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Original Article

A convenient spectrometric assay system for intracellular quantitative measurement of DNA glycosylase activity

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Cytosine methylation is a vital biology event. However, it is also the source of genomic instability due to deamination of 5′-methylcytosine by spontaneous hydrolysis, which produces thymine and results in G:T mismatches. Thymine DNA glycosylase and methyl-CpG-binding protein 4 are major DNA glycosylases involved in the mismatch repair progress, and their activities have been measured in many related researches. In this study, we developed a convenient spectrometric assay system for specific and quantitative measurement of intracellular DNA glycosylase activity. A G:T mismatch was introduced into the upstream region of firefly luciferase-coding sequence in the pGL3-control plasmid. Only if the G:T mismatches were repaired to G:C, will luciferase be expressed in transfected cells. By measuring luciferase activity, which is simple and convenient, the intracellular DNA glycosylase activity can be determined.

Keywords DNA glycosylase activity; intracellular; luciferase; thymine DNA glycosylase

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Introduction

DNA methylation is an important regulator of gene transcription and a common form of post-replicative DNA modification existed from bacteria to eukaryotes, and it plays an important role in growth control and differentiation [1]. In mammalian genomes, 5% of the cytosines are methylated and methylcytosines occur predominantly at CpG dinucleotides [2]. However, methylation is also the source of genomic instability due to deamination of 5′-methylcytosine (Me-C) by spontaneous hydrolysis, which produces thymine and results in G:T mismatches [3]. Such deamination of Me-C represents the most frequent cause of endogenous mutagenesis [4,5]. Whereas the deamination of 5-Me-C produces G:T mismatches, the deamination of cytosine produces G:U mismatches. The rate of deamination is highest for cytosine among the four standard nucleotides and it is estimated to occur at $1/10^7$ cytosine residues per day [6]. The product of this reaction is uracil, which results in G:U mismatches [7]. While U is a foreign base in DNA, it is easily recognized and repaired. In contrast, the correction of a deaminated MeC, i.e. T, requires a higher level of sophistication at damage recognition, since the ‘damage’ in this case is a perfectly normal DNA base, except that it is mispaired [8].

All organisms have evolved repair enzymes that remove these deaminated bases. G:T mismatches are mostly repaired by mismatch-specific thymine DNA glycosylase (TDG) and methyl-CpG-binding protein 4 (MBD4) [9,10], which are two main DNA glycosylases. The accurate and efficient repair of a G:U mismatch is mediated by uracil DNA glycosylase [11]. TDG and MBD4 also have strong capacity for G:U mismatch repair (MMR). Although structurally unrelated, TDG and MBD4 have similar enzymatic properties. They release T and U from G:T and G:U mismatches [9,12,13]. To what degree each of these glycosylases contributes to G:T processing in living cells is uncertain, but TDG and MBD4 are both indispensable to G:T MMR [8].

Incidences of G:T and G:U mismatches are very high and their MMRs are constantly conducted, thus activities of DNA glycosylases are frequently assayed in the related research field. The classical assay for DNA glycosylase activity is an *in vitro* method. Purified proteins or nuclear extracts [10,14–20] are incubated with end-labeled DNA substrates containing G:T or G:U mismatches *in vitro* and the ratio of cleavage products to intact DNA substrates was

