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Possible Role of DNA Polymerase beta in Protecting Human Bronchial Epithelial Cells Against Cytotoxicity of Hydroquinone[1]

DA-LIN HU[#,+], HUAN-WEN TANG[*], HAI-RONG LIANG[*], DONG-SHENG TANG[+], YI-MING LIU[#], WEI-DONG JI[#], JIAN-HUI YUAN[╪], YUN HE[#], ZHENG-YU ZHU[§], JIAN-PING YANG[#], DAO-KUI FANG[#], YAN SHA[#], XIAO-ZHI TU[#], AND ZHI-XIONG ZHUANG[#,╪,2]

[#]Department of Preventive Medicine, School of Public Health, SunYat-sen University, Guangzhou 510080, Guangdong, China; [╪]Shenzhen Center for Disease Control and Prevention, Shenzhen 518020, Guangdong, China; [+]Medical College, Foshan University, Foshan 528000, Guangdong, China; [*]School of Public Health, Guangdong Medical College, Dongguan 523808, Guangdong, China; [§]Department of Biochemistry and Molecular Biology, Sun Yat-sen University, Guangzhou 510080, Guangdong, China

Objective To explore the toxicological mechanism of hydroquinone in human bronchial epithelial cells and to investigate whether DNA polymerase beta is involved in protecting cells from damage caused by hydroquinone. **Methods** DNA polymerase beta knock-down cell line was established *via* RNA interference as an experimental group. Normal human bronchial epithelial cells and cells transfected with the empty vector of pEGFP-C1 were used as controls. Cells were treated with different concentrations of hydroquinone (ranged from 10 μmol/L to 120 μmol/L) for 4 hours. MTT assay and Comet assay [single-cell gel electrophoresis (SCGE)] were performed respectively to detect the toxicity of hydroquinone. **Results** MTT assay showed that DNA polymerase beta knock-down cells treated with different concentrations of hydroquinone had a lower absorbance value at 490 nm than the control cells in a dose-dependant manner. Comet assay revealed that different concentrations of hydroquinone caused more severe DNA damage in DNA polymerase beta knock-down cell line than in control cells and there was no significant difference in the two control groups. **Conclusions** Hydroquinone has significant toxicity to human bronchial epithelial cells and causes DNA damage. DNA polymerase beta knock-down cell line appears more sensitive to hydroquinone than the control cells. The results suggest that DNA polymerase beta is involved in protecting cells from damage caused by hydroquinone.

Key words: Human bronchial epithelial cells; RNA interference; Hydroquinone; Toxicology; DNA polymerase beta

INTRODUCTION

Hydroquinone is an important industrial material used as antioxidant, reducing agent, chemical intermediate, polymerization inhibitor, and drug material. Hydroquinone is also a natural ingredient of various agricultural products including fruits, grains, vegetables, tea, and beer. It has been reported that in male F344 rats, hydroquinone exposure causes renal tubule adenomas and exacerbates spontaneous chronic progressive nephropathy (CPN)[1-5]. Fetotoxicity, hematotoxicity, immunotoxicity, permanent corneal damage, and exogenous ochronosis have been shown to be related to hydroquinone exposure. However, its toxicological mechanisms, especially molecular toxicological mechanisms, are still unclear[6-8]. Furthermore, whether hydroquinone can cause damages to other tissues, organs, and systems remains unknown. Human DNA polymerase beta, the smallest eukaryotic DNA polymerase, 39kDa, is a single polypeptide chain enzyme. It has two specialized domains that contribute essential enzymatic activities to base excision repair (BER) and other DNA metabolic events involved in gap-filling DNA synthesis. The amino-terminal domain has a lyase activity which is essential for removing the intermediate of 5'-deoxyribose phosphate (dRP) generated in the process of BER. The larger polymerase domain has nucleotidyl transferase activity, which has several sub-domains that bind to double stranded DNA molecules, catalytic metals, and the correct nucleoside triphosphate in a template-dependent manner[9-12]. Our study showed that hydroquinone exposure could cause DNA

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[2]Correspondence should be addressed to Zhi-Xiong ZHUANG, Tel: 86-755-25639066. E-mail: zxzhuang@szcdc.net

Biographical note of the first author: Da-Lin HU, male, born in 1971, associate professor, Ph. D. candidate at the College of Public Health, Sun Yat-sen University, majoring in molecular toxicology.

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damage to human bronchial epithelial cells and DNA polymerase beta was involved in protecting human bronchial epithelial cells from damage caused by hydroquinone.

