行政院國家科學委員會專題研究計畫 成果報告

研究 p53R2 在輻射線誘發之 DNA 修補機制中所扮演之角色
研究成果報告(精簡版)

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附件一

行政院國家科學委員會補助專題研究計畫 □ 成 果 報 告 □期中進度報告

研究 p53R2 基因在輻射線誘發之 DNA 修補機制中所扮演之角色

計畫類別：■ 個別型計畫　　□ 整合型計畫
計畫編號：**96-2311-B-002-007-**
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計畫主持人：**蔡孟勳**
共同主持人：
計畫參與人員：

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：**國立臺灣大學生物科技研究所**

中　華　民　國　96　年　10　月　31　日

一、中英文摘要：

（一） 中文摘要。

p53R2 基因的蛋白質產物為核糖核苷酸還原酶(ribonucleotide reductase) 。 在 *p53R2* 基因第一個 intron 區域內含有抑癌基因 p53 蛋白結合的序列，並且已被證實 *P53R2* 直接受到 p53 的調控。當細胞受到游離放射線,或紫外線導致基因受損時，細胞會透過 p53 來誘導 *p53R* 基因。由於核糖核苷酸還原酶的作用在催化核糖核苷酸(ribonucleotide)轉化為去氧核糖核苷酸(deoxyribonucleotides)，p53R2 被認為在 DNA 傷害發生時負責 DNA 修復的功能上扮演重要的角色。先前的研究發現，在 p53 突變的細胞株 WTK1 對於游離放射線有很高的敏感性，受到輻射照射後的基因突變頻率(mutation frequency)提高很多。因此我們假設由於 p53 的缺失導致 *p53R2* 無法表現，*p53R2* 無法表現導致細胞無法提供去氧核糖核苷酸供 DNA 修補以至於基因的突變率增高。為了證明 WTK1 受輻射照射後的高突變率是由於 *p53R2* 無法表現所導致，我們將 *p53R2* 利用載體 *pcDNA3* 後(Invitrogen, USA)大量表現在 WTK1 細胞株內。目前進度已經利用 Tranfection 技術將載體 *pcDNA3* 送入 WTK1 細胞中，我們將挑選 Stable clone，得到具有大量表現 p53R2 的單一細胞株，此外，我們也將 p53R2 SiRNA 利用載體 *pSilencer* 4.1-CMV neo(Ambion, USA)送入 TK6 細胞株內，期望可以挑選到抑制 p53R2 表現的細胞株，也目前進度為挑選 Stable clone 的階段。未來計畫將此大量表現 p53R2 的 WTK1 細胞照射放射線後，觀察細胞之存活是否改變及基因突變率是否降低，以了解 p53R2 與基因突變率的關係。我們也將利用免疫沉澱法探討 p53R2 蛋白是否會何其他 DNA 修補相關的蛋白質作用，以了解 p53R2 在 DNA 修補功能上扮演的腳色。

（二） 英文摘要。

p53R2 encodes a 351- amino-acid peptide homologous to the human ribonucleotide reductase small subunit 2 (R2) which catalyzes the conversion of ribonucleotides (NTP) into deoxyribonucleotides (dNTP). *p53R2* has a p53-binding site in intron 1 and has been demonstrated to be regulated by p53. *p53R2* is induced when DNA damage by gamma-ray, ultraviolet irradiation and genotoxic chemicals such as adriamycin in a wild-type p53-dependent manner, and is suggested to function in DNA repair by increasing the dNTP pools needed for repair. p53R2 has been defined in the DNA repair pathway, however, its role in radiation mutagenesis remains to be elucidated. For proving WTK1 high mutation rate is because p53R2 couldn't expression,we over expression p53R2 on WTK1 cell line by vector pcDNA3. The

progress has already utilized tranfection technology to send the carrier pcDNA3 into WTK1 cells at present, we will select Stable clone, have a large number of single cell one which display p53R2. In addition, we sent p53R2 SiRNA into TK6 cell line by utilizing vector pSilencer 4.1 - CMV neos (Ambion, USA), and expected select cell line which could inhibit p53R2 display.We select the stage of Stable clone in progress at present too.Previous studies have shown that WTK1 cell line, which overexpresses a mutant form of p53, is much sensitive to spontaneously arising and radiation-induced mutation. To verify whether p53R2 responses to the high frequency of mutagenicity in the radiation treated WTK1 cells, we will construct a p53R2-overexpressed WTK1 cell line to determine the toxicity and mutagenicity of WTK1 cells with overexpressed p53R2 after ionizing radiation treatment. Furthermore, we will use immunoprecipitation coupled with western blot to identify the putative p53R2 interacting proteins and study the relationship between p53R2 and other DNA repair related proteins.