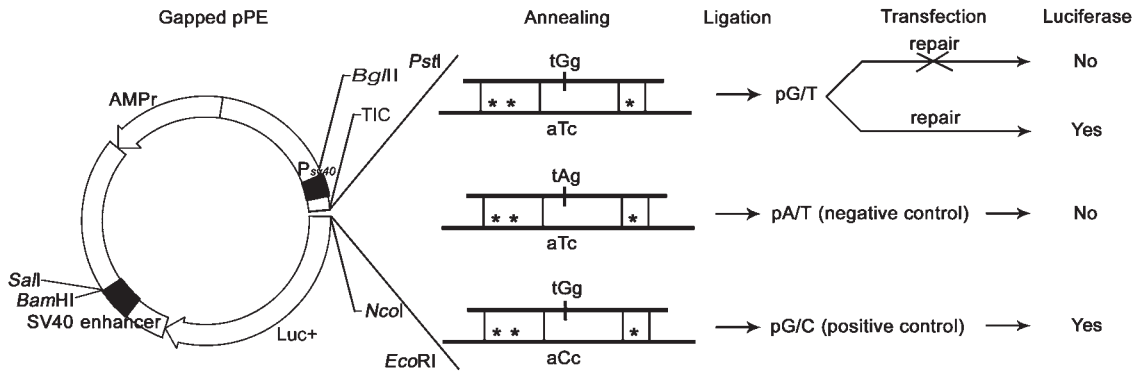

Figure 1 Illustration of protocol for intracellular DNA glycosylase activity assay pGL3-control vector was firstly modified to pPE with increasing *Pst*I and *Eco*RI sites. Three annealed DNA oligonucleotides were ligated to *Pst*I-*Eco*RI fragment of recombinant plasmid pPE to form pG/T, pA/T and pG/C. pG/T was transfected into cells, DNA glycosylase activity in live cells was evaluated using calibrated luciferase activity, and pA/T and pG/C were used as negative control and positive control, respectively. TIC means the translation initiation codon (ATG) of luciferase harbored in the *Nco*I site; **HA tag; **Bam*HI site.

used to evaluate the DNA glycosylase activity. This method is sensitive, reliable and feasible, but it cannot reflect the real intracellular situation. In fact, G:T or G:U MMR need base excision repair (BER) pathway *in vivo*. DNA glycosylases initiate BER through hydrolysis of the *N*-glycosidic bond of aberrant base to generate potentially harmful abasic site (AP site) in DNA. Then AP endonuclease (APE1) gains access to the AP site and forming DNA glycosylase-APE1-AP site complex. With the help of APE1, the DNA glycosylase separates from AP site, and then repair system channels into an appropriate BER pathway. These involve DNA strand incision 5′ to the AP site by APE1, trimming of the resulting 5′ end by an AP-lyase or an endonuclease, DNA resynthesis by a DNA polymerase and strand sealing by a DNA ligase [21–23]. *In vitro* assay of DNA glycosylase only shows the capability of producing AP site, but does not reflect other progressions. Furthermore, it cannot be used to measure the dynamics of MMR activity in live cells in response to the changes in the intracellular and/or extracellular environment.

In this study, we described a method for quantitatively evaluating the DNA glycosylase activity intracellularly. This method has been used to determine the G:T mismatch capacity of DNA glycosylases in several cells by measuring luciferase activity, and the results could be confirmed by western blot analysis. Our method can be used to monitor the regulation or dynamics of DNA glycosylase activity in live cells as they are subject to varying growth conditions. Hence, it will help to study whether and how DNA glycosylase or MMR activity is regulated under different physiological and/or pathological conditions.

Materials and Methods

Modification of PGL3-control vector

pGL3-control (Promega, Madison, USA) was first modified to increase the *Pst*I and *Eco*RI sites through *Nco*I site. Oligonucleotides R-*Pst*I and L-*Eco*RI (**Table 1**; Invitrogen, Shanghai, China) were designed and they can match each other to form the double-stranded DNA fragment with two cohesive *Nco*I ends as well as *Pst*I and *Eco*RI sites. The two oligonucleotides (10 pmol each) were annealed in annealing buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl) by heating at 90°C for 5 min, and allowed to cool off to room temperature over 2 h gradually. Then the double-stranded DNA fragment was inserted into *Nco*I site of pGL3-control vector and transformed into *E. coli* Top10. The positive clones were screened with *Bgl*II and *Eco*RI restriction enzyme analysis and then validated by DNA sequencing. The resulting construct was named as pPE.