MATERIALS AND METHODS

Materials, Strains, and Plasmids

Hydroquinone (AR) was obtained from Sigma (USA). *E. coli* strain DH5α was kindly provided by Dr. Heyun at Sun Yat-sen University. pSIREN-RetroQ vector containing human U6 promoter was purchased from BD Company (USA). pEGFP-C1 vector was bought from Clontech (USA). Restriction enzymes (*Bam*H, *Eco*RI, and Bgl Ⅱ), T4 DNA ligase, DNA marker (DL2000 and DL15000), MiniBEST plasmid purification kit, DNA agarose gel purification kit were from TaKaRa (Japan). Tryptone, yeast extract, and agarose were from OXID (England). Human bronchial epithelial cells were stored in our laboratory. DNA synthesis, sequencing, and dsRNA synthesis were commercially prepared by Sangon (China). DNA polymerase beta AB-1 (mouse monoclonal antibody) was from NeoMarkers (USA). Phototope-HRP Western blot detection kit was from NEB (England). DMEM and fetal bovine serum (FBS) were from GIBCO (USA). DMSO was from Invitrogen (USA). Lipofectamine 2000 kit, G418 was from Roche (Germany).

Design, Synthesis of dsRNA, Construction of Recombinant pEGFP-C1-U6-dsRNA

Following the cDNA sequence of polymerase beta gene published in Genbank and the provided universal criteria, sequences of dsRNA used for RNA interference were designed by dsRNA oligonucleotide designer, one of which was picked out and commercially synthesized (2OD value) with chemical methods in Sangon Company. The 19nts target sense sequence of dsRNA is 5'-CCATCATCAGCGAATTGGG-3', the target anti-sense sequence of dsRNA is 5'-CCCAATTCGCTGATGATGG-3', the hairpin construction between the target sense sequence and target anti-sense sequence is 5'-TTCAAGAGA-3'. The sites of restriction enzyme *Bam*HI and *Eco*RI lay respectively at each end of the dsRNA sequence, and five thymidine residues were added as termination signal, thus resulting in the following dsRNA sequence: 5'-GATCCG (19nts) TTCAAGAGA (19nts) TTTTTACGCGT-3'. DNA recombination technology was used to insert the up related dsRNA sequence into the vector of pSIREN-RetroQ, and then the pSIREN-RetroQ-dsRNA recombinant was obtained. After *E. coli* DH5α was transformed with the pSIREN-RetroQ-dsRNA recombinant and screened with 1 μg/mL ampicillin for positive clones. Plasmid was extracted and digested by *Eco*RⅠ and Bgl Ⅱ, the fragment of pU6-dsRNA was purified and confirmed by sequencing. The pU6-dsRNA fragment was cloned into the vector of pEGFP-C1 by recombination technology to produce recombinant of pEGFP-C1-pU6-dsRNA, the authenticity of which was identified by restriction endonuclease analysis and DNA sequencing using human U6 premier 5' -CACAAAAGGAAACTCACCCT-3'.

Cell Culture and Transfection

Human bronchial epithelial cells were routinely cultured in Dulbecco's modified Eagel's medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 mmol/L HEPES buffer, and maintained in 12-well cell culture plates at 37℃ in a humidified atmosphere containing 5% CO_2. The cells were transfected with recombinant of pEGFP-C1-pU6-dsRNA or empty vector of pEGFP-C1 using Lipofectamine 2000 according to the protocol provided by the manufacturer. At the same time normal control cells were cultured. Plasmid DNA (0.8 μg) was dissolved in 50 μL DMEM free from serum and antibiotics and mixed with 2 μL Lipofectamine 2000 dissolved in 50 μL serum and antibiotics-free DMEM, and incubated for 20 minutes at room temperature to produce DNA-liposome complexes. Five hundred μL serum and antibiotics-free DMEM and 50 μL DNA-liposome complex were added to the relative wells of culture plate in which human bronchial epithelial cells had 80% confluence. The cells were incubated at 37℃ in a CO_2 incubator for 24 hours. Then the medium was removed and fresh growth medium containing 800 μg/mL G418 was added to the cells for screening of positive clones.

Fluorescence Microscopy Imaging

After screened by G418, human bronchial epithelial cells derived from the positive clones were detected to identify whether they possessed green fluorescence at 430 nm under Axiotron fluorescence microscope with a UV filter block.