Key words: p53R2, radiation, mutagenicity.

二、前言

In our previous study[1], we have known that p53 responses to the hypermutability to ionizing radiation. The functions of p53 in regulation of mutational processes have been further investigated utilizing cDNA microarray analyses. In the microarray study, gene expression patterns among three related human cell lines with different p53 status: TK6 (wild-type p53), NH32 (p53-null) and WTK1 (mutant p53), were compared. Total RNA samples of the three cell lines were collected at 1, 3, 6, 9, and 24 h after 10Gy γ-irradiation. The results show that, in contrast to WTK1 and NH32, genes associated with DNA repair, such as p53R2, DDB2, XPC, PCNA, BTG2, and MSH2, were highly induced in TK6. p53R2 is a human ribonucleotide reductase (RR) small subunit 2 (R2) gene whose function in conversing ribonucleotides into deoxyribonucleotides (dNTP). The background mutation frequencies at the TK locus after 2Gy radiation in TK6 cells with p53R2 knock down by siRNA were about three times higher than those seen in TK6 cells without p53R2 knock down. These results indicate that p53R2 is induced by p53 protein and that p53R2 is implicated in protecting against radiation-induced mutagenesis.

三、研究目的、文獻探討。

Ionizing radiation has been known to cause a variety of cellular responses such as necrosis, DNA damage and repair, cell cycle arrests, and apoptosis[2-5]. Many genes involved in stress responsive pathways, such as the MAP kinase pathway[6, 7], EGFR gene family[8], cytoskeletal elements[9], cytokines[10, 11], DNA repair proteins[12, 13] and TP53-activated molecules[14-16], have been shown to alter the transcriptional levels after giving ionizing radiation, and are expected to participate in the radiation-induced responses.

p53R2 encodes a 351- amino-acid peptide with three domains, two putative nuclear localization signal sequences and one RR small subunit signature [17]. *p53R2* is homologous to the

human ribonucleotide reductase small subunit 2 (R2) which catalyzes the conversion of ribonucleotides (NTP) into deoxyribonucleotides (dNTP). Ribonucleotide reductases are essential for DNA synthesis and repair: R2 is important in DNA synthesis during cell division, whereas p53R2 is induced when DNA is damaged and is suggested to function in DNA repair by increasing the dNTP pools needed for repair. *p53R2* has a p53-binding site in intron 1 and has been demonstrated to be regulated by p53 [17]. Previous studies show that expression of p53R2, but not R2, was induced by gamma-ray, ultraviolet irradiation and genotoxic chemicals such as adriamycin in a wild-type p53-dependent manner [17-21]. Xue et al. [22] demonstrated that three human RR subunits, hRRM1, hRRM2 and p53R2, translocate from the cytoplasm to the nucleus in response to UV irradiation and that the capability of translocation is deficient in the presence of mutant p53. Furthermore, inhibition of p53R2 expression in cells that have a p53-dependent DNA damage checkpoint results in the reduction of RR activity, DNA repair, and cell survival after exposure to various genotoxins [17]. Based on these findings, it has been proposed that p53R2 mutations result in deficiency of DNA repair and cancer susceptibility.

Although p53R2 has been defined in the DNA repair pathway, however, its role in radiation-induced mutagenesis remains to be clarified. Previous study showed that RNR can be formed via two different pathways, the p53-dependent pathway that composed of p53R2 and R1, and the p53-independent pathway that composed of R2 and R1 [17]. Another study also showed that DNA damage caused increased levels of the R2 protein and dNTPs, that improved the survival of p53(-/-) cells [20]. This result suggests that DNA damage repair can process via a p53R2-independent pathway. However, p53R2 still play a pivotal role in DNA repair when DNA damage caused by UV light, radiation or adriamycin. According to a previous study by Tanaka et al.[17], inhibition of p53R2 with antisense DNA caused a significant decrease in incorporation of dNTPs into DNA, following DNA damage induced with UV light or adriamycin. Moreover, Kimura et al. (2003) [23] showed that dNTP pools were severely attenuated in p53R2-/- mice under oxidative stress, and p53R2 deficiency caused higher rates of spontaneous mutation in the kidney of p53R2-/- mice. Our previous studies[1] have indicated that suppression of p53R2 gene expression resulted in a significant increase in radiation-induced mutability, however, only slightly decreased survival of g-irradiated cells. This indicated that those cells with p53R2 knock-down presumably are deficient in the DNA repair mechanism when damaged by irradiation, but their survival is relatively unaffected. Based on those results, it appears that p53R2 plays a vital role in repairing DNA damage. A disruption of p53R2 gene expression, which would be expected to hinder the production of RNR, in turn reduces dNTP pools. Such damage might block the integration of dNTP into DNA and lead to accumulation of spontaneous or induced mutations.

