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博士論文

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小干擾核糖核酸治療神經外科疾病---以神經病變痛及神經腫瘤為例

Small interfering RNAs for treatment of neurosurgical diseases, specifically involving neuropathic pain and neural tumors.

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目錄

表目錄

圖目錄

Abbreviations(縮寫表)

Apo B	apolipoprotein B
Bcl-2	B-cell lymphoma 2
Bcl-xL	extra-large B-cell lymphoma
CCD	charge-coupled device
CNS	central nervous system
DC	dyskeratosis congenita
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DR5	death receptor 5
DREZ	dorsal root entry zone
DRG	dorsal root ganglion
dsRNA	double stranded ribonucleic acid
ELC	electrochemiluminescence
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum

FITC	fluorescein isothiocyanate
GDNF	glia derivated neurotrophic factor
HRP	horseradish peroxidaseNOXA
IBMFS	inherited bone marrow failure syndrome
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
Noxa	NAPDH oxidase activator
p53	tumor protein 53
PBS	phosphate buffered saline
PI	propidium iodide
PTGS	post-transcriptional gene silencing
Puma	p53-upregulated modulator of apoptosis
RB1	retinoblastoma 1 gene
RNAi	RNA interference
siRNA	small interfering Ribonucleic acid
Smac	second mitochondria-derived activator of caspases

TFF3 trefoil factor 3

TRAILR-2 tumor-neurosis-factor-related apoptosis-inducing ligand receptor-2

TRF1 telomeric repeat-binding factor 1

中文摘要

本篇論文研究分為三部分，第一部分是以緩激肽 B2 受體小干擾核糖核酸(Bradykinin B2 receptor siRNA)在大白鼠背根神經節阻斷疼痛傳導而達到鎮痛效果。第二部分是以端粒酶小干擾核糖核酸(telomerase siRNA)引導神經母細胞瘤癌細胞(neuroblastoma cell)產生核凝結及細胞週期停動；第三部分是以棉酚(Gossypol)引導經由 Smac, p53 及 caspase 途徑的視網膜母細胞腫瘤細胞凋亡。

小干擾核糖核酸(small interfering RNA; siRNA)是長度 20-25 個核甘酸的雙股 RNA，主要參與 RNA 干擾(RNAi)現象，以帶有專一性的方式調節基因的表達。小干擾 RNA 可經由多種不同轉染(transfection)技術導入細胞內，並對特定基因產生具專一性的基因表現減量(knock-out)效果，已經成為研究基因功能與開發新藥物目標的一項重要工具。

第一部分研究以大白鼠神經病變痛動物模式進行，將大白鼠麻醉後，左側腰椎第六條神經使用外科手術用鈦金屬夾夾住，造成神經損傷，因此就成為神經病變痛動物模式。大白鼠的接受手術後，在不同的足底觸覺刺激程度時觀察牠左腳收起時所需的時間，用來作為對疼痛感覺敏銳度的評估。在對照組是將神經夾起，實驗組則將神經夾起同時系列的注射緩激肽 B2 受體小干擾核糖核酸，實驗動物觀察到 2 周的時間，同時重複性的比較大白鼠雙下肢對於觸感刺激的下肢收縮時間，用於評估對疼痛的耐受度。根據實驗結果可以證實，使用緩激肽 B2 受體小干擾核糖核酸會使大鼠對於神經病變痛的耐受性顯著的增加,另外實驗後將大鼠的左右雙側腰椎第四、五及第六背根神經節取出，以 RT-PCR 檢測緩激肽 B2 受體的表現量，可以證實在給予小干擾核糖核酸之後緩激肽 B2 受體的表現量在實驗組有顯著的降低。由以上的結果可以證實緩激肽 B2 受體小干擾核糖核酸在動物模式可以顯著地降低疼痛的敏感度，達到鎮痛的效果。

第二部分研究的目標是神經母細胞瘤，我們選用人類神經母細胞瘤細胞(IMR-32)做實驗，在加入不同濃度的 siRNA 後，發現端粒酶的活性顯著降低(加入 100 μM 濃度時比對照組降低 80%活性)，檢測其端粒酶 RNA 發現加入 100 μM 組是對照組的 20%。

IMR-32 細胞的生存能力以 WST-1 測定法(WST-1 assay)評估，結果發現加入 10 μM 及 100 μM 濃度組別的 IMR-32 細胞生存能力顯著的比對照組低。

細胞凋亡的末期會有細胞核碎裂(fragmentation)，IMR-32 細胞培養時加以上不同濃度的端粒酶 siRNA，並以 DAPI 標記，DAPI 可以分辨出正常細胞與進行凋亡的細胞，結果發現 10 μM 及 100 μM 濃度組的細胞比 1 μM 濃度及對照組多了 10%的凋亡現象。

以流式細胞計量術(flow cytometry)劑量細胞週期不同期的細胞比率，一般而言，癌細胞為不死細胞，因為預估會有較少細胞在 sub-G1 及 G1 期；而較多的細胞在 S 及 G2/M 期。將 IMR-32 以不同濃度端粒酶 siRNA 處置後，結果顯示在 10 μM 及 100 μM 濃度組在 sub-G1 期細胞顯著的增加：在 100 μM 組則發現 G1 及 S 期的細胞顯著的減少。

第三部分研究的目標是視網膜母細胞瘤，我們選用人類視網膜母細胞 Y79 以及人類視網膜素色上皮細胞(ARPE)進行實驗。在細胞存活能力方面使用 MTT-測定法，發現 5,10 及 20 μM 濃度的棉酚顯著的抑制 Y79 的存活能力，但對 ARPE 細胞沒有影響。

Y79 細胞培養時加上不同濃度的棉酚，並以 DAPI 標記，發現 10 μM 及 20 μM 濃度組比對照組有顯著增加的細胞凋亡現象。

以流式細胞計量術計量細胞週期不同期的數目時，發現在 10 μM 及 20 μM 時，有顯著的細胞增加在 G0/G1 期，但在 G2/M 期則顯著的減少，表示棉酚除了可以

誘導凋亡外，也可以使細胞週期停動。

在棉酚導引細胞凋亡機制研究方面，以 20 μM 濃度組與對照組比較蛋白質表現發現凋亡蛋白質如 DR5, p53, Smac, caspase 8, caspase 9 及 caspase 3 在 Y79 外細胞組的提升 1.5-2 倍。而 Cytochrome C 則提升到 5.8 倍，顯示棉酚可使用多個機制導引 Y79 細胞凋亡，包括 1) TRAIL-媒介(DR5)途徑，導致 caspase 家族成員濃度提升，如 Smac, Cytochrome C 等。2) DNA 降解致 p53 提升及細胞週期停動。

以上的結果，可以推論若能通過未來進一步的臨床試驗，則可以在臨床治療上，使用緩激肽 B2 受體及端粒酶的小干擾核糖核酸分別治療重度神經疼痛及罹患神經母細胞瘤的病患。另外棉酚除了現有的男性避孕臨床用途外，可以經由臨床試驗將來或能在難治的視網膜母細胞的治療上扮演重要角色。

關鍵詞：神經病變痛，緩激肽 B2 受體，小干擾核糖核酸，端粒酶，神經母細胞瘤，細胞核凝集，細胞週期停動，棉酚，視網膜母細胞瘤，細胞凋亡

Abstract in English (英文摘要)

Keywords: Neuropathic pain, Bradykinin B2 receptor, siRNA, Telomerase, Neuroblastoma, Nuclear condensation, Cell cycle arrest, Gossypol, Retinoblastoma Apoptosis

RNA interference (RNAi) describes a conserved biological response to double stranded RNA (dsRNA), which results in the degradation of homologous messenger RNA. This process of sequence-specific, post-transcriptional gene silencing has become a key technique for rapidly assessing gene function in both plants and mammals. For target RNA recognition to occur, the small interfering RNA (siRNA) duplex unwinds, allowing binding of one siRNA strand to the target mRNA. The advantage of RNAi to an organism is that siRNA, which specifically binds to target mRNA, prevents damage to other tissues. Adopting this approach may increase a treatment's therapeutic effect and reduce side effects in patients receiving treatment.

The first part of this dissertation is RNA interference of bradykinin B2 receptor reducing the neuropathic pain caused by sciatic nerve injury. In the condition of cell injury, several inflammatory mediators release from damaged cells. Some of these

mediators cause local effect results in increased sensitivity to pain. The hypersensitivity of this sensation is partly due to inflammatory mediators such as prostaglandins, histamine, bradykinin, substance P, and serotonin, which cause a local effect of nociception, and partly some neurotrophic factors. Some mediators transmit the sensation of pain, induced by damage of surrounding cells or even nociceptive neurons. The sensitization of nociception caused by neural damage is normally known as neuropathic pain. Other mediators, such as nerve growth factor and other neurotrophic factors, regulate the progress of neuron regeneration. However, changes in expression of receptors for allogeneic substances such as bradykinin may also be involved, causing a long-term effect. The result shows the nociception caused by neuropathy was reduced by bradykinin B2 receptor siRNA. We therefore supposed that inhibit bradykinin B2 expression may reduce the nociceptive sensation caused by neuropathy.

In our preliminary studies, we screened several inflammatory mediators and found that the nociception caused by neuropathy can be decreased by blocking the transmission of bradykinin. We therefore constructed the RNA interference (RNAi) of bradykinin B2 receptor and applied on the neuropathic animal models. The spared nerve injury models will be used to demonstrate the neuropathic nociception and the

mechanical sensitivity behavior test were used to evaluate the degree of neuropathic nociception. Bradykinin B2 receptor expression was upregulated after sciatic nerve crush, while this upregulation was reversed by application of siRNA of bradykinin B2 receptor. This result confirmed that inhibit bradykinin B2 gene expression reduce the nociception caused by neuropathy.