Western Blot Analysis

Human bronchial epithelial cells transfected with the recombinant of pEGFP-C1-pU6-dsRNA, cells transfected with the empty vector of pEGFP-C1 and normal control cells were harvested and washed 3 times with cold PBS. Cells were treated with RIPA (0.1% SDS, 1% NP-40, 0.5% sodium, 50 mmol/L

Tris-HCl, 150 mmol/L NaCl) for 30 min on ice and centrifuged at 12 000×g for 25 min. Supernatant was collected and boiled for 5 min. After SDS-PAGE was done, proteins were transferred to PVDF membranes which were blocked with 5% free fat milk at 4℃ overnight in TBS-T (150 mmol/L NaCl, 0.05% Tween-20, 10 mmol/L Tris-HCl, pH 7.4) and washed at room temperature for 30 minutes with wash buffer changed 3 times and incubated for 2 hours at room temperature in blotting buffer containing mouse anti-human polymerase beta antibody diluted 1:1000. After washing, the membranes were treated with horseradish peroxidase-conjugated goat anti-mouse antibody diluted 1:14000 for 1 hour at room temperature. The result was developed on x-ray film.

Cells Attacked by Hydroquinone

At the logarithm growth stage, 1×10^4 normal control cells or cells transfected with the recombinants of pEGFP-C1-pU6-dsRNA or empty vector of pEGFP-C1 were respectively supplied to the relative wells of a 96-well culture plate, and cultured to adhere to the bottom of the plate. Then the cells were separately treated with DMEM containing different concentrations of hydroquinone (including 0 μmol/L, 10 μmol/L, 20 μmol/L, 40 μmol/L, 80 μmol/L, 120 μmol/L) for 4 hours for MTT assay.

MTT Assay

After treatment with hydroquinone, cells were washed with PBS slightly, 180 μL fresh complete DMEM and 20 μL MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbronide, 5 mg/mL] were added. Cells were incubated continuously for another 4 hours. Then the medium was removed and 150 μL DMSO was added to each well. The plate was shaken at 120 rpm for 10 min to completely dissolve the crystal. The optical density at 490 nm of each well was read on an automated microplate reader (Bio-Tec Instruments). The preliminary assay revealed that the absorbance of each well was directly proportional to the number of survived cells.

Comet Assay

During the logarithm growth period, 1×10^5 normal control cells or cells transfected with the recombinant of pEGFP-C1-pU6-dsRNA or empty vector of pEGFP-C1 were put into 25 cm^2 culture flasks respectively and incubated at 37℃ in a CO_2 incubator for 24 hours. Then the cells were treated with different concentrations of hydroquinone (including 0 μmol/L, 10 μmol/L, 20 μmol/L, 40 μmol/L, 80 μmol/L, 120 μmol/L) dissolved in complete DMEM for 4 hours. The treated cells were washed, detached, centrifuged and resuspended in ice-cold PBS. In environmental condition of dim light, comet assay was performed according to the protocol provided by Singh *et al*. One hundred μL of the mixture of cells and 0.5% low melting point agarose (LMA) in PBS were loaded to frosted microscope slides pre-treated with 0.5% normal melting point agarose (NMA), then covered by 120 μL of LMA and immersed in cold lysing solution and kept at 4℃ for an hour. After that, electrophoresis was performed at 25 V and 300 mA for 30 minutes with a horizontal gel electrophoresis instrument and all slides were neutralized in Tris buffer (pH 7.5) and stained with ethidium bromide (EB). A fluorescence microscope (Olympus B-60F5) was used to observe the comets, 150 cells per sample were scored and DNA damage was detected as total comet length (increase in DNA migration).

Statistical Analysis

The total length of DNA was presented as $\bar{x}\pm s$. The data were analyzed with ANOVA followed by Student-Newman-Keuls test. $P<0.05$ was considered statistically significant. Software of SPSS10.0 for windows was used.

RESULTS

Construction of Recombinant pEGFP-C1-U6-dsRNA

After the dsRNA sequence: 5'- GATCCG (19nts) TTCAAGAGA (19nts) TTTTTACGCGT-3' was inserted into the vector of pSIREN-RetroQ, the recombinant of pSIREN-RetroQ-dsRNA was obtained. One percent agarose gel electrophoretic analysis of pSIREN-RetroQ-dsRNA was performed to verify its authenticity, showing an electrophoresis strip at the predicted location between 7500 bp and 5000 bp (Fig. 1).