The human B-lymphoblast cell lines TK6 and WTK1 are derived from the same progenitor, WIL2. Both are heterozygous at the autosomal thymidine kinase (TK) locus [24-26], but differ in their p53 status. WTK1 [27, 28] overexpresses a mutant form of p53 (methionine to isoleucine substitution at codon 237), whereas TK6 is wild type for p53. Furthermore, two cell lines show different behaviors in response to irradiation. Compared to TK6, WTK1 shows a delayed and reduced X-ray-radiation-induced apoptosis and appears much sensitive to spontaneously arising

and radiation-induced mutation [24]. Our previous study[1], the mutation frequencies after radiation in TK6 cells with p53R2 knock down were significantly higher than those seen in TK6 cells without p53R2 knock down, that demonstrated p53R2 involves in DNA repair. Here we propose a hypothesis that lack of p53 causes deficiency in the presence of p53R2 and consequently enhances mutation frequency in the radiation-treated WTK1 cells. To verify p53R2 is implicated in the increase of mutation frequency in the WTK1 cells after radiation, we will construct a p53R2-overexpressed WTK1 cell line to investigate whether the overexpressed p53R2 protein could rescue the radiation-induced mutation frequency in transfected WTK1 cells. Furthermore, because p53R2 has a portal role in DNA repair, it may have interaction with other proteins that function in DNA repair system. Thus we will use immunoprecipitation and immunoblot analysis to identify the DNA repair related genes, such as PCNA, MSH2, DDB2, GADD153 or DNA-PK, that could interact with p53R2.

四、研究方法

Cell culture

Three human lymphoblastoid cells, WTK1 and TK6, will be maintained as exponentially growing cultures in RPMI 1640 supplemented with 10% horse serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells (1×10^8) will be grown exponentially in stationary culture in loosely capped tissue culture flasks at 37°C in 5% CO_2 at densities of $4–10\times10^5$ cells/ml. Cells will be treated with 10Gy -ray radiation and total protein samples will be extracted from the transfected WTK1 cells or the TK6 cells as well as the mock control after irradiation for Western blots.

Overexpression of p53R2

Stable cells lines expressing construct encoding recombinant p53R2 will be obtained as follows. WTK1 cells (5×10^5) in 60-mm dishes will be transfected using pCEN-p53R2 and pCEN vector (10 μg each DNA) and Lipofectin (Life Technologies, Gaithersburg, MD) as described previously (2). The medium containing 1 mg of G418 per ml (GIBCO/BRL) will be changed every 4 days, and single colonies will be picked after 21 days. To examine p53R2 expression, western blot analysis will be conducted.

Western blot analysis

Cells will be pelleted and washed twice with cold PBS, and lysed on ice for 20 min in 50mM Tris-HCl (pH 8.0), 150mM NaCl, 0.02% sodium azide, 100 mg/ml phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, and 1% NP40. The protein concentration of each sample will be quantified, and 50mg of protein from each sample will be loaded onto a 12% SDS polyacrylamide gel. After electrophoresis, proteins were transferred onto a nitrocellulose membrane. Filters will be probed with p53R2 antibody and followed secondary antibody. The signals were detected by the enhanced chemiluminescence system (PerkinElmer, Boston, MA, USA).

Determination of toxicity and mutagenicity of ionizing radiation

Following radiation treatment, lymphoblastoid cells will be seeded into 96-well microtiter plates at densities of 1–10 cells/well; after 12 days, colonies will be counted, and the Poisson distribution will be used to calculate the plating efficiency. The surviving fraction will be determined by dividing the plating efficiency of a transfected culture by the plating efficiency of the TK6 and mock control[29]. For mutagenicity analysis, cultures will be grown in nonselective medium for 3 days after treatment, then cells will be plated in 96-well plates in the presence of TFT (2.0 mg/ml) to select TK^- mutants. Cells from each culture will be also plated at 1 cell/well in the absence of TFT to determine plating efficiency. All plates will be incubated for 11 days prior to scoring colonies. Mutation plates will be refilled with fresh TFT medium and incubated for an additional 7 days to observe the appearance of any late-appearing mutants. The mutant fractions will be calculated with the Poisson distribution[29].