Construction and identification of plasmids

DNA primer pairs T-56/G-48, T-56/A-48 and C-56/G-48 (Invitrogen) listed in **Table 1**, were annealed to form three double-stranded oligonucleotides with *Pst*I and *Eco*RI cohesive ends. These double-stranded oligonucleotides were inserted between *Pst*I and *Eco*RI sites of pPE to form pG/T, pA/T, and pG/C plasmids (**Fig. 1**). The ligation reaction conditions were as follows: 1 × T4 DNA ligase buffer, 50 pmol gapped pPE, 500 pmol double-stranded oligonucleotide and 10,000 U T4 DNA ligase (TaKaRa, Dalian, China) in a final volume of 1 ml. Ligation was performed at 16°C overnight. The ligation products were purified by using agarose gel electrophoresis, and recovered using 3S Spin agarose gel DNA purification kit (Shenergy Biocolor, Shanghai, China), then concentrated and sterilized by ethanol precipitation. DNA concentration was determined by OD_{260} using Biophotometer (Eppendorf, Hamburg, Germany). Firstly, ligation products were identified using *Bam*HI digestion, and then pA/T and pG/C were transfected into Top10 alone, and one clone per plasmid was expanded and sequenced.

Table 1 Oligonucleotides used in this study

Name	Sequence	Purpose
R-*Pst*I	5′-catgcctgcagatatctagcgcttaggctgagaattc-3′	Sense
L-*Eco*RI	5′-catggaattctcagcctaagcgctagatatctgcagg-3′	Antisense
G-48	5′-p-tacccatacgatgttccggactacgcc**tG**gagcggatccgtcgacccg-3′	Sense
A-48	5′-p-tacccatacgatgttccggactacgcctagagcggatccgtcgacccg-3′	Sense
T-56	5′-p-aattcgggtcgacggatccgctc**T**aggcgtagtccggaacatcgtatgggtatgca-3′	Antisense
C-56	5′-p-aattcgggtcgacggatccgctccaggcgtagtccggaacatcgtatgggtatgca-3′	Antisense

The underlined sequences indicate the introduced restriction sites. The boxed sequences are coding sequences for HA tag. Mismatched bases are in capital.

pCI-HA-mTDG and pCI-HA-mMΔN [mMΔN means the catalytic domain (amino acids 367−554) of mouse MBD4 (GI:3800807, NCBI)] were previously constructed in our laboratory, the mTDG (GI:55250066, NCBI) and mMDN genes were amplified from mouse cDNA and inserted between the *Eco*RV and *Xho*I sites in the pCI-HA vector (gift from Dr. Zheng Xing of University of California at Davis, USA).

Cell culture and transfection

Mouse melanoma cells (B16F10) were purchased from American Type Culture Collection (ATCC, Manassas, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (PAA, Pasching, Austria), mouse embryonal carcinoma cells (P19) were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and cultured in α-minimal essential medium with 10% fetal calf serum. Working cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Lipofectamine 2000 reagent (Invitrogen) was used to transfect DNA into cultured cells. Cells were plated on 12-well plates at densities ranging from 0.4 to 4×10^4 cells/well depending on the cell type. In the following day, when cells reached 50−60% confluence, complete medium was replaced with serum-free medium. For one-step transfection procedure, 1 μg pCI-HA, pCI-HA-mTDG or pCI-HA-mMΔN was co-transfected into cells together with 0.6 μg of pG/T and 0.3 μg of *Renilla* luciferase reporter vector pRL-null. Eight hours later, the medium was replaced by complete medium. For two-step transfection procedure, pCI-HA, pCI-HA-mTDG or pCI-HA-mMΔN was firstly transfected into cells. Eight hours later, the medium was replaced by complete medium, 0.6 μg pG/T and 0.3 μg pRL-null were then transfected into cells.

Luciferase assay

To assess the luciferase activity, the Promega luciferase assay system was used. The cells were harvested in phosphate-buffered saline (PBS) 24 h post-transfection by centrifugation at 800 g at 4°C for 5 min, and the cells pellets were re-suspended and lysed in 60 μl of 1× passive lysis buffer (Promega) on ice for 30 min. Cell debris was removed by centrifugation for 5 min at 13,400 g at 4°C. The cell lysate (20 μl) was used to measure the luciferase activity with a luminometer (Turner Designs, Sunnyvale, USA) as described in the Dual-Luciferase TM reporter Assay System manual (Promega). Luciferase activity was calibrated using the ratio of the number of firefly luciferase activity to the number of *Renilla* luciferase activity. All the luciferase assays were performed at least five times and statistical significance was counted using Student's *t*-test analysis.

