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p53 Promoter-based Reporter Gene *in vitro* Assays for Quick Assessment of Agents with Genotoxic Potential

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Abstract The *p53* promoter-based green fluorescent protein (GFP) and luciferase reporter gene assays have been established for detecting DNA damage induced by genotoxic agents. To evaluate the system, NIH3T3 cells transfected with either pHP53-GFP or pMP53-GFP construct were treated with mitomycin or 5-fluorouracil. Expression of the GFP reporter gene was significantly and specifically induced in the cells exposed to mitomycin or 5-fluorouracil. Then we treated NIH3T3 cells harboring pHP53-Luc or pMP53-Luc vector with mitomycin, 5-fluorouracil or cisplatin at various concentrations. Similarly, exposure of the cells to these agents with genotoxic potentials resulted in a dose-dependent induction in luciferase reporter gene expression. Thus, these *in vitro* reporter gene assays could provide an ideal system for quick assessment or screening of agents with genotoxic potential.

Key words *p53* promoter; luciferase; green fluorescence protein (GFP); genotoxic agent

Generation of DNA damage is considered to be an important initial event in many pathological conditions. The most common example is that DNA damage, if not repaired or repaired incorrectly, will result in mutations capable of initiating carcinogenesis in somatic cells or heritable diseases in germ cells [1]. Therefore, DNA damage is a useful end-point for assessing the genotoxic effects of environmental chemicals on human health [2]. The current methods for detecting DNA damage include the *in vitro* micronucleus assay and the single cell gel electrophoresis (Comet) assay. However, these assays have limited use because they work on the principle that breakage of double-stranded DNA or chromosomes leads to fragmentation of the large DNA molecule or chromosome, and these DNA fragments can be stretched out by electrophoresis [3,4]. However, chemicals can damage DNA in several ways, including base alteration, adduct formation, strand breaks and cross-linkage. Thus, there is a great need for a new approach that is technically simple, sensitive, relatively fast, and more flexible for detecting agents that might have the ability to cause different types of DNA damage.

The p53 protein, also known as "guardian of the genome", plays a pivotal role in maintaining the integrity of the genome and the cellular response to a wide range of DNA damage-induced genotoxic stresses [5]. Previous studies indicate that the genotoxic stress response of *p53* is partly regulated at the transcriptional level, and the minimum human *p53* promoter region required for the genotoxic stress-responsive activity resides within the core promoter region extending from –78 to +12 [6]. Therefore, we hypothesized that the *p53* promoter sequence could provide a useful strategy for detecting DNA damage and assessing the genotoxic potential of chemicals.

We developed a *p53* promoter-based reporter gene *in vitro* assay for assessing the genotoxicity of various compounds with genotoxic potential on cells *in vitro* through examining the induction of reporter gene expression, luciferase or green fluorescence protein (GFP), in the presence of the compounds.

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Material and Methods

Chemicals and plasmids

Mitomycin (MMC), 5-fluorouracil (5-FU) and cisplatin (CIS) were purchased from Sigma-Aldrich (St. Louis, USA). pEGFP-1 was purchased from Clontech (San Jose, USA). pGL3-BASIC and pRL-TK were gifts from Dr. Andre BENSADOUN (Cornell University, Ithaca, USA). Plasmids were purified using the Endofree kit (Qiagen, Carlsbad, USA) according to the manufacturer's instructions.

Construction of reporter constructs

For construction of reporter constructs, a fragment containing human *p53* gene core promoter element was generated by amplifying a region from –128 to +12 of human genomic DNA, isolated from human lymphocytes, using 5′-gacaagcttcagTCGCTCGAGCAGGCGATTACTTGCCCTTACTT-3′ and 5′-tcgctcgagcagGCTCTAGACTTTTGAGAAGCTC-3′ as forward and reverse primers, respectively. Similarly, 5′-gacaagcttcagACTTAGGGGCCCGTGTTGGTTC-3′ and 5′-tcgctcgagcagCCATCTTCGTCGCCTGAGTC-3′ were used as another pair of forward and reverse primers, respectively, to amplify a 493 bp genomic DNA fragment containing mouse *p53* gene promoter (extending from –393 to +100) on mouse genomic DNA isolated from mouse tail tissue. The sequences in lowercase at the 5′-ends of the forward and reverse primers are recognition sites specifically designed for *Xho*I and *Hin*dIII restriction enzymes, respectively. After digestion with *Xho*I and *Hin*dIII and purification, the two fragments were cloned into *Xho*I and *Hin*dIII sites upstream of the firefly luciferase reporter gene in the pGL3-BASIC vector and the GFP reporter gene in the pEGFP-1 vector. The resultant plasmids were designated as pHP53-Luc, pMP53-Luc, pHP53-GFP and pMP53-GFP.

