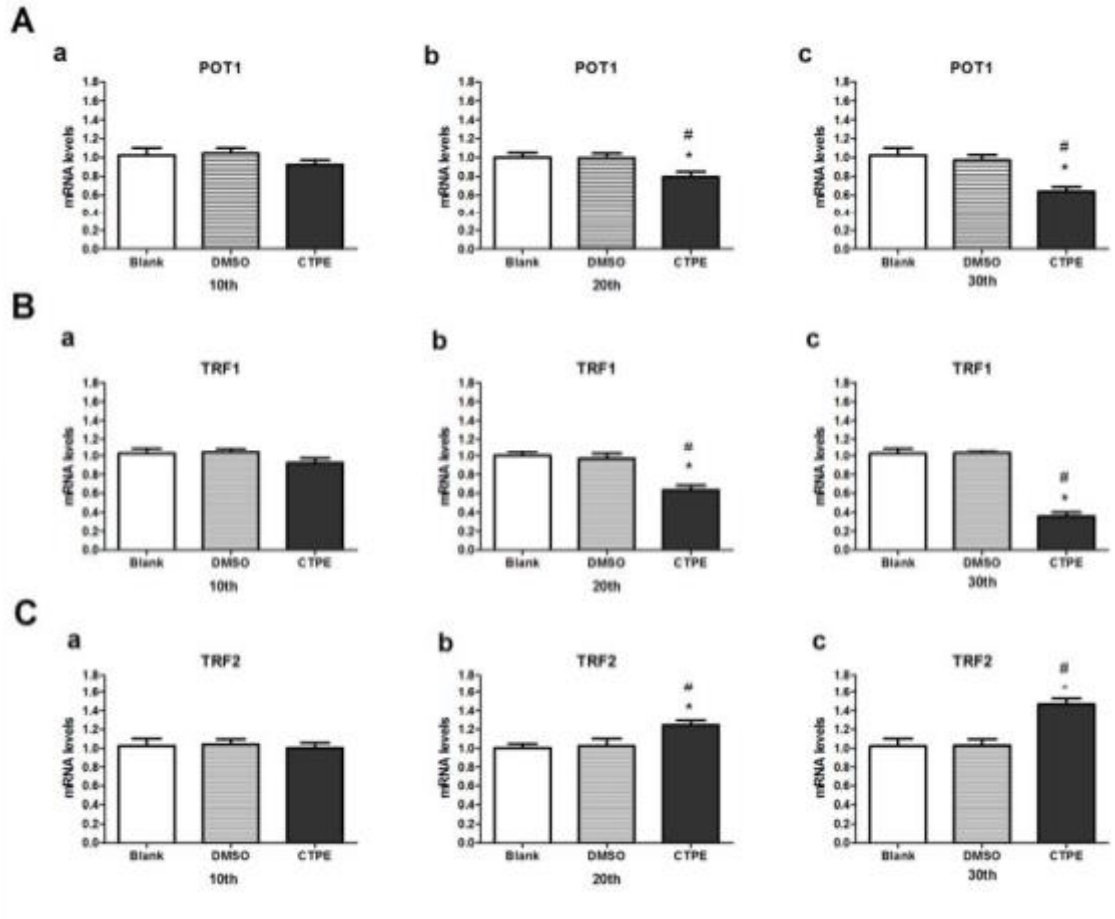

A
a
POT1
b
POT1
c
POT1
B
a
TRF1
b
TRF1
c
TRF1
C
a
TRF2
b
TRF2
c
TRF2
mRNA levels
Blank
DMSO
CTPE
10th
20th
30th