Western blot assay

After luciferase assay, the protein concentrations of the aliquots of cell lysate were determined using the Bradford method, and 80 μg of the cell lysate was subject to 10% SDS-PAGE and then electrophoretically transferred to PVDF membrane (Millipore, Billerica, USA). The membrane was blocked with 5% non-fat milk for 1 h and then incubated with anti-HA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, USA) at a final concentration of 0.2 μg/ml for 2 h at room temperature. After three washes with PBS Tween-20 (PBST), the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at 1:2000 dilution (Santa Cruz) for 1 h and washed again. The membrane was developed with enhanced chemiluminescence reagent (Cell Signaling, Beverly, USA) and exposure to Kodak X-Omat Blue film (NEN Life Science, Boston, USA). Quantitative analysis was performed using Grab-it 2.5 and Gelwork software. Western blot analysis was performed three times and statistical significance was calculated using Student's *t*-test.

Results

Strategy for intracellular assay of DNA glycosylase activity

The strategy used to measure the intracellular DNA glycosylase activity is shown schematically in **Fig. 1**.

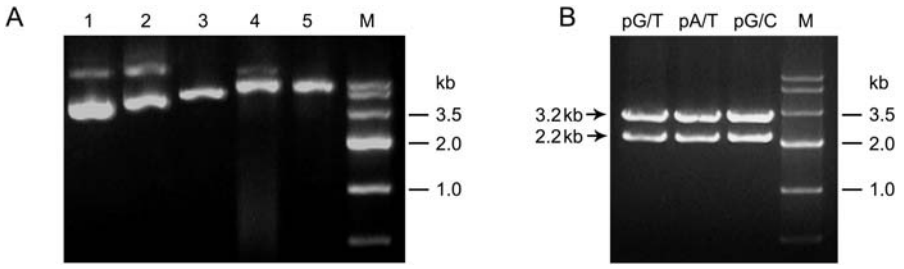

Figure 2 Construction and identification of ligation products (A) Construction and validation of pG/T. Lane 1, pGL3 control; lane 2, pPE; lane 3, gapped pPE digested with *Pst*I and *Eco*RI; lane 4, pG/T ligation product; lane 5, pG/T ligation product after gel purification; lane M, marker. (B) Ligation products, pG/T, pA/T and pG/C, were digested with *Bam*HI. Lane M, marker.

pGL3-control does not have proper restriction enzyme sites at the coding sequence upstream of the firefly luciferase except *Nco*I site harboring the ATG translation start codon. In order to assure the direction of the inserted DNA fragment and prevent vector self-ligation, pGL3-control vector was first modified to pPE. In comparison with pGL3-control, pPE has two more restriction enzyme sites, *Pst*I and *Eco*RI, locating in front of the start codon of luciferase. In addition, it is necessary that the 5′-terminus of oligonucleotides for annealing were phosphorylated during the oligonucleotide synthesis (**Table 1**) in order to ligate.

DNA replication needs to be avoided in our method since it will eliminate G:T mismatches. The pG/T plasmid in our research comes directly from ligation product, and it will never be transformed into *E. coli* to expand the plasmid. Because ligation product is a mixture, sequence cannot been performed. To identify ligation product, a *Bam*HI site is designed in the inserted double-stranded oligonucleotides. Combined with another *Bam*HI site located at 2442 bp in parent pGL3-control plasmid, the correct ligation product can be easily distinguished by the appearance of two bands of 2.2 and 3.2 kb resulted from *Bam*HI digestion (**Fig. 2**). Moreover, pA/T and pG/C, parallel operation with pG/T, can be transfected into Top10 to sequence in order to further verify construction.