Cell culture and transient transfection

NIH3T3 cells, obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Transfection of NIH3T3 cells was carried out by the LipofectAMINE PLUS method (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. NIH3T3 cells were grown in 24-well plates to 70%–80% confluence and subjected to transfection. Each well of cells was either transfected with 400 ng of GFP reporter plasmid DNAs or co-transfected with 400 ng of the luciferase reporter plasmid DNAs and pRL-TK *Renilla* luciferase vector (Promega, Madison, USA) (9:1) as an internal control. All the cells were subsequently grown in serum-free DMEM.

Exposure to genotoxic agents and luciferase assays

Twenty hours after transfection, cells were treated with 5-FU (5 μg/ml, 10 μg/ml, and 20 μg/ml), MMC (10 μg/ml, 20 μg/ml, and 30 μg/ml) and CIS (5 μg/ml, 10 μg/ml, and 20 μg/ml) for 24 h, respectively. The chemicals were dissolved to 1000× stock solution in water immediately prior to use. After 24 h of treatment, the cells transfected with GFP reporter vector were directly observed under fluorescence microscope. The cells transfected with luciferase reporter vector were processed for luciferase assays. The luciferase activities were assayed using the Dual Luciferase Assay System (Promega). After normalization to the *Renilla* luciferase control, the activity of each reporter construct was calculated and expressed as relative light units (RLU). The experiments were carried out three times independently with three replicates. The data are presented as the mean±SD of RLU.

Results

Construction of chimeric reporter vectors

Fig. 1 showed the schematic illustration of the plasmid structure. Four plasmids were constructed in total: two luciferase reporter vectors in which the luciferase reporter is under the control of the human or mouse *p53* gene promoter (pHP53-Luc and pMP53-Luc, respectively); and two GFP reporter vectors in which the GFP reporter is under the control of the human or mouse *p53* gene promoter (pHP53-GFP and pMP53-GFP, respectively). All the constructs were confirmed by restriction enzyme digestion and sequence analysis.

p53 promoter-driven GFP expression induced by genotoxic compounds

To evaluate the role of *p53* promoter in mediating expression of the GFP reporter gene induced by compounds with genotoxic potential, the NIH3T3 cells transfected with either pHP53-GFP or pMP53-GFP (co-transfected with pRL-TK as the internal control) were treated with 20 μg/ml of 5-FU or 30 μg/ml of MMC. Larger doses of the compounds were used for the treatment as a relatively high expression of GFP is required

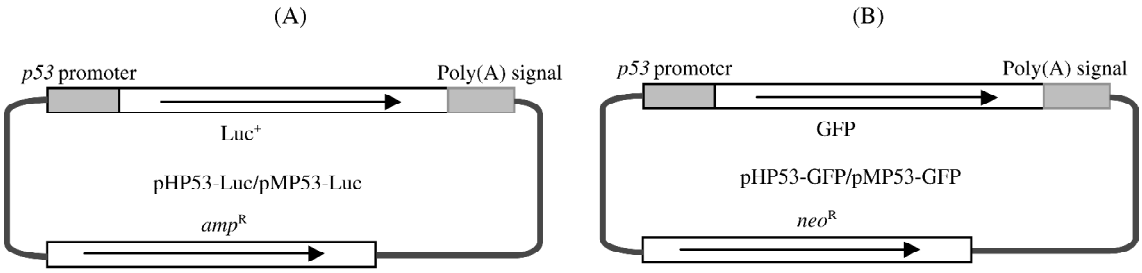

Fig. 1 Schematic maps of structure of chimerical reporter vectors constructs for this study

(A) Plasmids with the luciferase (Luc) reporter gene under the control of either human or mouse *p53* promoter (pHP53-Luc and pHM53-Luc, respectively). The vectors were generated by cloning a fragment containing either human or mouse *p53* core promoter regions into *Xho*I and *Hin*dIII sites upstream of the luciferase reporter gene in pGL3-BASIC. (B) Plasmids with the green fluorescent protein (GFP) reporter gene under the control of either human or mouse *p53* promoter (pHP53-GFP and pMP53-GFP, respectively). Similarly, the vectors were generated by cloning human or mouse *p53* promoter regions into *Xho*I and *Hin*dIII sites upstream of the GFP reporter gene in pEGFP-1.