The second part of this dissertation is nuclear condensation and cell cycle arrest induced by telomerase siRNA in neuroblastoma cells. Neuroblastoma is a type of malignant extracranial tumor that occurs in children. Advanced neuroblastoma, and tumors with MYCN amplification in particular, has poor prognoses. Therefore, it is important to find an effective cure for this disease. Small interfering RNA (siRNA) disrupts gene function by specifically binding to target mRNA. In this study, we used siRNA against telomerase to treat neuroblastoma, to evaluate any anti-proliferative effect on these cells. We evaluated cell viability by WST-1 assay on neuroblastoma cells treated with or without telomerase siRNA. Nuclear condensation, an indicator for apoptotic cells, was determined by DAPI labeling following siRNA treatment. The effectiveness of telomerase siRNA on altering the neuroblastoma cell cycle was detected by flow cytometry. Our results indicated that telomerase siRNA reduces the

viability of neuroblastoma cells and increases the percentage of cells in the cell cycle's sub-G1 phase. We found that telomerase siRNA increases the percentage of condensed DNA in neuroblastoma cells. In conclusion, using siRNA against telomerase could be further developed as a therapy for the treatment of neuroblastoma.

The third part of this dissertation is involvement of Smac, p53, and caspase pathways in induction of apoptosis by gossypol in human retinoblastoma cells. Retinoblastoma is a malignant tumor of the retina usually occurring in young children. To date, the conventional treatments for retinoblastoma have been enucleation, cryotherapy, external beam radiotherapy or chemotherapy. Most of these treatments, however, have possible side effects, including blindness, infections, fever, gastrointestinal toxicity and neurotoxicity. More effective treatments are therefore imperative. Gossypol has been reported as a potential inhibitor of cell proliferation in various types of cancers, such as prostate cancer, breast cancer, leukemia, and lung cancer. This study investigates the possible anti-proliferative effect of gossypol on retinoblastoma. The human retinoblastoma cells were cultured with various concentrations of gossypol and checked for cell viability with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Nuclear

condensation caused by cell apoptosis was detected by staining retinoblastoma cells with 4', 6-diamidino-2-phenylindole (DAPI), counting those with condensed nuclei, and determining the percentage of apoptotic cells. In addition, the stages of apoptosis and phases in cell cycles were examined with flow cytometry. The possible signal transduction pathways involved were examined with a protein array assay and western blot analysis. Our results indicated that after incubation, the cell survival rate was significantly lower after treatment with 5, 10, and 20 μM of gossypol. The maximum antisurvival effect of gossypol was observed at 20 μM, and the number of apoptotic cells was higher in the preparations cultured with 10 and 20 μM of gossypol. The results in flow cytometry indicated that at concentrations of 10 and 20 μM, gossypol increased the proportion of early- and late-apoptotic retinoblastoma cells and induced cell arrest of retinoblastoma cells at the same concentrations. This anti-proliferative effect was later confirmed by upregulating the expression of death receptor 5 (DR5), caspase 8, caspase 9, caspase 3, cytochrome C, tumor protein 53 (p53), and second mitochondria-derived activator of caspases (Smac) in the signal transduction pathways. We concluded that gossypol has an anti-proliferative effect on retinoblastoma cells.

Chapter 1 Introduction (緒論)

1.0 Small interfering RNA (siRNA) act as a role in post-transcriptional gene silencing (PTGS)

RNA interference (RNAi) is a naturally occurring mechanism for regulating gene expression which has been observed in several model organisms and is mediated by double stranded RNA (dsRNA) (Hammond et al, 2001; Tuschl, 2001; Sharp, 2001). dsRNA can initiate a cellular response that results in the sequence-specific degradation of homologous single-stranded RNA. This occurs in a wide variety of eukaryotic organisms tangling from protozoa to mammals, including plants. The process is called PTGS in plants and RNAi in animals. The term post-transcriptional indicates that RNA transcript gets specifically degraded so that the corresponding gene becomes silenced. The dsRNA is the key trigger, which gets processed by double-strand specific RNase to shorter RNA fragments of both polarities observed first by Hamilton and Baulcomb (Hamilton et al, 1999). This processing product is later found a 21-22 nucleotide dsRNA, which is also known as siRNA (Harborth et al, 2001; Elbashir et al, 2001). siRNA is later used in many fields silencing genes in specific occasions. For instances, Kosciolek and collogues used RNAi to inhibit the telomerase activity in cancer cell as

for therapy in cancer patients (Kosciolek et al, 2003). Since the discovery of the Nobel prize-winning mechanism of RNA interference (RNAi) on 2006, it has become a promising drug target for the treatment of multiple diseases. There have already been some successful applications of siRNA drugs in the treatment of age-related macular degeneration and respiratory syncytial virus infection.

1.1 RNA interference of bradykinin B2 receptor reduced the neuropathic pain caused by sciatic nerve injury

1.1.1 Role of bradykinin B2 receptor in pain.

Tissue damage or inflammation causes release of bradykinin, one of the most potent algogenic substances, by the action of the proteolytic enzyme kallikrein on a precursor protein, kininogen (Davis & Perkins, 1994; Woolf et al, 1994). Bradykinin is a potent agonist at the bradykinin B2 receptor. B2 receptors are expressed in nociceptive neurons (Seabrook et al, 1997; Steranka et al, 1988), and activation of these receptors by bradykinin causes action potentials in nociceptive nerve fibers (Koltzenburg, 1999;Kumazawa et al, 1996) and pain in human or animal subjects (Dray & Perkins, 1993). Segond Von Banchet et al. (Segond Von Banchet et al, 1996) found that B2

agonists bind to acutely isolated nociceptive neurons, and that after culture in the presence of nerve growth factor (NGF). We had also proved that the level of B2 mRNA is increased by NGF and to a lesser extent by glial derived neurotrophic factor (GDNF) (Lee et al, 2002).

1.1.2 Animal model for study of neuropathic pain.

In order to understand the clinical neuropathic pain, animal models that reflect similar syndromes have been used for several decades. Neuropathic pain often does not respond to traditional analgesics, it is therefore important to seek for a solution for it. There are many different types of animal models been used in the studies of mechanism of therapy in neuropathic pain. Most neuropathic pain patients have a partial nerve injury and indeed, a complete lesion is seen usually only after amputation, where the pain of a special type, phantom limb pain. Several animal models have been made to mimic the partial nerve injury. Chung and colleagues developed a model using a tight ligation of two spinal segmental nerves, L5 and L6, close to the dorsal root ganglion, leaving the L4 component of the sciatic nerve intact as well as the saphenous nerve (L3 root) (Kim & Chung, 1991; Kim & Chung, 1992). This model had been supported to be

the most ideal model for neuropathic pain until the spared nerve injury model developed. The spared nerve injury model has proved to be robust, with substantial and prolonged changes in mechanical sensitivity and thermal responsiveness that closely mimic many features of clinical neuropathic pain (Decosterd & Woolf, 2000).

1.1.3 Hypothesis: Down-regulation of bradykinin B2 receptor expression at dorsal root ganglion is able to reduce neuropathic pain.

Interruption of ascending transduction of nociception to the central nervous system has been used in clinical pain management. The methods include neurectomy, ablation of dorsal root ganglion (DRG), lesioning on dorsal root entry zone (DREZ) et al. The effective of pain reduction is around 50 % in average and that why multidisciplinary approaches in still the main stream for treatment of intractable neuropathic pain. According to the primer review, we hypothesize that application of bradykinin B2 siRNA to the DRG of rat would be able to reduce the neuropathic pain.

1.2 Nuclear condensation and cell cycle arrest induced by telomerase siRNA in neuroblastoma cells.

1.2.1 Critical points on the treatment for pediatric malignancies.

The overall outlook for children with cancer has improved greatly over the last half-century. In 1975, just over 50 percent of children diagnosed with cancer before age 20 years survived at least 5 years. In 2004-2010, more than 80 percent of children diagnosed with cancer before age 20 years survived at least 5 years. Although survival rates for most childhood cancers have improved in recent decades, the improvement has been especially dramatic for a few cancers, particularly acute lymphoblastic leukemia, which is the most common childhood cancer. Improved treatments introduced beginning in the 1970s raised the 5-year survival rate for childhood acute lymphoblastic leukemia from less than 10 percent in the 1960s to about 90 percent in 2003-2009. Survival rates for childhood non-Hodgkin lymphoma have also increased dramatically, from less than 50 percent in the late 1970s to 85 percent in 2003-2009. By contrast, survival rates remain very low for some cancer types, for some age groups and for some cancers within a site. For examples, median survival for children with diffuse intrinsic pontine glioma is less than 1 year from diagnosis.

However, despite the overall decrease in mortality, nearly 2,000 children die of cancer each year in the United States, indicating that new advances and continued

research to identify effective treatments are required to further reduce childhood cancer mortality.

1.2.2 General information about neuroblastoma

Neuroblastoma, a malignant neoplasm of neurocrest origin, is a common solid extracranial tumor in children and accounts for 8–10 % of all cancers in children; approximately 600 new cases are diagnosed in the United States each year (Gatta et al. 2002; Heck et al. 2009), and it is the leading cause of childhood cancer-related mortality and morbidity. Approximately one-third of cases are diagnosed during the child's first year, and nearly 90 % of cases are diagnosed by 5 years of age. Only 2 % of cases occur in people over the age of 10 years, including some adults. In 60–70 % of cases, the disease remains undiagnosed before spreading to distant organs (Schor et al. 2009). Of the several factors that affect the management of these tumors, one of the main challenges is the immaturity of the central nervous system (CNS) in children (Abdullah et al. 2008), which increases the complexity of the management of childhood brain tumors. Most patients with neuroblastoma are young; the median age at diagnosis is 18 months and is commonly present with metastatic disease. More than 60 % of patients

have high-risk tumors that are likely incurable. The advent of combinations of surgery, chemotherapy, radiation therapy, and high-dose chemotherapy with stem cell rescue has provided significant improvements in survival rates for advanced neuroblastoma. However, the prognosis for advanced neuroblastoma, and particularly for tumors with MYCN amplification, remains poor (Kawa et al. 1999; Matthay et al. 1999). More than 50 % of children with high-risk neuroblastoma relapse because of a drug-resistant residual disease (Goldsby et al. 2004; Perez et al. 2000).