A T:G mismatch, whose sense strand was TGG and corresponding anti-sense strand CTA, was introduced upstream of firefly luciferase coding sequence in the pGL3-control plasmid. Negative control (pA/T), containing constant TAG stop codon and positive control (pG/C), containing constant TGG instead of TAG stop codon were designed in our synthesized double-stranded oligonucleotides. For pA/T plasmid, translation will be stopped by the TAG stop codon after synthesis of the first 12 amino acid residues. So luciferase protein cannot be expressed. But for pG/C, because TAG was replaced with TGG, translation will proceed smoothly and luciferase protein with HA tag could be expressed. For pG/T, only if the G:T mismatch was repaired to G:C, the mismatch region sequence will be rectified from TAG stop codon to TGG to express functional luciferase. By directly measuring the luciferase activity, it is simple to assess the DNA glycosylase activity. In addition, HA tag was introduced upstream of the mismatch region in frame with luciferase coding sequence, so the expressed luciferase protein would be endowed with HA tag at its N-terminus. Western blot assay could be easily performed using HA antibody to detect the luciferase protein level, confirming the results from luciferase activity assay.

Overexpression of mTDG or mMΔN significantly increases G:T MMR activity in live cells

In this intracellular assay system, the tested reporter gene and DNA glycosylase enzyme gene were cloned and expressed in different plasmid. Thus, we first compared the influence of different transfection procedures on the assay system. The tested pG/T plasmid was co-transfected into cells together with the pCI-HA control vector, pCI-HA-mTDG or pCI-HA-mMΔN at the same time for one-step transfection procedure. For two-step transfection protocol, pG/T plasmid was introduced into cells 8 h after pCI-HA, pCI-HA-mTDG or pCI-HA-mMΔN was transfected. The results showed that overexpression of mTDG or mMΔN in cells could lead to about 70% increase in calibrated luciferase activity in one-step transfection, while the calibrated luciferase activity of two-step transfection protocol could increase about 140% [**Fig. 3(A)**]. That means overexpression of mTDG or mMΔN in cells could significantly increase G:T MMR activity and the increase of G:T MMR activity from two-step transfection protocol is more significant than that of one-step transfection procedure. Thus the two-step transfection was adopted in the following experiments.

pA/T and pG/C were transfected into cells as control. Compared with pGL3-control, pGL3-basic (Promega) has neither SV40 promoter nor SV40 enhancer and it was also used as a negative control. The cells transfected with pGL3-basic from large-scale preparation and pA/T had almost no calibrated luciferase activity, the cells transfected with pG/C had the highest calibrated luciferase activity and the cells transfected with pG/T had intermediate calibrated

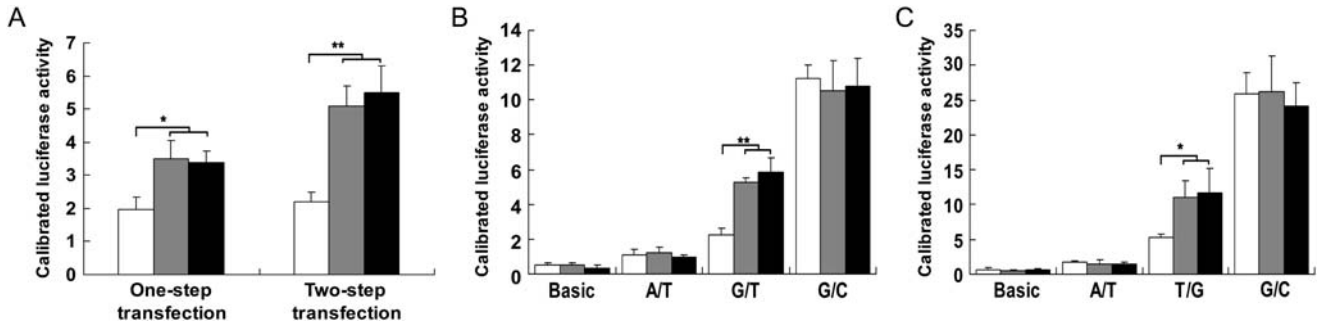

Figure 3 DNA glycosylase activity assay intracellularly (A) One-step and two-step transfections were used to evaluate the influence of overexpression of mTDG or mMΔN on the G/T MMR activity in B16F10 cell. $*P < 0.05$ and $**P < 0.01$. (B) DNA glycosylase activity assay in B16F10 cells. pGL3-basic (Basic), pA/T (A/T), pG/T (G/T) or pG/C (G/C) plasmid was introduced into cells together with pRL-null vector 8 h after pCI-HA, pCI-HA-mTDG or pCI-HA-mMΔN plasmid was transfected. (C) DNA glycosylase activity assay in P19 cells. Transfection were performed the same as B16F10 cells.