for direct microscopic visualization. As shown in **Fig. 2**, the expression of the GFP reporter gene was highly induced by the two compounds in cells transfected with either pHP53-GFP or pMP53-GFP vector. Thus, the *p53* promoter-driven GFP reporter assay provides a simple and sensitive method for qualitatively detecting the capability of chemicals to damage DNA molecules in cells.

p53 promoter-driven luciferase expression induced by genotoxic compounds

To further validate the *p53* promoter-based strategy for detecting DNA damage induced by chemicals, the NIH3T3 cells were co-transfected with pRL-TK (as the internal control) and pHP53-Luc or pMP53-Luc vectors, then treated with 5-FU, MMC, or CIS at various concentrations. To minimize the stimulatory effects of serum on the *p53* promoter, cells were exposed to these compounds under serum-free conditions. The luciferase activities were determined using the Dual Luciferase Assay System (Promega). As shown in **Fig. 3**, 5-FU significantly and reproducibly induced the expression of the luciferase reporter gene in a dose-dependent manner. Similarly, induction of luciferase expression was observed after treatment with MMC and CIS (**Fig. 3**). Therefore, the *p53* promoter-driven luciferase reporter assay could be an ideal system for quantitative or qualitative assessment of the level of DNA damage and the genotoxic potential of chemicals.

Discussion

Genotoxicity assessment is the evaluation of agents for their ability to induce DNA damage or mutations. Compared with the detection of mutation, the detection of DNA damage provides a rapid and sensitive way of documenting exposure. The current methods for detecting DNA damage include the *in vitro* micronucleus assay and the single cell gel electrophoresis (Comet) assay. However, the use of these might be limited because they are basically designed for detecting chromosome aberrations or DNA strand breaks [3,4]. Therefore, a more versatile or flexible method is demanded for detecting various forms of DNA damage with high sensitivity.

The *p53* gene plays a crucial role in the cellular response to various genotoxic stresses induced by DNA damage [7]. Activation of *p53* by DNA damage occurs at two levels, the transcriptional and the post-translational (stabilization of p53 protein), which leads to the induction of several cellular processes including cell cycle checkpoints, DNA repair or apoptosis [5]. A previous study indicated that the human *p53* core promoter region (from –78 to +12) is sufficient for *p53* genotoxic stress-responsive promoter activity [6]. Thus, the *p53* core promoter sequence would be very useful in developing a reporter gene assay for detecting DNA damage. GFP and luciferase are the most versatile and common reporter genes, and are regularly used to study a wide range of biological events in cultured cells. The common advantages of both reporter gene assays are their high sensitivity, versatility, rapidity and low costs. Therefore, the reporter gene assays in which the luciferase or GFP reporter gene is placed under the control of the *p53* promoter could be an ideal system for quantitatively or qualitatively detecting DNA damage in mammalian cells with high sensitivity. In this report, we provide evidence to support this hypothesis.

The four constructs, pHP53-Luc, pHP53-GFP, pMP53-Luc and pMP53-GFP, were generated by cloning either a