RNA interference (RNAi) describes a conserved biological response to double-stranded RNA (dsRNA), which results in the degradation of homologous messenger RNA. This process of sequence-specific, post-transcriptional gene silencing has become a key technique for rapidly assessing gene function in both plants and mammals. For target RNA recognition to occur, the small interfering RNA (siRNA) duplex unwinds, allowing binding of one siRNA strand to the target mRNA. Depletion of endogenous TFF3 by sienna decreases the oncogenicity and invasiveness of mammary carcinoma cells (Kannan et al. 2010). In a study on gp120-mediation, inhibition of gp120 resulted in increased IL-8 production in astrocytes in the NF-kappaB pathway (Shah et al. 2010). The advantage of RNAi to an organism is that

siRNA, which specifically binds to target mRNA, prevents damage to other tissues. Adopting this approach may increase a treatment's therapeutic effect on neuroblastoma, and reduce side effects in patients receiving treatment.

1.2.3 Hypothesis: Telomerase siRNA has the anti-proliferative effect in the neuroblastoma cell line IMR-32.

A telomere is a region of repetitive DNA at the end of a chromosome; telomere regions protect genes near the ends of chromosomes from deterioration by allowing for short- ending of the chromosome, which necessarily occurs during chromosome replication (Chan et al. 2004). The telomere-shortening process normally limits cells to a fixed number of divisions, and animal studies suggest that this is responsible for aging at the cellular level and sets an upper limit to lifespan (Chan et al. 2004). The telomeres, nucleotide repeats, and protein complex at the ends of chromosomes are required for chromosomal stability and are important markers of aging (Butler et al. 1998; Friedrich et al. 2000). Telomeres protect a cell's chromosomes from fusing with each other or rearranging; these are abnormalities that can lead to cancer, and therefore, cells are destroyed when their telomeres are consumed (Chan et al. 2004). Most cancers are the

result of ‘‘immortal’’ cells that have developed ways of evading this programmed destruction. Patients with dyskeratosis congenita (DC), an inherited bone marrow failure syndrome (IBMFS), bore mutations in telomere biology genes, and had extremely short telomeres (Gadalla et al. 2010). Telomeres in patients with DC were significantly shorter than the telomeres of other IBMFS patients (Gadalla et al. 2010); aberrations in telomere length are associated with cancer. To help maintain telomere homeostasis, the telomeric protein TRF1 and other associated proteins inhibit human telomerase elongation of telomeres (Soohoo et al. 2011).

Telomerase is an enzyme that adds DNA sequence repeats (‘‘TTAGGG’’ for all vertebrates) to the 3’ end of DNA strands in human telomere regions (Njajou et al. 2010). High level of telomerase activity was detected in stem cells and cancer cells, thereby avoiding telomere shortening and maintaining cell division (Liu et al. 2012; Hiyama et al. 1995; Kar et al. 2012; Holt et al. 1996). Numerous studies reported that about 90 % of human cancer specimens or malignant tumors showed activities of telomerase (Hoare et al. 2001; Kim et al. 1994; Zhang et al. 2012; Shay et al. 2011). Hiyama et al. (Hiyama et al. 1997) also found that telomerase activity may be the prognostic indicator in neuroblastoma, and some other studies exhibited that telomere

length (Ohali et al. 2006) may also be the biomarkers of neuroblastoma. On the other hand, the somatic cells expressed low level of telomerase activity, and the telomerase-based therapies may present greater specificity on cancer cells (Shay et al. 2011; Ge et al. 2011; Durant et al. 2012). Thus, to reduce the proliferation of neuroblastoma cells, we propose using siRNA against telomerase. In the present study, we aimed to identify the anti-proliferative effect of telomerase siRNA in the neuroblastoma cell line IMR-32.

1.3 Involvement of Smac, p53, and caspase pathways in induction of apoptosis by gossypol in human retinoblastoma cells

1.3.1 General information about retinoblastoma

Retinoblastoma is the most common intraocular malignant tumor in infants and children, where it might occur unilaterally or bilaterally. Most cases of unilateral retinoblastoma involve somatic nonhereditary retinoblastoma 1 gene (RB1) mutations, which account for 60% of all retinoblastoma cases (Melamud et al. 2006). Approximately 40% of retinoblastoma patients carrying a germline mutant of RB1 have a bilateral retinoblastoma tumor (Melamud et al. 2006), and these patients are more

likely to develop secondary tumors. The morbidity of retinoblastoma is approximately 1 per 20,000 to 1 per 15,000 live births in the United States (Young et al. 1999), and approximately 10 new cases are reported each year in Taiwan (Chen et al. 2010). Retinoblastoma is usually recognized in patients younger than 5 years by leukocoria, strabismus, and pain (Melamud et al. 2006; Balmer et al. 2007). To date, retinoblastoma has conventionally been treated with enucleation, cryotherapy, external beam radiotherapy or chemotherapy, depending on the condition and stage of tumor development and the location and size of the primary tumor (Lin et al. 2009; Gombos et al. 2007). However, most of these treatments have possible side effects, including blindness, infections, fever, gastrointestinal toxicity and neurotoxicity. Therefore, more effective treatments are imperative for improving patients' prognoses. Increasing the rate of complete cure from this disease has long been a priority in ophthalmologic and pediatric clinics.

1.3.2 Hypothesis: Gossypol has an anti-proliferative effect on retinoblastoma cells.

Gossypol is a polyphenolic extract of cottonseeds. The biologic effects of gossypol are attributable to 6 hydroxyl and 2 aldehyde groups, which also make the molecule

soluble in organic solvents. To date, gossypol has had a wide range of uses. In agricultural applications, it reduces larval weight and the metabolic rate in invertebrate pest species by inhibiting mitochondria ATPase activity (Wang et al. 2009). Gossypol has also been suggested as a male contraceptive that reduces energy production in spermatozoa, and therefore, their motility and concentration, without affecting testosterone levels (Shandilya et al. 1982). In curative medicine, the anticancer properties of gossypol have been investigated extensively. In studies on prostate cancer, gossypol-induced B-cell lymphoma 2 (Bcl-2)-dependent autophagy and apoptosis through an increase in the level of the tumor protein 53 (p53) upregulated modulator of apoptosis (p53-upregulated modulator of apoptosis [Puma] and NAPDH oxidase activator [Noxa]) (Lian et al. 2010; Meng et al. 2008; Volate et al. 2010). Gossypol was also found to induce autophagy in breast cancer (Gao et al. 2010), and to suppress colon cancer by downregulating cyclin D1 expression, causing G_0/G_1 phase arrest and activating death receptor 5 (DR5; tumor-necrosis-factor-related apoptosis-inducing ligand receptor-2, or TRAILR-2)–mediated apoptosis (Wang et al. 2000; Sung et al. 2010). In some cases, gossypol inhibited extra-large B-cell lymphoma (Bcl-xL) in large cell lymphoma and non-Hodgkin lymphoma (Li et al. 2008; Mohammad et al. 2005),

and in others gossypol suppressed nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) related signaling in the U937 leukemia cell line (Moon et al. 2008). Studies have further indicated that gossypol inhibits lung cancer, ovarian cancer, and pancreatic cancer (Chang et al. 2004; Mohammad et al. 2005). We therefore supposed that gossypol may also be a potential candidate for treating retinoblastoma.

Chapter 2. Methods and materials(研究方法與材料)

2.1 RNA interference of bradykinin B2 receptor reduced the neuropathic pain caused by sciatic nerve injury

2.1.1 Animals

Male Sprague-Dawley rats, 200-250 g, were used in this study. Animals were maintained in a 12h-light / 12h-dark pathogen-controlled environment and were allowed access to food and drink ad libitum. Laboratory chow and water were available to the rats ad libitum. Animal was separated into two groups. One of the groups is control (left L5 ligation) and the other is experiment (left L5 ligation and supply for siRNA). The animals were housed in the Animal Care Facility at the Fu-Jen Catholic University under standard animal care guidelines. All animal protocols were in accordance with Fu-Jen's Standard protocols for Laboratory Animals in Research and approved by the animal care and use committee in Fu-Jen Catholic University.

2.1.2 Surgery for neuropathic pain model

One week after acclimatization to laboratory conditions, baseline behavioral measurements are made and surgery is performed as previously described by Chung et

al. (Chung et al, 1992). Animals were anesthetized with chloral hydrate (RDH, 0.4 g/ml i.p.). The sciatic nerve and its three main branches: sural, common peroneal and tibial nerves are exposed in the left thigh. Then the left L5 nerve was clamped by a premium surgiclip and the wound was closed. The lesion to the left L5 root of the rat was clip by Premium surgiclip instead of silk ligation in order to get uniform lesioning (Figure.1) After wound closure, animals recover from anesthesia on a heating blanket. At the fourth days after surgery, animals are tested with behavior test for evaluation of nociceptive sensation.

2.1.3 RNA interference construction

Small interfering RNA (siRNA) oligos targets to bradykinin B2 receptor were purchased from Dharmacon (USA). The single stranded siRNAs (a mixture of sequences SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4) (Figure 2.), which are formed by chemical synthesis, were suspended in physiological saline. The cultured neural cells (PC12 cells) was used to confirm the effectiveness of bradykinin B2 receptor siRNA transfection (Figure 3.) The stock concentration of siRNA is 20 μM, and suspended in the buffer, which they provided. Dilute to 200 nM in

autoclaved normal saline for animal injection. For construct transfection, siRNA was diluted with 155 ul of optimum medium (Invitrogen) with 7.5 ul of oligofectamine (Invitrogen), and incubate for 24 hours.

2.1.4 Administration of siRNA

The experimental group rats (n=5) received small interfering RNA (siRNA). Using 26 G needle (TERUMO), drug were injected in 200 µl volume into left lumbar vertebra on 0, 4, 6, 8, 11 and 13 days.