Immunoprecipitation

Cell lysates will be prepared in lysis buffer (50 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 1 mM EDTA, 0.1% Triton X-100, and 0.1% [wt/vol] SDS), containing protease inhibitor. Cell lysates containing 300 μg proteins will be treated with the p53R2 primary antibodies overnight at 4°C with gentle shaking. Immunocomplexes will be collected by incubation with protein G-Sepharose beads (GE Healthcare, Piscataway, NJ) at 4°C with gentle shaking for 2 hours. The beads will be washed, suspended in Laemmli sample buffer, and then analyzed by SDS-PAGE, followed by Western blot analysis. Western blot will be probed with the antibody of DNA repair related proteins and followed secondary antibody. The signals were detected by the enhanced chemiluminescence system.

Transfection

Cells diluted to 1 x 106/ml were transfected with 1 μg/ml p Silencer 4.1-p53R2 plasmid or pcDNA3-p53R2 plasmid control using DMRIE-Reagent (Invitrogen) in Opti-MEM I reduced serum (Invitrogen). For later experiments, 6μg/ml p Silencer 4.1-p53R2 plasmid or pcDNA3-p53R2 plasmid in siPORT XP-1 (Ambion, Austin, TX) and RPMI 1640 medium mixture were used for transfection. After incubation at 37˚C for 5 hours, the medium was removed and replaced with fresh RPMI 1640 medium supplemented with 15% horse serum. Transfection was repeated two days after first transfection for both TK6 and WTK1.

五、結果與討論

For over-expression of p53R2, the experiment were designed primers p53R2FL (3'-atgggcgacccgga) and p53R2FR (ttaaaaatctgcatccaaggtga). Using RT-PCR, a 1055bp cDNA fragment was amplified from TK6 cells. This fragment were then cloned into the pcDNA3 vector (Invitrogen, USA , Fig 2) by cloning technique. Sequencing validation was also performed. The sequence confirmed plasmid was then transfected into WTK1 cells by lipofectamine

transfection and cultured in RPMI medium with G418 to select the stable transfection WTK1 cells. At present, we have screened eight 96 well plates and only got 8 clones can grow in RPMI medium contained G418. These clones were further tested by real-time RT PCR to validate p53R2 mRNA expression level. Unfortunately, real-time PCR results showed no significant p53R2 expression level among these 8 clones. We will keep trying to find the p53R2 over-express stable WTK1 cells for further radiation induced mutation assay.