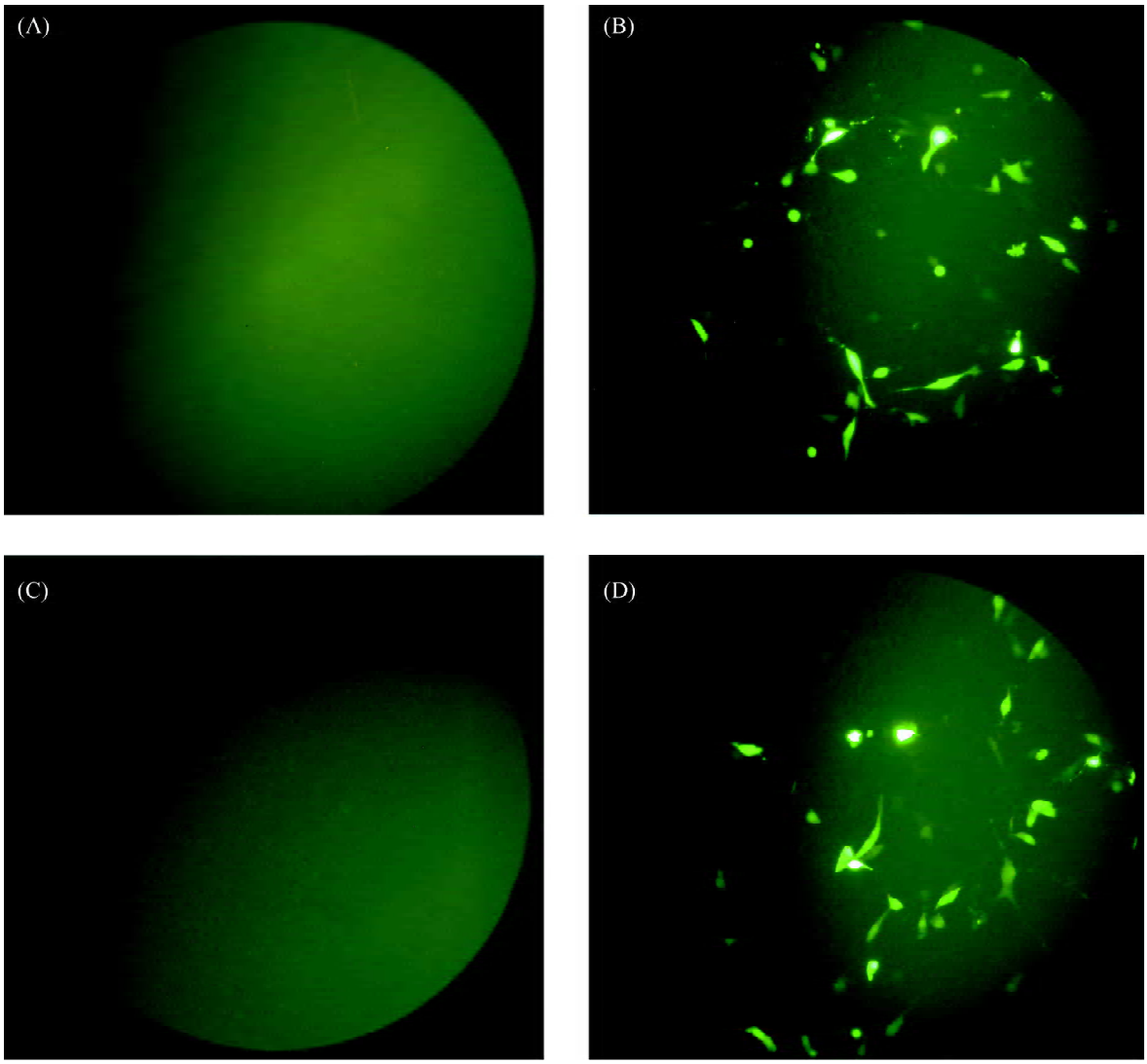

Fig. 2 Fluorescence induction by mitomycin (MMC) in NIH3T3 cells transiently transfected with construct harboring green fluorescent protein (GFP) reporter gene under control of *p53* promoter

NIH3T3 cells transfected with pHP-GFP plasmid were exposed for 24 h to serum-free medium (control) (A) or to 30 μg/ml MMC (B). Similarly, NIH3T3 cells transfected with pMP-GFP plasmid were exposed for 24 h to serum-free medium (control) (C) or to 30 μg/ml MMC (D). All the cells fluoresced green were directly visualized and photographed under a fluorescent microscope. Similar results were observed for the cells treated with 20 μg/ml of 5-fluorouracil (photographs not shown). Magnification, 100×.

140 bp fragment containing human *p53* core promoter, extending from −128 to +12, or a 493 bp fragment containing the mouse *p53* promoter region, from −393 to +100, into *Hin*dIII and *Xho*I sites in the pGL3-BASIC and pEGFP-1 vectors. To validate the use of the reporter gene assays and to examine the role of *p53* promoter in mediating GFP or luciferase induction in cultured mammalian cells, we tested the GFP reporter system by exposing NIH3T3 cells, transfected with pHP53-GFP or pMP53-GFP, to MMC or 5-FU. MMC and 5-FU are widely used anti-tumor agents that are given as clinical treatment for several types of cancers. Many studies reveal that they have genotoxic properties in bacteria, in mammalian cells *in vitro* and in mammals *in vivo*, inducing DNA damage such as cross-linking, mutations, chromosomal aberrations, micronuclei and sister chromatid exchanges [8–10], and treatment of mammalian cells with MMC results in a significant increase in the cellular *p53* level [11–13]. Theoretically speaking, DNA damage resulting from exposure of the cells to MMC or 5-FU would lead to activation of the *p53* gene promoter and induction of the GFP reporter gene under the control of the *p53* promoter.