2.1.5 Behavioral analysis

Animals are habituated to the testing environment daily for 3-7 days before baseline testing. The room temperature and humidity remained stable for all experiments. The Electronic von Frey Anesthesiometer (IITC Life Science, 2390 series) was used in the study to measure mechanical allodynia. The monofilament fiber used in this experiment was rigid tip which maximum value is 800 gm. This method was performed to test the sciatic nerve innervation area of the rat hind paws. Rats were placed on a wire grid floor in a plastic cage. Before testing, wait for 5-10 minutes in a quiet place. A threshold stimulus was determined by withdrawal which was induced by

poking the hind padded of the middle Tibial zone. We poked both hind paws and the right one first. Rats may lick or bit of their hind paw or center of gravity move to opposite if they feel pain in the poked leg. We scored and repeated up to 11 times.

For testing mechanical sensitivity, animals are put in plastic boxes (11 x 13 x 24 cm) on an elevated metal mesh floor and allowed 30 min for habituation, and mechanical allodynia is tested using von Frey filaments (Stoelting, Wood Dale, IL). Hind paws are pressed with one of series von Frey hairs with logarithmically incremental stiffness (0.6, 1, 1.4, 2, 4, 6, 8, 10, 15, and 26 gram, Stoelting), presented perpendicular to the plantar surface for 5-6 seconds for each hair (Chaplan et al, 1994). Testing is initiated with the 2.0-g hair, in the middle of the series. Stimuli are present in a consecutive fashion, whether ascending or descending. In the absence of paw withdrawal response to the initial selected hair, a stronger stimulus is presented; in the event of paw withdrawal, the next weaker stimulus is chosen. In cases in which threshold fell outside the range of detection, i.e., continuous positive or negative responses are observed to the limit of the available stimuli, values of 0.25 and 15.0g are assigned, respectively. Otherwise, threshold are calculated by noting the stimulus level at which the first change in behavior occurred and collecting four additional responses

to the continued presentation of stimuli in the above up-and-down manner.

2.2 Nuclear condensation and cell cycle arrest induced by telomerase siRNA in neuroblastoma cells.

2.2.1 Cell culture

Human neuroblastoma cells (IMR-32, the Bioresource Collection and Research Center, Hsinchu, Taiwan) were cultured in Eagle's minimum essential medium (HyClone Laboratories, Inc., Utah, USA) supplemented with 10 % heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, USA). Cells were grown at 37 ℃ in a 5 % humidified CO_2 incubator.

2.2.2 Preparation of siRNA

We dissolved siRNA in diethylpyrocarbonate (DEPC) water. The desired dsRNA was obtained by annealing telomerase siRNA hTR-F (5'-UUGUCUAACCCUAACUGAG TT-3') and hTR-R (3'-TTAACAGAUUGGGAUUGACUC- 5') using an annealing buffer. Before beginning experiments, the siRNA was heated to 94 ℃ for 2 min and then allowed to cool to room temperature.

2.2.3 Transfection of siRNA

Transfection of siRNA was performed with X-tremeGENE siRNA transfection reagent (Roche, Germany). First, the siRNA was diluted with serum-free medium at different concentrations, and a 5-time volume of the transfection reagent was added into the diluted siRNA. The mixture of transfection reagent and siRNA was incubated at room temperature for 20 min before adding to cell cultures. To confirm the knockdown effect of transfected siRNA, telomerase activity assay was performed after the transfection. The telomerase activity was measured by the TRAPeze XL Telomerase Detection Kit (S7707, Millipore, Billerica, MA, USA). In brief, a master mix of TRAPeze reaction mix and Taq polymerase was added to the cell extracts, and PCR amplification was used to examine the expression of telomerase.

2.2.4 Quantitation of telomerase RNA

Total RNA was extracted using an RNA isolation kit (GE healthcare, Uppsala, Sweden), and telomerase RNA was quantified by a reverse transcriptase PCR with a real-time PCR machine (Light Cycler, Roche, Darmstadt, Germany). The primers used were 5'-CTGGGAGGGGTGGTGGCC ATTT-3' and

5’-CGAACGGGCCAGCAGCTGACAT-3’. The reaction program was used as 94 ℃ for 20 s, 55 ℃ for 20 s, and 72 ℃ for 30 s for 30 cycles.

2.2.5 Cell proliferation assay

Cell viability was assessed by WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay (Roche, Germany). IMR-32 cells (1 x 10^4) were seeded in 0.1 ml of culture medium per well in a 96-well plate. Cells were incubated overnight to allow them to adhere. Cells were then treated with or without siRNA at the indicated concentrations (0, 10, or 100 nM) for 24 h. The medium was replaced with a fresh culture medium and the cells were incubated for a further 48 h. After culturing, the WST-1 reagent (10 μl in 100 μl medium) was added to each well. The cells were incubated for 2 h, and then agitated for 1 min on a shaker prior to analysis. Absorbance was measured at 450 nm with an ELISA reader (Beckman Coulter AD200, USA).

2.2.6 Labeling DNA fragments of apoptotic cells

Coverslips were rinsed alternately with 70 % ethanol and absolute alcohol and then air-dried and placed in a 24-well plate before use. For cell preparation, 2 x 10^4 IMR-32

cells were seeded on a coverslip in 1 ml of medium per well in a 24-well plate, and then incubated for 24 h to allow cells to adhere. Cells were then treated with various concentrations of siRNA (0, 1, 10, or 100 nM). After 24 h, the medium was replaced with fresh culture medium and the cells were incubated for a further 48 h. Cells were washed twice with phosphate buffered saline (PBS) and fixed with 4 % paraformaldehyde in PBS at room temperature for 30 min. After fixation, cells were washed once with PBS and stained with DAPI (40,6-diamidino-2-phenylindole dihydrochloride; Sigma, St. Louis, MO, USA) for 20 min. The staining solution was removed and the cells were washed twice with PBS and once with ddH_2O. The coverslips were mounted on a slide with an anti-fading agent (DAKO North America, Inc., CA, USA). Cells were observed using an upright fluorescence microscope (Leica DM2500, Germany) with a cool CCD camera (Cool SNAP EZ, Roper, Germany). A maximum of 500 DAPI-stained cells were counted on each coverslip and the apoptotic rate was then calculated.

2.2.7 Flow cytometry

IMR-32 cells (1×10^6) were seeded in 2 ml of medium per well of a 6-well plate

and incubated for 24 h to allow cells to adhere. The cells were then treated with various concentrations (0, 1, 10 or 100 nM) of siRNA. After 24 h, the medium was replaced with fresh culture medium, and the cells incubated for a further 48 h. After treatment, cells were collected and washed with ice-cold PBS, fixed with 3 ml of ice-cold ethanol while gently vortexing, and then stored at -20 ℃ for 24 h. Cells were then washed with ice-cold PBS, suspended in a DNA-extracting buffer, and stood at room temperature for 15 min. Prior to analysis, cells were suspended in a staining solution containing RNase A (10 mg/ml), triton X-100 (10 %; 1 in 100), and propidium iodide (PI) (10 μg/ml) for 30 min. Data were collected from 1 x 10^4 cells for each preparation using a CyFlow ML flow cytometer (Partec, Germany). Data were analyzed by Partec Flomax Operating and Analysis Software (Partec, Munster, Germany).

2.2.8 Statistical analysis

The software program SPSS (version 17; Chicago, IL, USA) was used for statistical analysis. One-way analysis of variance (ANOVA) with Bonferroni or Duncan's multiple comparison analysis was used to compare differences between groups. P value of <0.05 was considered significant.

2.3 Involvement of Smac, p53, and caspase pathways in induction of apoptosis by gossypol in human retinoblastoma cells.

2.3.1 Preparation of gossypol

Gossypol (Tocris Bioscience, Bristol, UK) was dissolved in dimethyl sulfoxide (DMSO) to make a 25 mM stock solution. The stock solution was diluted to concentrations of 0.5, 1, 3, 5, 10, and 20 mM in DMSO before the experiments were started, and then added (1 in 1,000) to the cell culture medium. Cell culture: The human retinoblastoma Y79 (HTB-18) and the human retinal pigmented epithelium (ARPE) cell lines were purchased from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). Cells were cultured in RPMI 1640 medium (Gibco, Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The cells were incubated at 37 °C with 5% CO_2, and the culture medium was changed every 3 days.

2.3.2 Cell viability assay

Cell viability was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Y79 cells

(5×10^4 cells/well) were cultured in 24-well plates with 1 ml RPMI 1640 medium and 0.5, 1, 3, 5, 10, or 20 μM of gossypol for 6 to 48 h. The preparations for the control group were cultured without gossypol but with the same amount of DMSO as the experimental groups. After incubation, 100 μl of MTT was added in each well, and incubated at 37 °C for another 2 h. Triton (10 μl, 10%) solution was then added into the preparations to dissolve the purple formazan from each cell. The amount of soluble formazan was measured with an enzyme-linked immunosorbent assay (ELISA) plate reader (AD200; Beckman Coulter, Brea, CA) at 550 nm absorption.

2.3.3 Assay for nuclear condensation

Y79 cells were cultured in each well of a 24-well culture plate (5×10^4 per well) with 1 ml RPMI 1640 medium, and treated with 0, 1, 10, or 20 μM of gossypol for 24 h. After incubation, cells were collected and centrifuged at 92.5 xg for 5 min at room temperature, washed twice in phosphate-buffered saline (PBS), fixed with 4% formaldehyde at room temperature for 30 min, and washed with PBS twice again after fixation. These fixed cells were further incubated with 4,6-diamidino-2-phenylindole (DAPI; 1 μl/ml) for 30 min at room temperature, avoiding light exposure. After DAPI staining, the cells were

washed with PBS twice to remove the redundant fluorescent dye, the remaining liquid was removed, and the preparation was completely suspended in the antifading mounting medium (Dako, Produktionsvej, Denmark). The preparation was then transferred to glass slides for observation under a fluorescence microscope (DM2500; Leica, Wetzlar, Germany), and images were taken and counted using a cooled charge-coupled device (CCD) camera (CoolSNAP EZ; Roper Scientific, Martinsried, Germany).