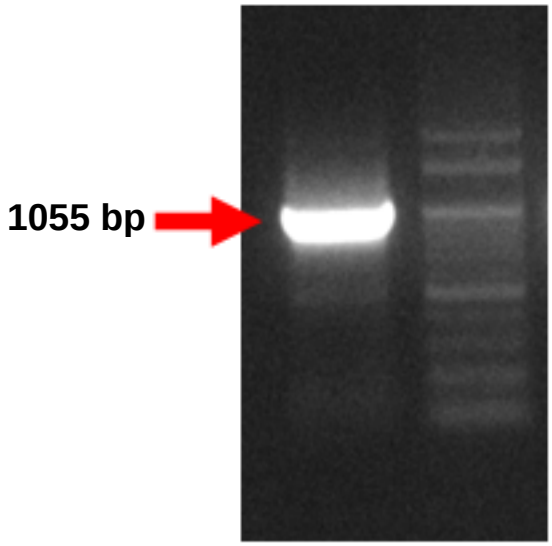

Fig. 1 p53R2大量表現PCR產物

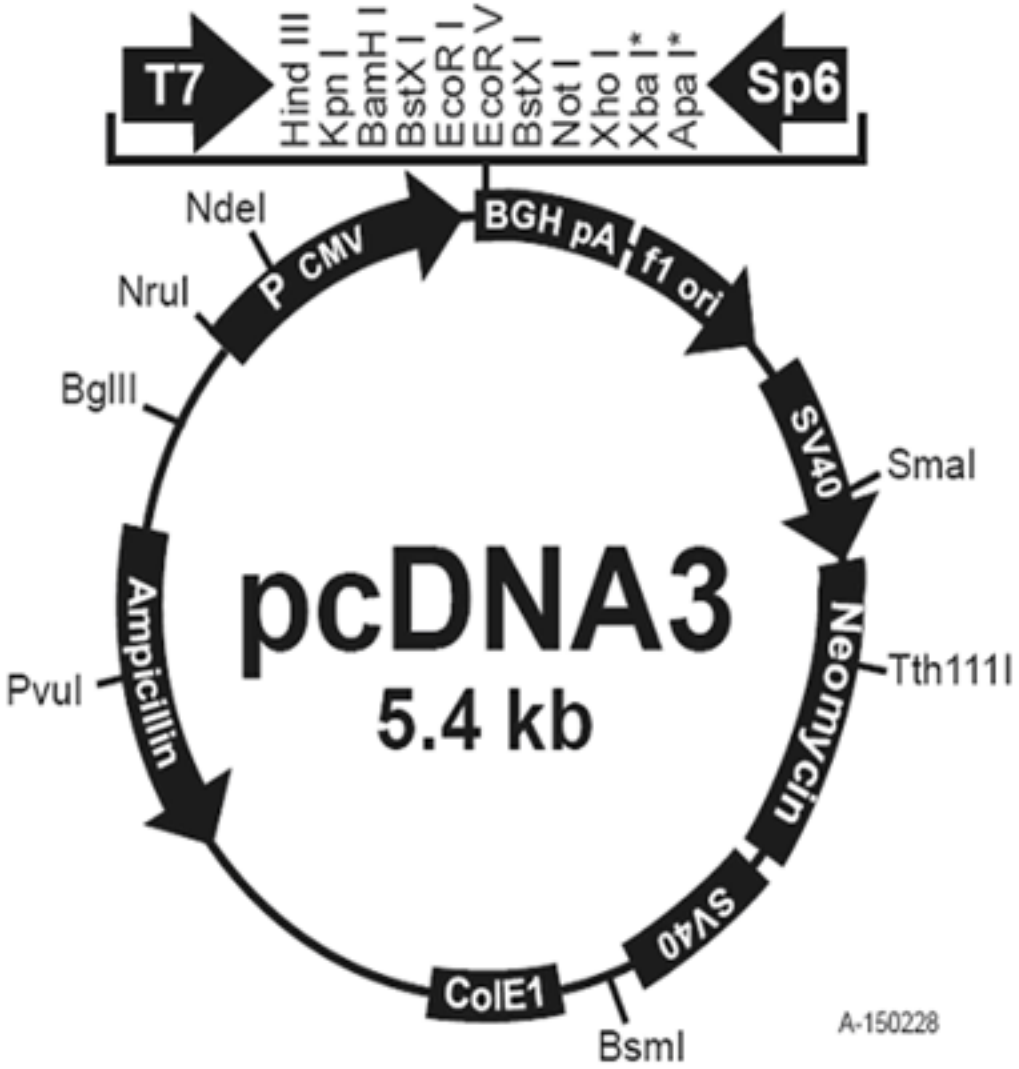

Fig. 2 pcDNA3大量表現載體

In addition, we also designed the anti-p53R2 RNAi plasmid (Fig.3) and transfected the plasmid into TK6 cells to knock down p53R2 gene. Moreover, to know the change of p53R2 level when p53 gene was knocked down was interesting. Therefore, anti-p53 RNAi plasmid (from National RNAi core, Taiwan) (Fig. 4) was also used to transfect into TK6 cells for knocking down p53 gene. p53 siRNA transfection showed significant reduction of p53 expression compared to control plasmid (anti-luciferase RNAi plasmid) after 48 hours transfection in TK6 cells (Fig. 5). We then continue selecting both anti-p53R2 and anti-p53 RNAi plasmid transfected stable TK6 cells.

We will further study the relationship between p53R2 and p53, gene mutation rate, or cell survived rate at radiation exposed TK6 cells.