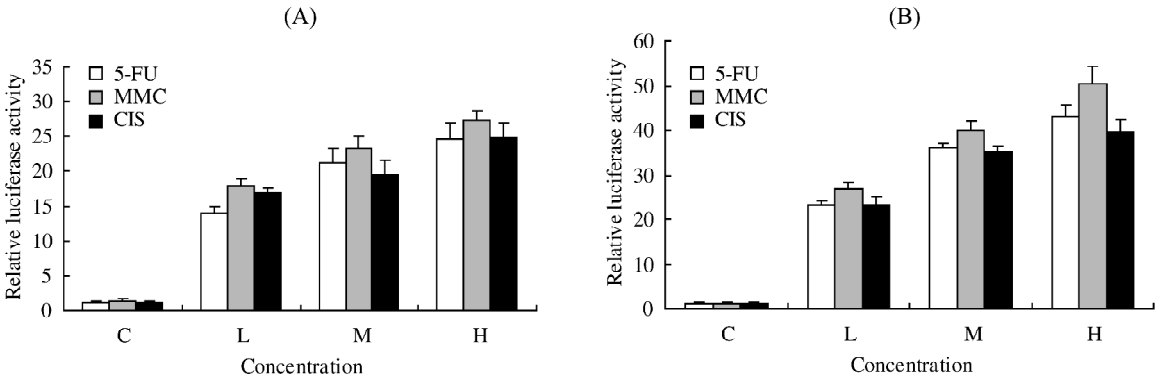

Fig. 3 Induction of *p53* promoter-driven luciferase reporter gene expression by chemotherapeutic chemicals with genotoxic potential

Relative luciferase activities of extracts of NIH3T3 cells transiently transfected with construct harboring luciferase reporter gene under the control of the *p53* promoter. (A) NIH3T3 cells were transfected with pHP-Luc plasmid harboring a luciferase reporter gene under the control of human *p53* promoter (−128 to +12). (B) NIH3T3 cells were transfected with pMP-Luc plasmid harboring a luciferase reporter gene under the control of the mouse *p53* promoter (−393 to +100). C, cells were exposed for 24 h to serum-free medium (control); L, cells were exposed for 24 h to low concentrations of compounds (5 μg/ml 5-FU; 10 μg/ml MMC; 5 μg/ml CIS); M, cells were exposed for 24 h to medium concentrations of compounds (10 μg/ml 5-FU; 20 μg/ml MMC; 10 μg/ml CIS); H, cells were exposed for 24 h to high concentrations of compounds (20 μg/ml 5-FU; 30 μg/ml MMC; 20 μg/ml CIS). To correct for differences in transfection efficiency, cells were co-transfected with pRL-TK plasmid expressing *Renilla* luciferase. Luciferase activity was assayed using the Dual Luciferase Assay System (Promega). The relative luciferase activity was calculated by taking the ratio of the firefly to *Renilla* luciferase. Values represent the mean±SD of three observations. The experiment was carried out three times with similar results. 5-FU, 5-fluorouracil; MMC, mitomycin; CIS, cisplatin.

The cells producing GFP will fluoresce green under blue light. The results in **Fig. 2** provide evidence for this working hypothesis. The cells exposed to MMC fluoresced green under the fluorescent microscope, whereas the cells treated with serum-free medium (control) did not, suggesting that the *p53* promoter-driven GFP reporter gene was dramatically induced by MMC or 5-FU and might be an ideal system for quick assessment of genotoxic potential of agents. In order to further validate the *p53* promoter-based strategy for detecting DNA damage induced by chemicals, NIH3T3 cells transfected with luciferase reporter vectors were exposed to 5-FU, MMC, or CIS at various concentrations. After 24 h of treatment, the cells were processed for luciferase assay. As shown in **Fig. 3**, all three compounds produced significant transcriptional activation in the luciferase reporter gene assays in a dose-dependent manner, suggesting that the system can be used for the quantitative detection of agents with various genotoxic potential. As no obvious difference was observed between the assays driven by the human *p53* promoter or by the mouse *p53* promoter, we believe that both systems are applicable to all mammalian cells.

In conclusion, we are the first to develop the *p53* promoter-driven luciferase and GFP reporter gene assay, which provides an alternative strategy for quick assessment of agents with genotoxic potential. Compared with other DNA damage-based genotoxicity assays, the advantages of this system include: (1) versatility and flexibility in detecting chemicals that cause different types of DNA damage; (2) low cost; and (3) ease of use. We acknowledge, however, that these findings might be strengthened if more cell lines or animal models were studied, and the GFP gene expressions were measured quantitatively with statistical significance and a time-course description. Therefore, additional experiments are underway, in our group, to further validate these observations and to explore whether it is possible to extend the *p53* promoter-based reporter gene assays to a transgenic model of zebra fish or other cell lines.

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