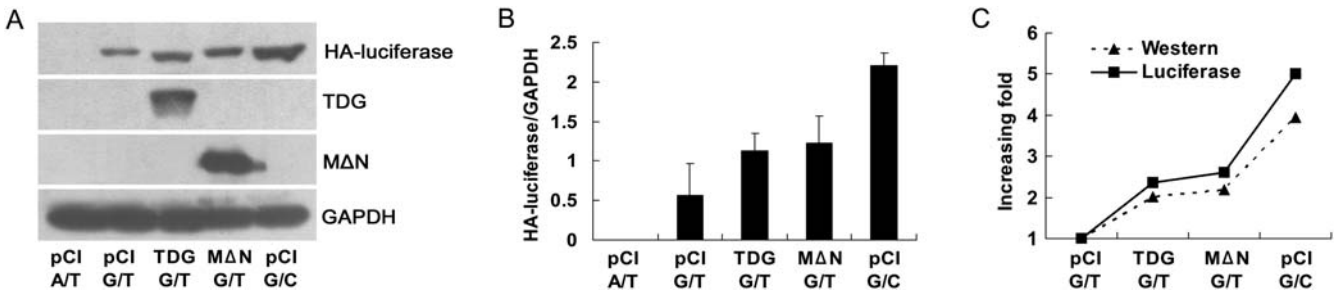

Figure 4 Western blot analysis further confirms the result from luciferase activity assay (A) The expression of HA-luciferase was assayed by western blot using the residual supernatants from B16F10 cell lysate after luciferase activity measure. (B) Quantitative analysis of western blot. (C) Increasing fold of luciferase activity and western blot assay based on the pCI-HA and pG/T cotransfection.

luciferase activity. Moreover, overexpression of mTDG or mMΔN did not change calibrated luciferase activity in the cells transfected with pGL3-basic, pA/T or pG/C, but can cause about 140 or 160% increase in calibrated luciferase activity in the cells transfected with pG/T [**Fig. 3(B)**]. Similar results have been observed in P19 cells [**Fig. 3(C)**].

HA tag was designed to be introduced at 5′ end of luciferase reporter gene, so the expression of luciferase protein could be monitored by the HA tag at its N-terminus. Thus western blot analysis could be performed using HA antibody to confirm the results of luciferase activity assay. As shown in **Fig. 4(A,B)**, the luciferase expression in the pG/T-transfected B16F10 cells was between the expressions of pA/T-transfected B16F10 cells (no expression) and pG/C-transfected B16F10 cells (stronger expression); and the B16F10 cells transfected with pCI-HA-mTDG or pCI-HA-mMΔN had relative stronger luciferase expression than the B16F10 cells transfected with pCI-HA control vector. These results were consistent with the luciferase analysis.

To sum up, the above data strongly support that our developed luciferase activity assay system is a reliable reporter or measurement system for intracellular DNA glycosylase enzyme activity in the living cells.

Discussion

The methods currently used to measure the DNA glycosylase activity can basically be grouped into two categories. The first one is an *in vitro* method and this classical method is generally used for *in vitro* DNA glycosylase activity assay, but it cannot reflect the real intracellular situation. The second category method for DNA glycosylase activity is intracellular assay, mismatched or heteroduplex DNA, including both plasmid and viral DNA, is introduced into cells and then later retrieved for analysis [24–29]. In this case, the retrieval process is usually laborious and tedious or less specificity. In these intracellular methods, two methods are relatively good for assaying MMR activity. Waters *et al.* [27] previously reported a method of assessing repair activity of G:T mispair *in vivo*, but in their method the *supF* reporter need to be finally assayed in bacteria, thus reflecting the DNA repair ability of mammalian cells indirectly. The other method was developed by Lei *et al.* [28] and Zhou *et al.* [29] using green fluorescent protein as reporter for *in vivo* MMR activity assay, but the cloning process of their reporter plasmids was complicated. More importantly, fluorescent microscopic observation and flow cytometric analysis could only measure the EGFP positive cell population, but could not

provide accurate quantitation of EGFP protein. Moreover, their method could neither measure the transfection efficiency nor reflect EGPF protein level, both of which are very important for the accuracy of evaluating DNA repair capability in different cells. In contrast, by measuring luciferase activity using dual-reporter system, our method can reflect reporter protein level accurately via calibration and quantification using dual-luciferase reporters, eliminating the interference introduced by variance of transfection efficiency during different samples or experiments.