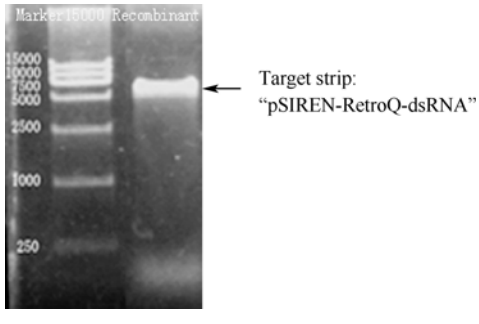

FIG. 1. One percent agarose gel electrophoretic analysis of pSIREN-RetroQ-dsRNA. Marker 15000: DNA marker DL15000. Recombinant: constructed oligonucleotide "pSIREN-RetroQ-dsRNA".

After *E. coli* DH5α was transformed with the

pSIREN-RetroQ-dsRNA recombinant and screened with ampicillin for positive clones, plasmid was extracted and digested by *Eco*RⅠ and BglⅡ. The fragment of pU6-dsRNA was purified and 1% agarose gel electrophoretic analysis was performed again, an electrophoresis strip could be found at the predicted location between 250 bp and 500 bp (Fig. 2), then the fragment of U6-dsRNA was obtained and purified. DNA sequencing was performed to verify its authenticity, showing that the sequence was consistent with the target sequence.

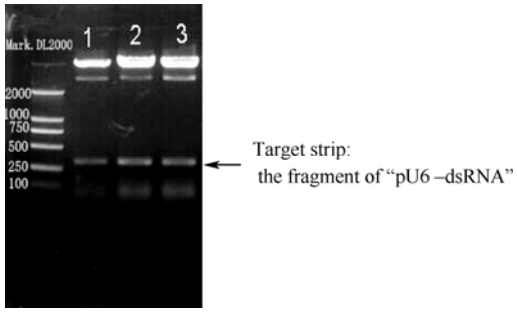

FIG. 2. One percent agarose gel electrophoretic analysis of "pSIREN-RetroQ-dsRNA" digested by *Eco*RI and BglⅡ. DNA marker DL 2000. Lanes1-3: three parallel samples of "pSIREN-RetroQ-dsRNA" digested by *Eco*RI and BglⅡ.

The fragment of pU6-dsRNA was cloned to the vector of pEGFP-C1. After transformation, expansion, screening and purification, the recombinant of pEGFP-C1-pU6-dsRNA was obtained. One percent agarose gel electrophoretic analysis showed that the strip of pEGFP-C1-pU6-dsRNA was located at the correct location of 5000 bp (Fig. 3). *Eco*RⅠ and BglⅡ were used to digest the recombinant of pEGFP-C1-pU6-dsRNA and a strip at the predicted location between 250 bp and 500 bp was found (Fig. 4). DNA sequencing showed that its sequence was in accordance with the target sequence.

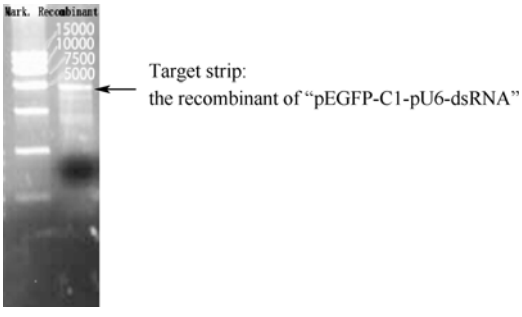

FIG. 3. One percent agarose gel electrophoretic analysis of "EGFP-C1-pU6-dsRNA". DNA marker DL15000. Recombinant: constructed recombinant of "pEGFP-C1-pU6-dsRNA".

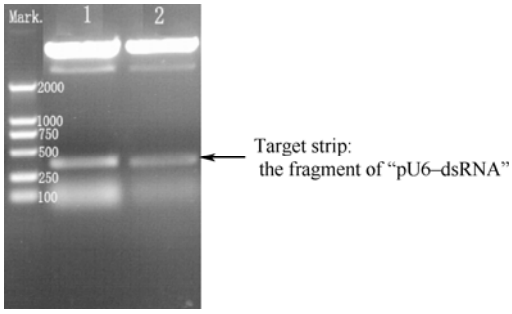

FIG. 4. One percent agarose gel electrophoretic analysis of "pEGFP-C1-pU6-dsRNA" digested by *Eco*RI and BglⅡ. DNA marker DL 2000. Lanes 1, 2: two parallel samples of "pEGFP-C1-pU6-dsRNA" digested by *Eco*RI and BglⅡ.