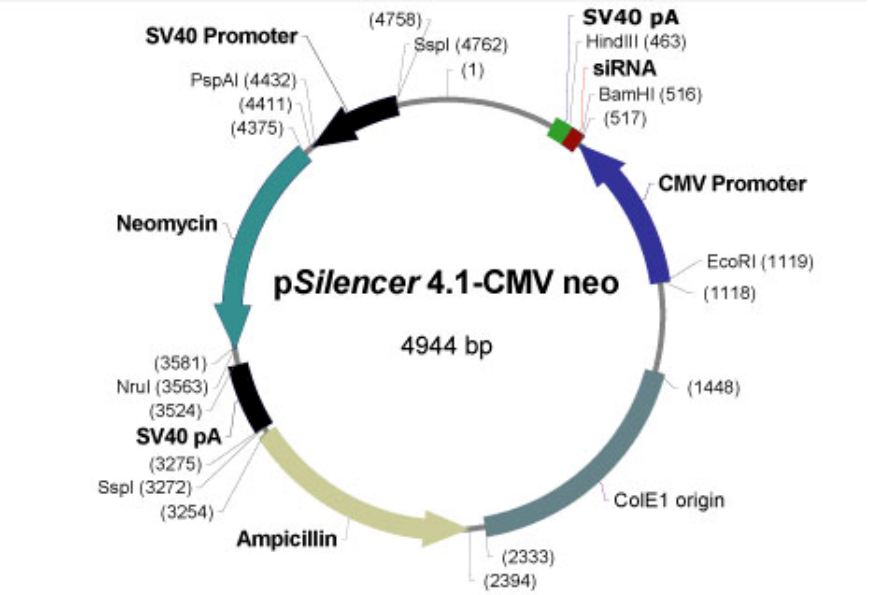

Fig. 3 p53R2載體

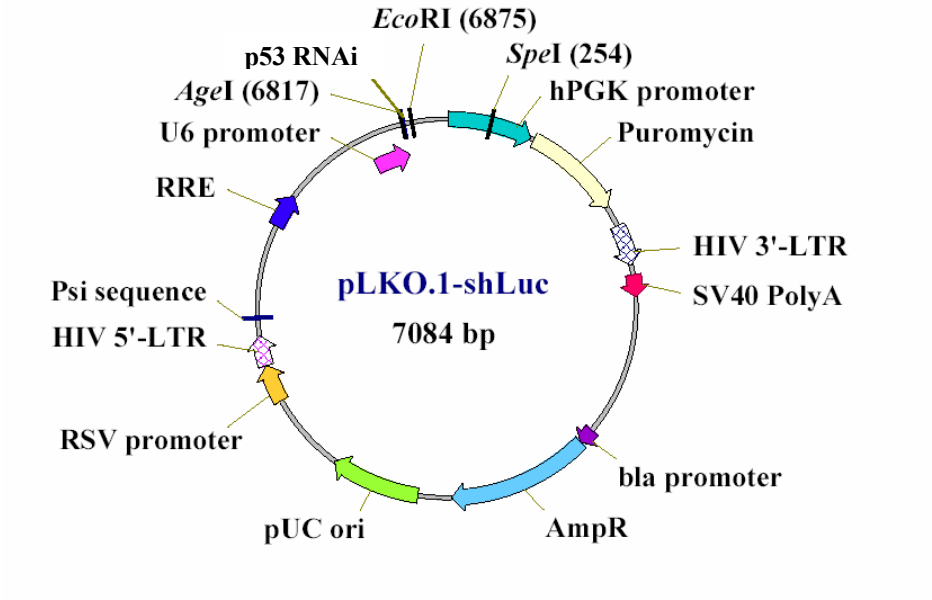

Fig. 4 p53載體

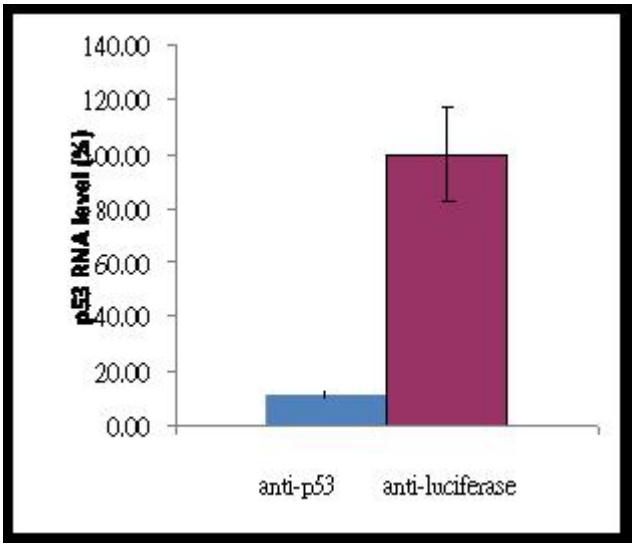

Fig. 5 以 real-time RT-PCR 檢測 anti-p53 RNAi 抑制 p53 gene 效果

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