2.3.4 Annexin V-fluorescein isothiocyanate and propidium iodide staining

After treatment with different concentrations of gossypol, cells were collected under the same conditions as described earlier, except that they were stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI), instead of DAPI. Cells were washed twice with PBS and then suspended in 500 μl of binding buffer; 5 μl of annexin V-FITC and 5 μl of PI (Annexin V-FITC Apoptosis Kit; BioVision, Milpitas, CA), and then incubated at room temperature for at least 10 min in the dark. After staining, the preparations were washed and transferred to glass slides for observation under a fluorescence microscope (DM2500; Leica) with a cool CCD camera (CoolSNAP EZ; Roper Scientific).

2.3.5 Flow cytometry

Y79 cells (1×10^6) were cultured in six-well plates with 2 ml of RPMI 1640 medium (Gibco) and 0, 1, 10, and 20 μM of gossypol for 24 h. After incubation, these cells were collected and washed in PBS, and then stained using an annexin V-FITC and PI kit (Annexin V-FITC Apoptosis Kit; Bio Vision). The preparations were transferred into the sample tube and diluted with PBS (the total sample solution contained 500 μl of binding buffer, 5 μl of annexin V-FITC, 5 μl of PI, and 490 μl PBS). For cell cycle study, the cells were stained with PI only. Data were collected from 4×10^4 cells in the gated region of each preparation.

2.3.6 Western blot analysis

Y79 cells were treated with 20 μM of gossypol for 24 h before collection. After treatment, cells were washed and suspended in protein extraction solution (iNtRON Biotechnology, Gyeonggi-do, Korea) with 0.1% EDTA and then stored at −20 °C. Total cellular protein extract was resolved using 10 or 12% sodium dodecyl sulfate–PAGE and electrophoretically transferred to Hybond-P polyvinylidene fluoride membrane (Amersham, Buckinghamshire, UK). The blot was blocked with 5% nonfat dry milk in

TBST (Tris-buffered saline [TBS] buffer, 20× liquid [AMRESCO Inc., Solon, OH]) containing 0.1% Tween-20) for 1 h at room temperature, and then incubated with primary antibodies (actin, DR5, caspase 9, caspase 8, caspase 3, cytochrome C, second mitochondria-derived activator of caspases [Smac], and p53) in blocking solution at 4 °C overnight. After incubation, the blot was washed in TBST, incubated for 1 h at room temperature, and then incubated with horseradish peroxidase (HRP)-linked secondary antibody. The binding antibodies were detected using an electrochemiluminescence (ECL) assay.

2.3.7 Statistical analysis

Experimental data were analyzed by using SPSS version 18 (IBM, Armonk, NY). The Student t test and one-way ANOVA with the Bonferroni multiple comparisons were used to evaluate statistical significance.

Chapter 3. Results (結果)

3.1 RNA interference of bradykinin B2 receptor reduced the neuropathic pain caused by sciatic nerve injury.

3.1.1 Analgesic effect of bradykinin B2 receptor siRNA

After administration, siRNAs of bradykinin B2 receptor was transfected into the cell bodies of primary nociceptive neurons in DRGs in the animal models. The Modified Chung model was used to demonstrate the neuropathic nociception. The mechanical sensitivity behavior test was used to evaluate the effect of siRNAs in reducing degree of neuropathic nociception (Table. 1). The result shows the nociception caused by neuropathy was reduced by bradykinin B2 receptor siRNA (Figure. 4,5). We therefore supposed that inhibit bradykinin B2 expression may reduce the nociceptive sensation caused by neuropathy.

3.1.2 Real-time PCR study of bradykinin B2 receptor expression.

The expression of bradykinin B2 receptor expression and GAPDH in dorsal root ganglia of naïve (no any treatment), control (nerve clipped) and experimental (nerve clipped + B2 receptor gene siRNA) animals were also examined by real-time PCR to

confirm our hypothesis. Bradykinin B2 receptor expression was upregulated after sciatic nerve clipping, while this upregulation was reversed by application of siRNA of bradykinin B2 receptor gene (Figure. 6). This result confirmed that inhibit bradykinin B2 gene expression reduce the nociception caused by neuropathy.

3.2 Nuclear condensation and cell cycle arrest induced by telomerase siRNA in neuroblastoma cells.

3.2.1 Activity of telomerase

The siRNA of telomerase was expected to decrease both the expression of telomerase RNA and telomerase activity in the neuroblastoma cells. It was observed that the activity of telomerase in the transfected cells decreased gradually along with the increase of siRNA concentrations from 10 to 100 nM (Figure 7A). In addition, the telomerase RNA expressed in the siRNA transfected cells decreased between 12 and 20 % of the untreated cells (Figure 7B). These experiments confirmed that the siRNA of telomerase effectively inhibited the activity of telomerase in neuroblastoma cells.

3.2.2 Cell viability

The viability of neuroblastoma cells in the presence of telomerase siRNA was

assessed by WST-1 assay (Figure 8). IMR-32 cells were transfected with telomerase siRNA (1, 10, and 100 nM), or without telomerase siRNA (control group), for 24 h. Viable cells were detected by WST-1 assay. Mitochondrial dehydrogenase cleaves the tetrazolium salt WST-1 to formazan. The proliferation of viable cells resulted in an increase in the overall activity of mitochondrial dehydrogenase, and increased formazan levels. The cells transfected with 10 and 100 nM telomerase siRNA had low viability compared to the control group. (Figure. 8)

3.2.3 Nuclear condensation of apoptotic cells

DNA fragmentation appears during the late stages of cell apoptosis. To examine the effects of telomerase siRNA on cellular apoptosis of human neuroblastoma cells, IMR-32 cells were treated with various concentrations of telomerase siRNA (0, 1, 10, and 100 nM) before DAPI labeling. DAPI is a fluorescent stain that binds to double-stranded DNA. It can pass through an intact cell membrane, albeit with reduced efficiency in live cells. Thus, we can differentiate live cells from cells undergoing apoptosis. DNA fragmentation was observed by fluorescence microscopy in Figure. 9, which showed DNA fragments indicated by arrows (Figure. 9A-D). In the experimental

groups, fluorescence increased with increasing numbers of cells. After counting DAPI-labeled cells, apoptotic rates were calculated (Figure. 9E). Apoptotic rates in the 10 and 100 nM group were 10 %greater than for the control and 1 nM treatment groups. This result indicates that telomerase siRNA effectively blocks the proliferation of IMR-32 cells.

3.2.4 Cell cycle study

We used flow cytometry to assess cell cycling and to determine the apoptotic induction effect of telomerase siRNAs. Cancer cells are generally immortal; they divide uncontrollably. Thus, we expected to see decreases in the sub-G1 or G1 phases, and increases in S and G2/M phases. We treated IMR-32 cells with siRNA at the indicated concentrations (0, 1, 10, and 100 nM). Data from 1 x 10^4 cells were analyzed (Figure. 10). The results show that telomerase siRNA (10 and 100 nM treatments) significantly increased the percentage of cells in the sub-G1 phase. However, 100 nM siRNA treatments additionally caused decreases in the G1 and S phases (Figure 11). Thus, we assume that treatment with telomerase siRNA at concentrations greater than 10 nM results in cellular arrest or apoptosis.

3.3 Involvement of Smac, p53, and caspase pathways in induction of apoptosis by gossypol in human retinoblastoma cells.

3.3.1 Effective concentrations of gossypol

Considering the proven anticancer properties of gossypol, it might be supposed to mitigate human retinoblastoma as well. The present study therefore sought to determine the concentrations at which gossypol would effectively retard proliferation in the Y79 cell line. In test samples of 5, 10, and 20 μM gossypol, cell viability after 24 h of incubation was observed to be inversely proportional to the concentration of gossypol, with the ED50 calculated as 8.4 μM (Figure 12A). Identical dosages of gossypol were found to have no effect on APRE cells (Figure 12B).

In time-course trials, although cell viability was first seen to decrease at 6 h in the 10- and 20-μM samples, the effect remained insignificant after as long as 12 h, even at a higher concentration (Figure 12C). Accordingly, a 24-h incubation period was chosen for the subsequent investigations, which were conducted using gossypol in concentrations of 0, 1, 10, and 20 μM.

3.3.2 Apoptotic studies of Y79 cells treated with gossypol

For the apoptotic studies, Y79 cells were incubated in solutions of 0, 1, 10, or 20 μM of gossypol for 24 h. The cells were then harvested and stained with DAPI or by using an annexin V/PI apoptosis kit to observe nuclear condensation and stages of cell apoptosis. As shown in Figure 13, the rate of condensed DNA, which indicates cell apoptosis, increased in conjunction with gossypol concentrations (Figure 13A). Figure 13B-E shows microimages of DAPI-stained cell nuclei exposed to different concentrations of gossypol. The arrows indicate the onset of nuclear condensation, which became obvious as the concentration of gossypol increased. In addition, there was a higher incidence of cells stained with annexin V and/or PI in the samples treated with higher concentrations of gossypol than in those treated with lower or zero concentrations (Figure. 14).

Flow cytometry was used to calculate the distribution of cells in normal, early, and late stages of apoptosis. Compared to control groups, significant increases in the proportion of cells in late apoptosis were observed in groups treated with 10 and 20 μM of gossypol for 24 h (Figure 15A). However, in preparations treated for 12 h, only the 20-μM groups showed significantly more cells in the late apoptosis stage (Figure 15A, B). Cell distributions after 12-h and 24-h treatments are shown in Figure 15 C, D where

Q3 represents the normal cell population, Q4 represents cell in early apoptosis stage, and Q2 represents cells in late apoptosis stage.