The approach used to evaluate our intracellular assay system based on the fact that overexpression of DNA glycosylase enzymes, TDG or MBD4, in cells should increase the cellular G:T repair activity. However, endogenous cellular G:T repair capacity can also repair the G:T mismatch target introduced by transfection. When pCI-HA-mTDG or pCI-HA-mMΔN and pG/T were co-transfected into cells at the same time, the G:T mismatch transfected would firstly be repaired by endogenous G:T repair capacity before the mTDG or mMΔN were expressed. Thus the relative contribution to G:T repair from overexpressed mTDG or mMΔN would be decreased. To verify this assumption and possibility, we had used one-step transfection and two-step transfection individually in our assay system. By comparison, overexpression of mTDG and mMΔN can increase calibrated luciferase activity more obviously during two-step transfection. Thus we are prone to the procedure that pG/T is transfected into cells 8 h after pCI-HA-mTDG or pCI-HA-mMΔN is transfected.

The results showed that calibrated luciferase activity from pG/T-transfected cells was just between negative control and positive control [**Fig. 3(B)**], and overexpression of mTDG and mMΔN could increase calibrated luciferase activity of the cells transfected with pG/T, but no effect on the cells transfected with pA/T and pG/C. Moreover, western blot assay further assured these results [**Fig. 4(A, B)**]. Based on pCI and pG/T cotransfection, increasing fold of mTDG and pG/T, mMΔN and pG/T, pCI and pG/C cotransfections can be calculated from calibrated luciferase activity [**Fig. 4(C)**]. Semi-quantification of western blot results [**Fig. 4(C)**] also showed similar trend [**Fig. 4(C)**]. These results indicated the reliability, rationality and feasibility of our method.

The ligation of an odd number of annealed double-stranded oligonucleotides can also be *Pst*I and *Eco*RI cohesive ends and can be inserted between *Pst*I and *Eco*RI sites of pPE, and these ligation products cannot be detected by *Bam*HI digestion. But multiple insertions have extremely low probability and some multiple insertions can be filtered (**Fig. 2**) by recovering the main band in the gel purification process. In addition, even if the ligation product has multiple ligations, they do not influence our system. If G:T mismatches were not repaired, luciferase cannot be expressed, and only if the G:T mismatches were repaired to G:C, luciferase will be likely to be expressed. Moreover, most multiple insertions change luciferase open reading frame.

DNA replication will eliminate G:T mismatch, so it should be avoided. The plasmid with SV40 replication origin can be replicated in the cells containing large T antigen. In this paper, SV40 replication origin from pGL3-control was not deleted in the construction process of pG/T, so pG/T could not be transfected into the cells containing large T antigen. Of course, this question will be avoided if the SV40 replication origin is deleted from the plasmid during DNA cloning.

Transfection efficiency is a bottleneck to our system. Because ligation product is a closed loop DNA, it is relatively difficult to be transfected and high-efficiency transfection reagent is required. We tried calcium phosphate transfection method, but ligation product cannot been transfected into cells successfully. In addition, the final product is obtained through two-step recovery from agarose gel, so high recovery efficiency from gel is important. We performed this procedure using 3S Spin agarose gel DNA purification kit carefully with 50–60% recovery efficiency. It is worthy to explore other efficient recovery approaches.

In conclusion, we developed an assay system to evaluate the intracellular DNA glycosylase activity effectively and specifically in live cells. It is rapid, reliable and less labor-intensive and might be applicable to monitor the dynamics or regulation of intracellular DNA glycosylase activity in live cells under various culture conditions. This method will facilitate the investigation of regulation and structure–function relationship studies of DNA glycosylase and similar studies.

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