Morphological Observation and Fluorescence Microscopy Imaging

After transfection with the recombinant of pEGFP-C1-pU6-dsRNA or empty vector of pEGFP-C1 using Lipofectamine 2000 kit according to the protocol provided by the manufacturer, human bronchial epithelial cells were observed under normal light microscope (Fig. 5). When observed under Axiotron fluorescence microscope with a UV filter block at 430 nm, cells possessed green fluorescence, while nothing but a dark background was seen in the fields of normal control cells (Fig. 6).

FIG. 5. Human bronchial epithelial cells under normal light microscope 24 h after transfection: 1: Normal human bronchial epithelial cells; 2: Human bronchial epithelial cells transfected with the recombination of "pEGFP-C1-pU6-dsRNA"; 3: Human bronchial epithelial cells transfected with the empty vector of "pEGFP-C1".

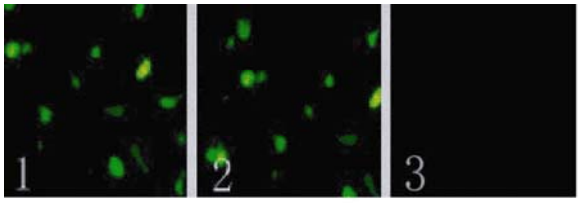

FIG. 6. Human bronchial epithelial cells under fluorescence microscopy 24 h after transfection. 1: Human bronchial epithelial cells transfected with the recombination of "pEGFP-C1-pU6-dsRNA"; 2: Human bronchial epithelial cells transfected with the empty vector of "pEGFP-C1"; 3: Normal human bronchial epithelial cells.

Expression Level of DNA Polymerase beta Protein

Western blot analysis was performed to detect the express levels of DNA polymerase beta in human bronchial epithelial cells transfected with the recombinant of pEGFP-C1-pU6-dsRNA or empty vector of pEGFP-C1 and in normal control cells. The image scanning system revealed that the expression level of DNA polymerase beta in the cells transfected with the recombinant of pEGFP-C1-pU6-dsRNA was about 9.7%, while the expression level of DNA polymerase beta was almost similar in normal control cells and cells transfected with empty vector (Fig. 7).

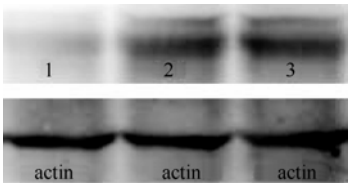

FIG. 7. Western blot analysis of polymerase beta protein in human bronchial epithelial cells 72 hours after transfection. 1: Human bronchial epithelial cells transfected with the recombination of "pEGFP-C1-pU6-dsRNA"; 2: Human bronchial epithelial cells transfected with the empty vector of "pEGFP-C1"; 3: Normal human bronchial epithelial cells. Actin: β-actin.

MTT Assay

MTT assay was used to evaluate the cytotoxicity of hydroquinone to human bronchial epithelial cells. In preliminary assay, a standard curve was generated, showing a linear response relation between cell number and absorbance at 490 nm. Cells were treated with hydroquinone at different concentrations. The number of survived cells was calculated by the absorbance value at 490 nm. A statistical chart was drawn with hydroquinone concentration on X axis and cell absorbance on Y axis (Fig. 8). The chart revealed that the number of survived cells transfected with the recombinant of pEGFP-C1-pU6-dsRNA was less than that of the two control groups, while there was no difference between the two control groups. At the same time, dose-response relation was also found in each group of cells.

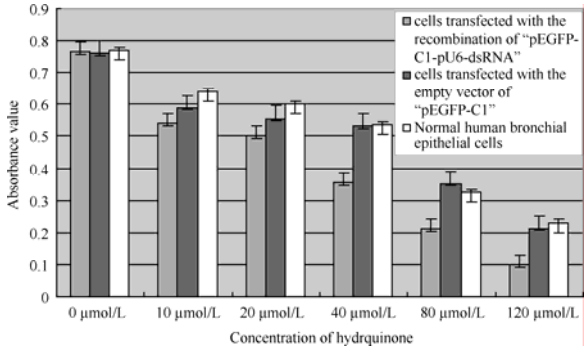

FIG. 8. The number of survived human bronchial epithelial cells and different concentrations of hydroquinone (MTT). Inset: the legend showing different groups of cells.

Comet Assay

Comet assay was performed to detect DNA damage caused by different concentrations of hydroquinone. The total length of DNA was measured and presented as $\bar{x} \pm s$ (Table 1).