The effects of gossypol on cell cycles in the Y79 cell line are shown in Figure 16. At concentrations of 10 and 20 μM, significantly more cells are in the G0/G1 phase but significantly fewer are in the G2/M phase than in the control groups. These results indicate that, in addition to provoking apoptosis in Y79 cells, gossypol also causes cell arrest in the cell cycles.

3.3.3 Signal transduction pathway of gossypol-mediated apoptosis of Y79 cells

Considering the potential of gossypol in treating human retinoblastoma, it is important to investigate the signaling transduction pathway via which gossypol causes apoptosis in Y79 cells. Comparison of the signal intensity of various apoptosis-related antibodies shows that the expressions of apoptotic proteins such as DR5, p53, Smac, caspase 8, caspase 9, and caspase 3 were upregulated 1.5 to twofold in the 20 μM gossypol-treated groups, compared to the control group (Figure 17). The expression of cytochrome C was upregulated by up to 5.8 fold (Figure 17B). Two signaling pathways may therefore be involved: (1) a TRAILR-mediated (death receptor 5) pathway in which

the upstream caspase family members are upregulated, causing Smac and cytochrome C to be released from the mitochondria; and (2) DNA degradation causing p53 upregulation and then cell cycle arrest.

Chapter 4. Discussion (討論)

4.1. RNA interference of bradykinin B2 receptor reduced the neuropathic pain caused by sciatic nerve injury.

The epidemiological studies showed more than 50% of patients consult doctors for the problem of pain. The pain has two main patterns, nociceptive and neuropathic pain. The nociceptive pain is caused by injuries to body tissues, such as inflammation, fracture, osteoarthritis or visceral pain. The neuropathic pain is initiated by a primary lesion or dysfunction in the nervous system such as postherpetic neuralgia, trigeminal neuralgia, diabetic peripheral neuropathy, poststroke pain and phantom limb pain et al. A severe cancer-related pain can have both nociceptive and neuropathic pain. When the pain is severe and prolonged, it can jarpodize the quality of life of these patients.

The patient would be unhappy, depressed, anxious and sleepless. Many mechanistic approaches are used for pain treatment such as decrease inflammatory response, decrease pain conduction, prevent central sensitization, increase descending inhibition and modify pain expression. There are multidisciplinary ways in the management of pain; however, the effectiveness is still not enough. That seeking new

ways for pain-control is mandatory.

Tissue injury causes severe inflammation mediators released from damage cells. Some of these mediators cause local effect results in increased sensitivity to pain. The prostaglandins, histamine, bradykinin, substance P and serotonin cause a local effect of nociception. Some mediators transmit the sensation of pain, induced by damage of surrounding cells or even nociceptive neurons. The sensitization of nociception caused by neural damage is normally known as neuropathic pain. Other mediators such as nerve growth factor (NGF) and other neurotrophic factors regulate the progress of neuron regeneration. However, changes in expression of receptors for algogenic substances such as bradykinin may also be involved, causing a long-term effect.

The bradykinin receptor B2 is a 9 amino acid bradykinin peptide elicits many responses in humans including vasodilation, edema, smooth muscle spasm and pain fiber stimulation. In this study, we have used Spragne-Dawley rats as modified Chung's neuropathic animal model. For we used premium surgiclip to clip the rat L5 spinal nerve instead of silk ligation of the original Chung's model. The clip can apply uniform force to the ligated nerve that has made the rat present the uniform degree of nerve

damage.

We have constructed the siRNA of bradykinin B2 receptor and deliver to the cell in dorsal ganglion of the neuropathic animal models. The mechanical sensitivity behavior test indicates that giving bradykinin B2 receptor siRNA significantly reduced the nociception caused by neuropathy.

The real-time PCR was used to examine gene expression in the dorsal root ganglion. The result demonstrated significantly decreased gene expression after giving the siRNA bradykinin B2 siRNA. This study has provided a potential analgesic therapy in the clinical practice.

4.2. Nuclear condensation and cell cycle arrest induced by telomerase siRNA in neuroblastoma cells.

We evaluated the effect of telomerase siRNA on a human neuroblastoma cell line using WST-1 assay, which measures mitochondrial and cellular viability. Cell viability decreased after treatment with telomerase siRNA; telomerase siRNA may have an anti-proliferative effect on neuroblastoma IMR-32 cells, inducing cell death at a concentration of 10 and 100 nM.

Cell death occurs by two processes: apoptosis and necrosis. Necrosis is a form of traumatic cell death, which results from acute cellular injury and produces an inflammatory response. Apoptosis, or programmed cell death, involves the death of individual cells, and does not lead to inflammation (Pezzino et al. 2011; Munoz-Pinedo et al. 2012). We hypothesized that telomerase siRNA may cause the death of human neuroblastoma cells by apoptosis (Nishimura et al. 2007). We examined IMR-32 cells undergoing apoptosis using DAPI labeling and flow cytometry analysis. This allowed us to evaluate evidence for apoptosis, such as DNA fragmentation and changes in the cell cycle for different telomerase siRNA concentrations. As shown in Figure. 9, treatment with telomerase siRNA produced more DNA fragments than the control group, and induced more cells to enter the resting phase of the cell cycle. Thus, we confirmed that the anti-proliferative effect of telomerase siRNA on IMR-32 cells occurs through activation of cell apoptosis. The cell cycle is a process that involves cellular reproduction, DNA replication, and cell division (Pajalunga et al. 2008). The cycle consists of 4 distinct stages: the G1, S, G2, and M phases. DNA replication occurs during the S phase, and division occurs in the G2 and M phases. Our study confirmed the role of telomerase siRNA in inducing apoptotic cell death among human

neuroblastoma cells. We also evaluated changes in the cell cycle phases of telomerase siRNA-treated IMR-32 cells. Our results showed that a greater percentage of cells remained in the sub-G1 phase of the cell cycle following telomerase siRNA treatment.

We presented the consistent dose-dependent finding in our results. In the WST-1 assay, both 10 and 100 nM treatment groups reduced cell numbers. In the DNA fragmentation and cell cycle analysis, the 10 and 100 nM treatment groups were both significantly different from the control group. We assumed that during the late stages of apoptosis, cells break into apoptotic bodies. In DNA fragmentation and cell cycle analysis, fluorescent dyes bind to DNA; therefore, we can evaluate cell death during late stage apoptosis.

We have demonstrated that telomerase siRNA has an anti-proliferative effect on human neuroblastoma cells via the cell apoptosis pathway. Moreover, telomerase siRNA treatment decreases the number of cells entering the interphase of the cell cycle. This study provides a potentially effective therapy for human neuroblastoma.

4.3. Involvement of Smac, p53, and caspase pathways in induction of apoptosis by gossypol in human retinoblastoma cells.

The present study found that the polyphenol molecule gossypol induced apoptosis in Y79 cells at concentrations of 5, 10, and 20 μM. This effect was not seen in non-cancer retinal pigmented epithelial cells. The proportion of nuclear condensed cells and the population of apoptotic cells were also found to increase significantly as the dose of gossypol increased. Cells treated with gossypol were found more likely to enter late apoptosis and to undergo cell arrest than cells in the control group.

A gossypol dose of 20 μM was used to study the signal transduction pathway by which gossypol has its anti-proliferative effect on Y79 cells. The p53, Smac, and caspase family pathways are all involved in the induction of cell apoptosis (Figure 18). The results of apoptosis protein studies through western blot analysis revealed that gossypol upregulates DR5 and activates caspase 8 to cleave procaspase 3 into activated caspase 3, thus inducing apoptosis of the cell. Gossypol also initiates activation of caspase 9, leading to the release of Smac and cytochrome C from the mitochondria. Because gossypol is lipid-soluble, it might have the ability to pass directly through the cell membrane and enhance mitochondrial or endoplasmic reticulum stress.

Earlier studies have shown gossypol to be a small molecule non-peptide

BH3-domain-binding inhibitor that blocks Bcl-2, Bcl-xL, and myeloid cell leukemia (Mcl-1) (Kitada et al. 2003; Goldsmith et al.2005; Reed et al. 2005). Gossypol also blocks the Bcl-2-homology-3 (BH3)-binding site of many antiapoptotic Bcl-2 family members that bind with other proapoptosis proteins such as Bcl2 interacting mediator of cell death (Bim), BH3 interacting domain death agonist (Bid), Bcl2-associated agonist of cell death (Bad), Puma, and Noxa, thus destabilizing the mitochondria membrane and effectuating apoptosis. Some research has shown gossypol to have a downregulation effect on Bcl-2, Bcl-xL, and Mcl-1. However, our results indicate no significant change in the expression of Mcl-1, Bcl-2, and Bcl-xL after gossypol treatment. Changes in these antiapoptotic proteins have been postulated by other researchers (Meng et al. 2008; Mohammad et al. 2005). The mechanism of the upregulation of Mcl-1 is thought to be caspase independent (Meng et al. 2008), and there is evidence that gossypol directly interferes with antiapoptotic protein function rather than expression level (Mohammad et al. 2005). Gossypol might also block the BH3-binding site of endoplasmic reticulum-associated proteins such as Beclin 1 and induce autophagy in cells. We found no evidence showing a similar effect of gossypol on Y79 cells.

The upregulation of mitochondria-related apoptotic proteins observed in the present

study suggests that gossypol molecules induce mitochondrial stress, thereby increasing mitochondrial membrane permeability and causing Smac and cytochrome C to be released through the membrane to the cytoplasm. This might lead to procaspase 3 cleavage and subsequent cell apoptosis. Some researchers have suggested that this protein release mechanism shares the same pathway as Bak- and Bax-dependent cytochrome C release (Buron et al. 2010). Because of the present results showing that gossypol initiates upregulation of p53, we supposed that gossypol may also mediate nuclear degradation directly via this pathway.