Figure 4

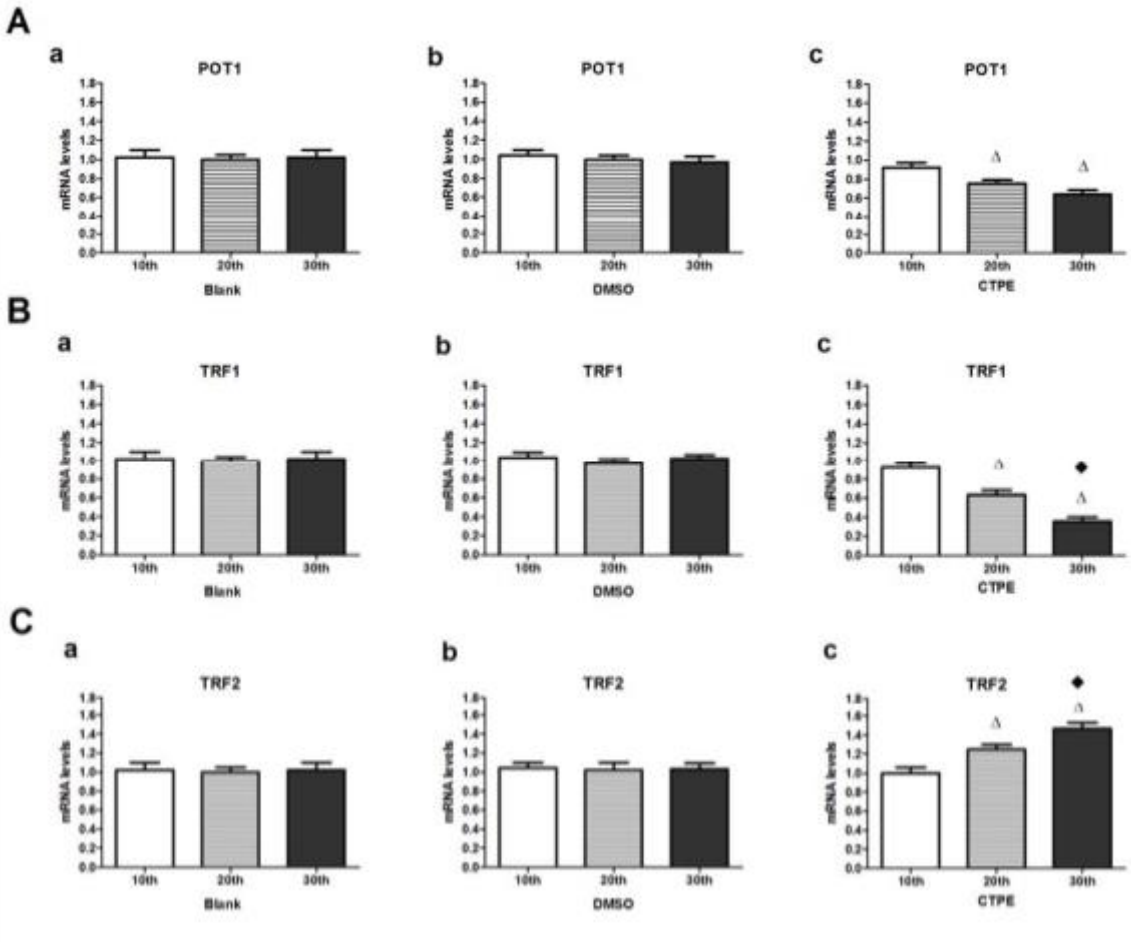

A
a
POT1
mRNA levels
10th
20th
30th
Blank
b
POT1
DMSO
c
POT1
CTPE
B
a
TRF1
Blank
b
TRF1
DMSO
c
TRF1
CTPE
C
a
TRF2
Blank
b
TRF2
DMSO
c
TRF2
CTPE