TABLE 1

Effect of Hydroquinone on the Extent of DNA Degradation in Human Bronchial Epithelial Cells, Measured by Comet Assay (Expressed as Mean Comet Length ± SD, in μm)

Treatment	Groups			*P* Value
	Cells Transfected With "pEGFP-C1-U6-dsRNA"	Cells Transfected With "pEGFP-C1"	Normal Control Cells	
Complete DMEM	44.37±9.11	45.10±9.01	44.23±8.09	>0.05
10 μmol/L Hydroquinone	49.12±11.23	47.32±9.67	47.09±9.39	<0.05
20 μmol/L Hydroquinone	55.36±10.69	50.36±8.92	51.76±10.84	<0.05
40 μmol/L Hydroquinone	59.79±12.14	54.37±13.17	53.27±11.64	<0.05
80 μmol/L Hydroquinone	63.36±11.68	58.39±13.65	59.04±12.25	<0.05
120 μmol/L Hydroquinone	77.37±13.97	69.89±12.98	67.31±13.59	<0.05
P Value	<0.05	<0.05	<0.05	

Note. Analyzed by one-way ANOVA with software of SPSS10.0 for windows

DISCUSSION

Cells have the ability to protect genomic DNA from damage due to their intrinsic multiple based excision repair (BER) pathways, including DNA polymerase beta-dependent single nucleotide BER and long-patch (2-10 nt) BER[18-21]. BER, a predominant pathway involved in DNA repair, is rather efficient in mammalian cells. However, when change occurs in the quantity or nature of polymerase beta, a certain amount of damage may inevitably escape, resulting in abnormal death or exacerbation of cell damage.

In our study, three groups of cells, including polymerase beta knock-down cells, control cells transfected with empty vector of pEGFP-C1 and

normal control cells, were treated with various concentrations of hydroquinone (including 0 μmol/L, 10 μmol/L, 20 μmol/L, 40 μmol/L, 80 μmol/L, 120 μmol/L) for 4 hours. MTT assay showed that after treatment with various concentrations of hydroquinone (certainly not including 0 μmol/L), polymerase beta knock-down cells significantly decreased absorbance at 490 nm compared to the relative normal control cells and cells transfected with empty vector of pEGFP-C1, the death rate in polymerase beta knock-down cells was significantly increased compared to the two control groups, while there were no significant differences in the two control groups. At the same time, a dose-response relationship was found (Fig. 8). Our results suggest that hydroquinone is cytotoxic to human bronchial epithelial cells, and polymerase beta plays an important role in protecting the cells against the cytotoxicity caused by hydroquinone.

Comet assay showed that hydroquinone could induce DNA damage in a dose-dependant manner. In addition, the degree of DNA damage in DNA polymerase beta knock-down cells was significantly higher than that in the normal control cells and cells transfected with empty vector of pEGFP-C1, while no significant difference was found between the two control groups. It is well known that DNA damage could induce a high rate of diseases. For instance, in male F344 rats, hydroquinone exposure is capable of inducing renal tubule adenoma and exacerbating spontaneous chronic progressive nephropathy (CPN)[7,22-25]. Therefore, DNA damage caused by hydroquinone may contribute to its carcinogenesis, and it seems necessary to maintain normal expression level of polymerase beta to accomplish the secondary prevention of hazard caused by hydroquinone.

In order to suppress polymerase beta by RNAi, we constructed the target recombinant of pEGFP-C1-pU6-dsRNA, which has the following merits. (1) Human bronchial epithelial cells transfected with this vector may provide a convenient GFP marker which is easily observed under a florescence microscope (Fig. 6). (2) The retroviral vector of pSIREN-RetroQ universally used in RNAi at present might produce viral supernatant and contain potentially hazardous recombinant virus and thus is potentially harmful to the research workers, while in a theoretical sense, pEGFP-C1-pU6-dsRNA construct is biologically safe. (3) This vector contains human U6 promoter (P_{U6}; RNA Pol Ⅲ-dependent), expressing the insert and a G418 resistance gene for the selection of stable transfectants. Western blot analysis showed that DNA polymerase beta in pEGFP-C1-pU6-dsRNA transfected cells was down regulated by about 90% compared to the control cells (Fig. 7), suggesting that it might be an efficient siRNA to suppress gene expression in mammalian cell lines.

In conclusion, RNAi technology can be used to establish polymerase beta stably knock-down human bronchial epithelial cell line. It might act as an important research tool in toxicological study on hydroquinone. Hydroquinone has obvious cytotoxicity to human bronchial epithelial cells. DNA polymerase beta plays an important role in protecting human bronchial epithelial cells from damage caused by hydroquinone.

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