In our preliminary protein array screening assay, the expression of insulin-like growth factor-binding protein 6 (IGFBP-6) increased more than twofold in the gossypol treated preparations. Other studies have indicated that Y79 occasionally uses autocrine signaling through insulin-like growth factor (IGF) types I and II to stimulate cell growth (Giuliano et al. 1996). IGFBP-6 has the highest affinity to IGF II of all IGFBP members, which could mean that in gossypol-treated preparations, IGF II could compete with insulin-like growth factor receptors (IGFRs) and prevent IGF-mediated signaling activation (Bach et al. 2005). IGFBP-6 has an anti-proliferative and apoptotic effect on many types of IGF II-dependent tumors (Bach et al. 2005), and our results suggest a

similar effect on this pathway in Y79 cells.

Gossypol has been shown to have antiangiogenesic (Karaca et al. 2008; Benz et al. 1991) and antimetastatic (Zhang et al. 2010; Chang et al. 1993) effects in many other cancers. This study concludes that gossypol induces cell apoptosis by inducing mitochondrial stress, DNA damage, and cell arrest in retinoblastoma cells, and that gossypol does not damage non-cancer retinal pigmented epithelial cells. Gossypol may therefore have a clinical application in anticancer therapy; however, further investigations with in vivo studies are required.

Chapter 5. Prospective (展望)

5.1. RNA interference of bradykinin B2 receptor reduced the neuropathic pain caused by sciatic nerve injury.

The purpose of this study is to seek a potential new way of analgesia that can help patients who suffered from severe pain badly. The conclusion of this study showed the siRNA of bradykinin B2 receptor has the potential to reduce the neuropathic pain.

We have passed the patent of Republic of china with the title of “A use of RNA interference for treating or reducing pain” on 2012/11/16 and also the patent of USA with the title of “Use of RNA interference for treating or reducing pain” on 2013/5/21 (Figure. 19, 20).

Our goal is to follow the clinical trial procedure then offer this method as a new therapy for patient with neuropathic pain.

However, significant barriers still exist on the road to clinical applications of siRNA drugs, including poor cellular uptake, instability under physiological conditions, off-target effects and possible immunogenicity. The successful application of siRNA as a new therapy requires the development of clinically suitable, safe and effective drug

delivery systems. The design criteria include chemical modifications, lipid-based nanovectors, polymer-mediated delivery systems, conjugate delivery systems, and others. However, several barriers still exist on the road to siRNA clinical use for therapy. Firstly, siRNA is unstable under physiological conditions. When siRNA traffics through the blood, it is easily digested by nucleases in the serum. The ideal administration route of siRNA is systemic injection, so that siRNA can reach the target cells more efficiently. After injection into the blood, siRNA is easily enzymatically degraded by endogenous nucleases, filtered by the kidney, taken up by phagocytes and aggregated with serum proteins. One of the first biological barriers encountered by administered siRNA is the nuclease activity in plasma and tissues. In addition, the kidney plays a key role in siRNA clearance; several studies in animals have reported that the biodistribution of siRNA shows the highest uptake in the kidney. In addition to circulating nuclease degradation and renal clearance, a major barrier to *in vivo* delivery of siRNA is uptake by the reticuloendothelial system (RES). The RES is composed of phagocytic cells, including circulating monocytes and tissue macrophages, the physiological function of which is to clear foreign pathogens and to remove cellular debris and apoptotic cells. Tissue macrophages are most abundant in the liver (where they are called Kupffer cells)

and the spleen, tissues that also receive high blood flow and exhibit a fenestrated vasculature. siRNA uptake after standard i.v. tail vein injection or intraperitoneal (i.p.) injection has been noted in the liver, spleen, kidney and bone marrow at 4 h, but the overall signal was weak. (Xu et al, 2015).

Soutschek et al. reported that chemically modified siRNA could silence an endogenous gene encoding apolipoprotein B (apo B) after intravenous injection in mice (Soutschek et al, 2004). We expected that the biological barrier could be concurred in the near future that the siRNA of bradykinin B2 receptor can be applied to the patient by intravenous injection. On the other way, the siRNA of bradykinin B2 receptor might be applied locally to the skin, subcutaneous tissue and dorsal root ganglion for reducing pain.

5.2. Nuclear condensation and cell cycle arrest induced by telomerase siRNA in neuroblastoma cells.

We have demonstrated that telomerase siRNA has an anti-proliferative effect on human neuroblastoma cells via the cell apoptosis pathway. Moreover, telomerase siRNA treatment decreases the number of cells entering the interphase of the cell cycle. This study provides a potentially effective therapy for human neuroblastoma.

The process of clinical trial is mandatory to confirm the security in clinical use and to assess its effectiveness. The surgery plays an important role in the treatment of neuroblastoma, not only for pathological confirmation but also for prompt removal of the tumor if feasible. One of the probable roles is to add on local application of telomerase siRNA at operation.

5.3. Involvement of Smac, p53, and caspase pathways in induction of apoptosis by gossypol in human retinoblastoma cells.

Gossypol has been shown to have antiangiogenesic [59,60] and antimetastatic [61,62] effects in many other cancers. This study concludes that gossypol induces cell apoptosis by inducing mitochondrial stress, DNA damage, and cell arrest in retinoblastoma cells, and that gossypol does not damage non-cancer retinal pigmented epithelial cells. Gossypol may therefore have a clinical application in anticancer therapy; however, further investigations with in vivo studies are required.

Gossypol has been used clinically as a male contraceptive in China. If the security work-up at clinical trial were proved, it would be an add-on anti-cancer treatment for retinoblastoma. Bai SW et al reported silencing the Pax 6 gene with siRNA resulted in

an inhibited growth and an increased apoptosis of cultured human retinoblastoma cells (Bai et al, 2004). We can try to the combined use of gossypol and siRNA for Pax 6 gene the control of retinoblastoma cells.

Chapter 6. Summary in Chinese (中文簡述)

核糖核酸干擾 (RNA interference, RNAi) 是生物體內控制基因表現的方法之一，在許多真核生物中皆有發現，包含：酵母菌、果蠅、線蟲、哺乳動物等。

核糖核酸干擾以微型核糖核酸 (microRNA, miRNA)和小干擾核糖核酸 (small interfering RNA, siRNA)這兩種小片段核糖核酸為最主要的干擾形式，主要是干擾蛋白質的表現，以達到抑制基因表現的結果，稱為稱為基因後轉錄的沈默作用 (post-transcriptional gene silencing). 核糖核酸是基因的直接產物，而這些小片段的核糖核酸可以藉由和特定的核糖核酸結合以抑制特定基因的表現。核糖核酸干擾在對抗入侵的外來的寄生性基因，例如病毒、跳躍子 (transposons) 和一般個體發育過程中扮演重要的角色。

克雷格・梅洛（Craig Cameron Mello）是美國麻薩諸塞州大學醫學院分子醫學教授。2006 年因與史丹福醫學院病理學和遺傳學教授安德魯・法厄(Andrew Zachary Fire) 發現 RNA 干擾現象而共同獲得 2006 年諾貝爾生理學或醫學獎。

小干擾核糖核酸是長度 20-25 個核甘酸的雙股核糖核酸，可經由多種不同轉染(transfection)技術導入細胞內，以帶有專一性的方式調節基因的表達，對特定基因產生具專一性的基因表現減量(knock-out)效果，已經成為研究基因功能與開發新藥物的一項重要工具。

本篇論文研究分為三部分，第一部分是以緩激肽 B2 受體小干擾核糖核酸 (Bradykinin B2 receptor siRNA)在大白鼠背根神經節阻斷疼痛傳導而達鎮痛效果。第二部分是以端粒酶小干擾核糖核酸(telomerase siRNA)引導神經母細胞瘤癌細胞

(neuroblastoma cell)產生核凝結及細胞週期停動；第三部分是以棉酚(Gossypol)引導經由 Smac, p53 及 caspase 途徑的視網膜母細胞腫瘤細胞凋亡。

神經病變痛(neuropathic pain)是神經系統異常所造成的一種疼痛。引起疼痛的原因很多，包括神經發炎、壓迫或受傷等因素。例如，感染疱疹病毒造成神經發炎之帶狀疱疹，這種神經痛常持續到疱疹皮症好了以後仍然存在，又稱為疱疹後神經痛。此外，三叉神經痛、糖尿病、腦中風、脊髓外傷、周邊神經受傷、截肢後、以及腕隧道症候群的病人，都可能引發程度不一的神經性疼痛。神經受壓迫或受傷，例如骨頭斷裂、椎間盤凸出（坐骨神經痛）或是腫瘤壓迫亦可能造成神經痛。神經病變性疼痛對傳統止痛劑常發生反應不佳的困擾。另一方面，由於長期慢性疼痛，不僅有疼痛擾人，還會合併失眠、焦慮、憂鬱等症狀，不但會影響工作表現，同時也會造成病人社會功能與生活品質的退化，除了個人的病痛外，對家庭與社會更是沉重的負擔。

疼痛是一種複雜的神經反應，身體或內臟神經末端的傷害感受器，能夠感覺物理性、溫度、化學性的刺激。傷害性刺激，例如炎症、組織壞死、缺血、缺氧等情況發生時，組織會釋放一些激素或體液物質來激活痛覺受體，引起疼痛。緩激肽即是身體出現發炎反應時所生成的一種胜肽，會強烈刺激痛覺受器(nociceptor)，屬於疼痛的強刺激物。研究發現，緩激肽是表現於痛苦感受神經元之緩激肽 B2 受體的促效劑，B2 受體經由緩激肽作用會促使痛苦感受神經纖維中 B2 受體的活化，而使人類或動物個體感到疼痛。若能有效阻斷緩激肽 B2 受體，即可進一步防止這些受體造成痛覺受器之活化，而且不會影響其他觸覺、溫度感覺、壓覺或其他發炎因子的生理功能。