Figure 5

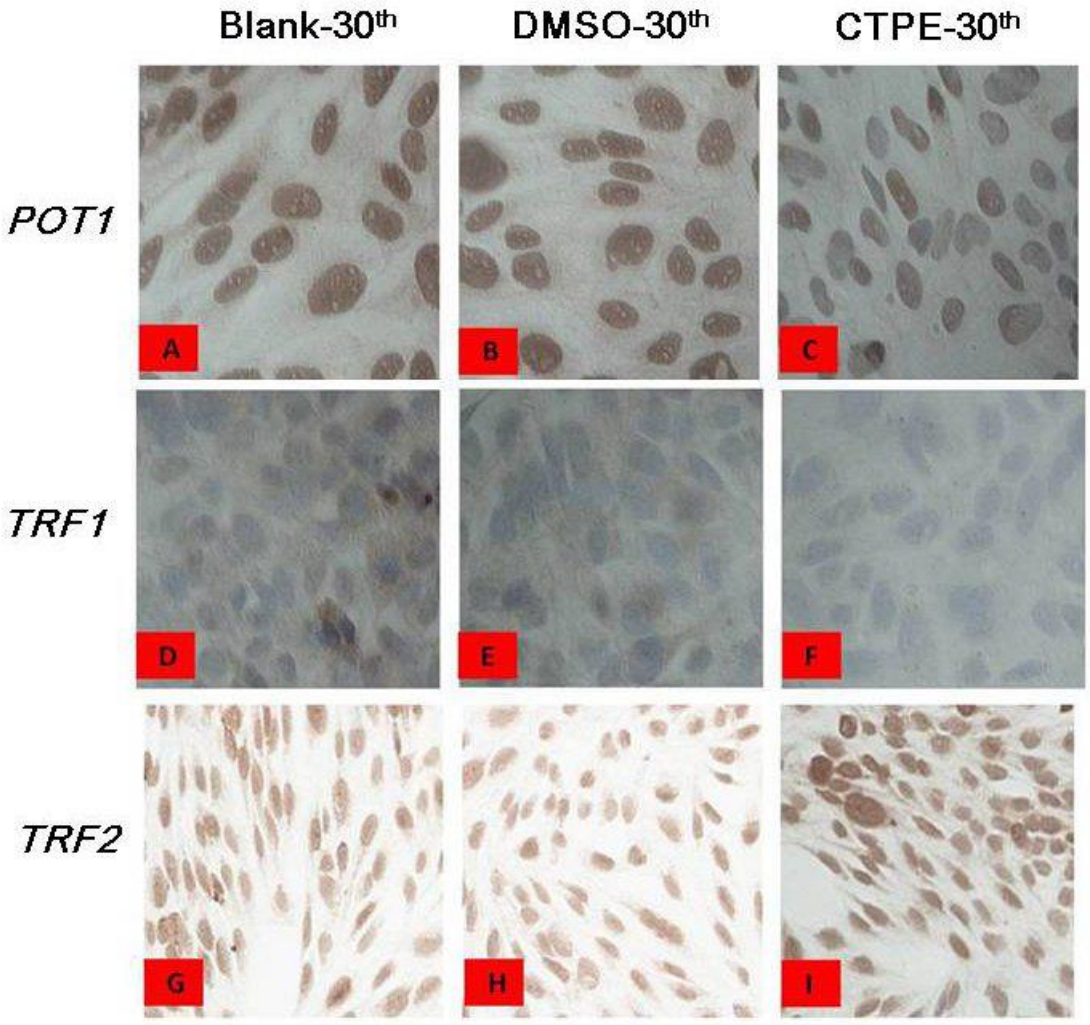


Figure 6

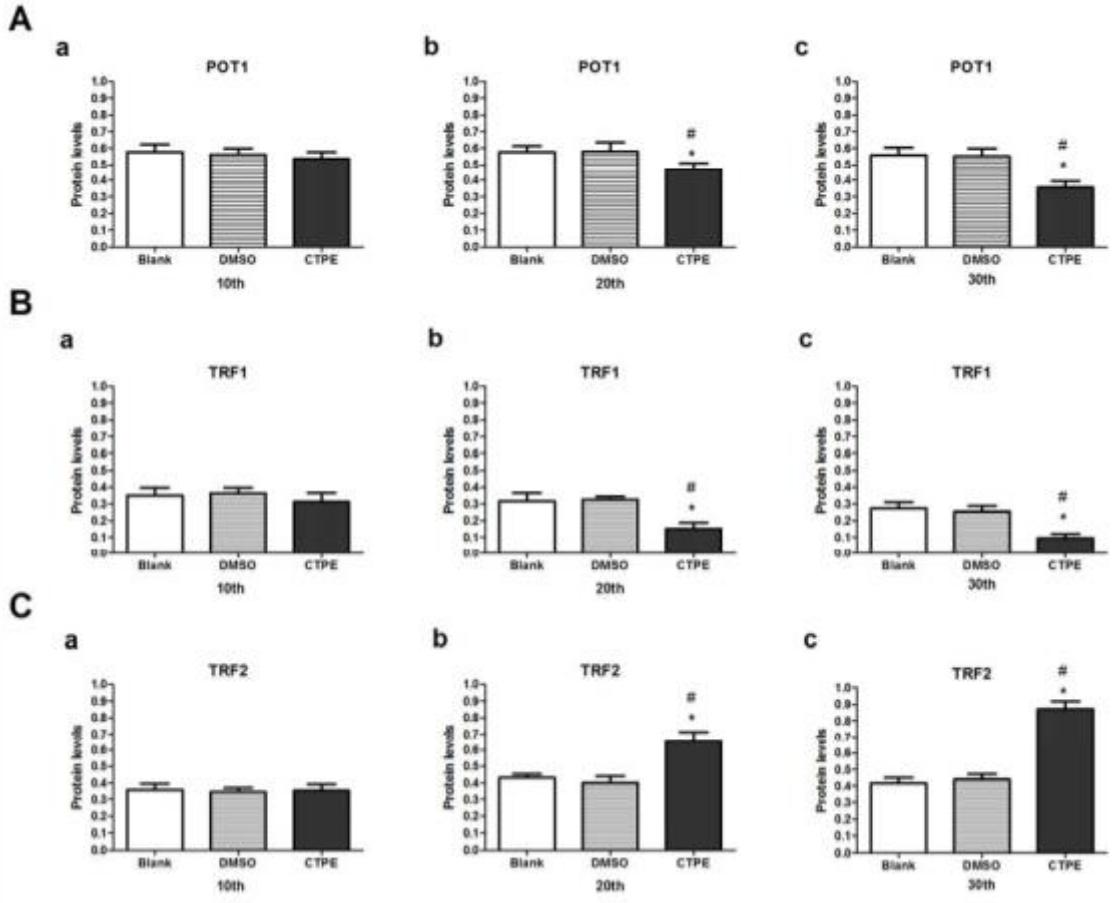
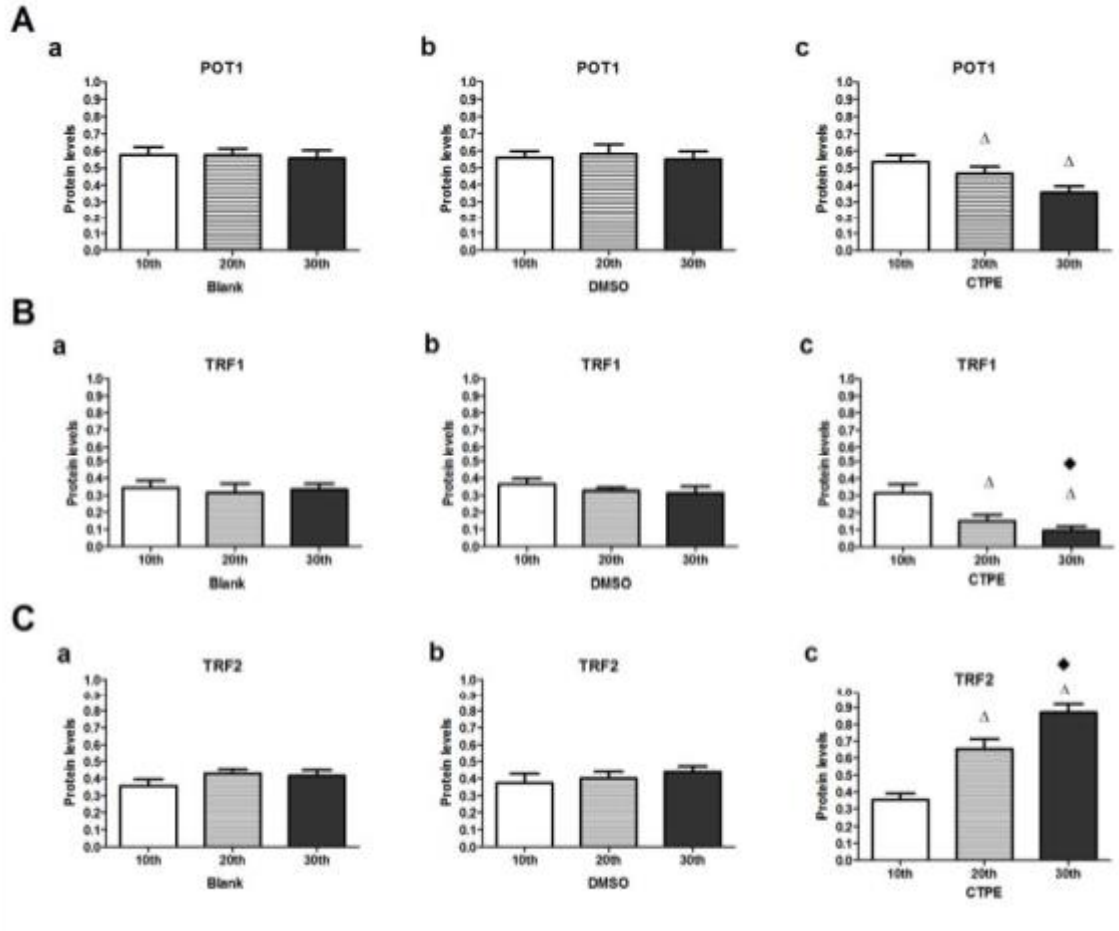

A
a
POT1
Protein levels
Blank
DMSO
CTPE
10th
b
POT1
20th
c
POT1
30th
B
a
TRF1
10th
b
TRF1
20th
c
TRF1
30th
C
a
TRF2
10th
b
TRF2
20th
c
TRF2
30th

Figure 7


A
a
POT1
Protein levels
10th
20th
30th
Blank
b
POT1
DMSO
c
POT1
CTPE
B
a
TRF1
Blank
b
TRF1
DMSO
c
TRF1
CTPE
C
a
TRF2
Blank
b
TRF2
DMSO
c
TRF2
CTPE