第一部份研究以大白鼠神經病變痛動物模式進行，將大白鼠麻醉後，左側腰椎第五條神經使用外科手術用鈦金屬夾夾住，造成神經損傷，因此就成為神經病變痛動物模式。大白鼠接受手術後，在不同的足底觸覺刺激程度時觀察牠左腳收起時所需的時間，用來作為對疼痛感覺敏銳度的評估。在對照組是將神經夾起，實驗組則將神經夾起同時系列的注射緩激肽 B2 受體小干擾核糖核酸，實驗動物觀察到 2 周的時間，同時重複性的比較大白鼠雙下肢對於觸感刺激的下肢縮腳迴避的時間，用於評估對疼痛的耐受度。根據實驗結果可以證實，使用緩激肽 B2 受體小干擾核糖核酸會使大鼠對於神經病變痛的耐受性顯著的增加，另外實驗後將大鼠的左右雙側腰椎第四、五及第六背根神經節取出，以 RT-PCR 檢測緩激肽 B2 受體的表現量，可以證實在給予小干擾核糖核酸之後緩激肽 B2 受體的表現量在實驗組有顯著的降低。由以上的結果可以證實緩激肽 B2 受體小干擾核糖核酸在動物模式可以顯著地降低疼痛的敏感度，達到鎮痛的效果。

依據最近的美國醫學資料，兒童罹患惡性腫瘤的 5 年存活率，在 1975 年大約 50%；最近半世紀以來由於診斷技術進步、多元治療方法的開發以及團隊治療的建立，使其 5 年存活率提升到 80%。然而並非不同的惡性腫瘤平均的提升；其中進步最多的是急性淋巴母細胞白血病，由 1960 的小於 10%進步到 2010 年的 90%；但是進步最少的是瀰漫性橋腦膠原細胞瘤，病患很難存活超過 1 年。總括來看，在美國於 20 歲因惡性腦瘤而死亡的人數每 10 萬人每年由 1975 年的 5 人降到 2010 年的 2.3 人。雖然已有進步，但是每年仍有約 2000 病患因而死亡，因此，持續的醫學探索研究以求改善仍是需要繼續進行的。

第二部分研究的目標是神經母細胞瘤，發生率佔兒童惡性腫瘤的 8-10%，也居兒童因惡性腫瘤死亡的首位。大約有三分之一病童在 1 歲前確認診斷，但也高

達 60-70%病患在診斷確立時已有遠端轉移。兒童時期中樞神經系統的發育尚未臻成熟是治療選擇困難的重要因素，也影響預後。近年來結合手術、放射治療及化學治療已經使得治療成效改善，但依據美國的報告，5 年存活率與臨床分級有關，分級依據腫瘤的手術可切除率，是否多發性，有無淋巴侵犯、遠端轉移，腫瘤細胞有無 MYCN 擴增(MYCN amplification)。5 年存活率在低、中及高風險族群分別大約為 95%，90%及 40-50%。

端粒(telomere)是染色體末端的 DNA 重複序列，作用是保持染色體的完整性和控制細胞分裂週期。細胞分裂一次，由於 DNA 複製的方向必須從 5’方向到 3’方向，DNA 每次複製端粒就縮短一點。一旦端粒消耗殆盡，細胞將會立即激活凋亡機制。大部分的惡性腫瘤細胞會發展出規避前述凋亡機制的方法而癌化為不死細胞。

1985 年，Gueider 等發現端粒酶(telomerase)，可用於端粒 DNA 加尾，它可以增加 DNA 重複序列(大部分哺乳類為”TTAGGG”)到人類細胞端粒區。

許多研究報告大約 90%的人類惡性腫瘤細胞有端粒酶活性。Hiyama 等發現端粒酶的活性大小是神經母細胞瘤重要的預後指標。另外有研究指出正常體細胞的端粒酶活性較癌細胞為相對的低。因此調控端粒酶活性成為癌症治療的方向之一。我們的假設是以端粒酶 siRNA 可以降低神經母細胞瘤的顆粒酶活性，達到降低腫瘤細胞成長分化的能力。

我們選用人類神經母細胞瘤細胞(IMR-32)做實驗，在加入不同濃度的 siRNA 後，發現端粒酶的活性顯著降低(加入 100 μM 濃度時比對照組降低 80%活性)，檢測其端粒酶 RNA 發現加入 100 μM 組是對照組的 20%。

IMR-32 細胞的生存能力以 WST-1 測定法(WST-1 assay)評估，結果發現加入 10 μM 及 100 μM 濃度組別的 IMR-32 細胞生存能力顯著的比對照組低。

細胞凋亡的末期會有細胞核碎裂(fragmentation)，IMR-32 細胞培養時加以上不同濃度的端粒酶 siRNA，並以 DAPI 標記，DAPI 可以分辨出正常細胞與進行凋亡的細胞，結果發現 10 μM 及 100 μM 濃度組的細胞比 1 μM 濃度及對照組多了 10%的凋亡現象。

以流式細胞計量術(flow cytometry)計量細胞週期不同期的細胞比率，一般而言，癌細胞為不死細胞，因為預估會有較少細胞在 sub-G1 及 G1 期；而較多的細胞在 S 及 G2/M 期。將 IMR-32 以不同濃度端粒酶 siRNA 處置後，結果顯示在 10 μM 及 100 μM 濃度組在 sub-G1 期細胞顯著的增加：在 100 μM 組則發現 G1 及 S 期的細胞顯著的減少。

由以上的結果，我們得到推論，端粒酶 siRNA 可以進一步研究期能成為神經母細胞瘤病患的新治療方法之一。

第三部分研究的目標是視網膜母細胞瘤，是最常見的兒童眼眶內惡性腫瘤，發生率大約佔兒童惡性腫瘤的 2%。

視網膜母細胞瘤大部分為單側，少數為雙側，及少數併有腦內的松果體母細胞瘤(trilateral retinoblastoma)；有些病患腫瘤細胞會擴散到淋巴結或遠端轉移。單側患者的 60%會有 retinoblastoma 1 gene(RB1)基因突變。

大多數病患在 5 歲前確診，主要的臨床表現為白色瞳孔、斜視及眼眶疼痛。

目前的診療方法包括手術摘眼術、冷凍療法、放射治療及化學治療。雖然 5

年存活率可達 90%以上，但相關治療會引發副作用，例如視力缺損、感染、發燒、胃腸機能障礙及神經毒性。因此持續探索新的治療方法有其必要性。

棉酚(gossypol)是棉花種子萃取物，它有廣泛的用途，在農業上可以經由抑制 ATPase 活性而當殺蟲劑用；它也可以降低精蟲的能量製造，抑制其活力，但不影響睪丸激素濃度而用於男性避孕。其他研究發現透過細胞凋亡機制的調控，棉酚可以用來治療攝護腺癌、淋巴癌、乳癌、肺腺癌、卵巢癌及胰臟癌。

我們的假設是棉酚是否可以透過調控細胞凋亡機制用以治療視網膜母細胞瘤。我們選用人類視網膜母細胞 Y79 以及人類視網膜素色上皮細胞(ARPE)進行實驗。

在細胞存活能力方面使用 MTT-測定法，發現 5,10 及 20 μM 濃度的棉酚顯著的抑制 Y79 的存活能力，但對 ARPE 細胞沒有影響。Y79 細胞培養時加上不同濃度的棉酚，並以 DAPI 標記，發現 10 μM 及 20 μM 濃度組比對照組有顯著增加的細胞凋亡現象。 以流式細胞計量術計量細胞週期不同期的數目時，發現在 10 μM 及 20 μM 時，有顯著的細胞增加在 G0/G1 期，但在 G2/M 期則顯著的減少，表示棉酚除了可以誘導凋亡外，也可以使細胞週期停動。

在棉酚導引細胞凋亡機制研究方面，以 20 μM 濃度組與對照組比較蛋白質表現發現凋亡蛋白質如 DR5, p53, Smac, casepase 8, caspase 9 及 caspase 3 在 Y79 細胞組的提升 1.5-2 倍。而 Cytochrome C 則提升到 5.8 倍，顯示棉酚可使用多個機制導引 Y79 細胞凋亡，包括 1) TRAIL-媒介(DR5)途徑，導致 caspase 家族成員濃度提升，如 Smac, cytochrome C 等。2) DNA 降解致 p53 提升及細胞週期停動。由以上的結果，我們得到推論，棉酚可以抑制人類視網膜母細胞的繁殖，作為進一步研究是否可以用於病患治療的方法之一。

綜合以上的研究可以得知在動物模式及實驗室細胞研究方面可以了解緩激肽 B2 受體小干擾核醣核酸可用來鎮痛，端粒酶小干擾核醣核酸可用來抑制神經母細胞瘤的成長，未來的研究方向希望將這兩部分進行一系列的臨床試驗，若能得到正面的結果，則希望能用於臨床上對於重度神經病變痛病人及神經母細胞瘤的病患增加治療的選項達到良好的預後。有關小干擾核醣核酸的臨床使用則需要進一步研究可否由靜脈注射或者周邊組織局部使用就可達到臨床療效，以增加臨床使用的方便性，這部份還是需要進一步的臨床研究。至於棉酚用在治療視網膜母細胞部分，將來計畫研究棉酚加上 Pax 6 基因小干擾核糖核酸對於視網膜母細胞瘤是否可以有加成治療的效果，若能有正面的結果將可以對視網膜母細胞瘤的臨床治療有更進一步的貢獻。

Chapter 7. References (參考文獻)

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Chapter 8. Figures and Tablets (圖表)

Days after surgery	Resistance (mean±SEM)		
	Control group	Neuropathic group	Neuropathic+RNAi group
4	102.7±3.4%	52.8±4.9%	64.4±5.4%
5	99.9±4.3%	55.4±3.5%	71.4±2.4%
6	99.6±3.2%	56.5±3.6%	68.2±2.7%
7	97.2±2.8%	55.7±3.6%	69.6±3.9%
8	99.0±2.9%	57.2±4.0%	68.7±3.2%

Table. 1. Mechanical pressure test (von Frey's test) on neuropathic pain models. The percentage of resistance to pain was measured in comparison to the mean of the control group.

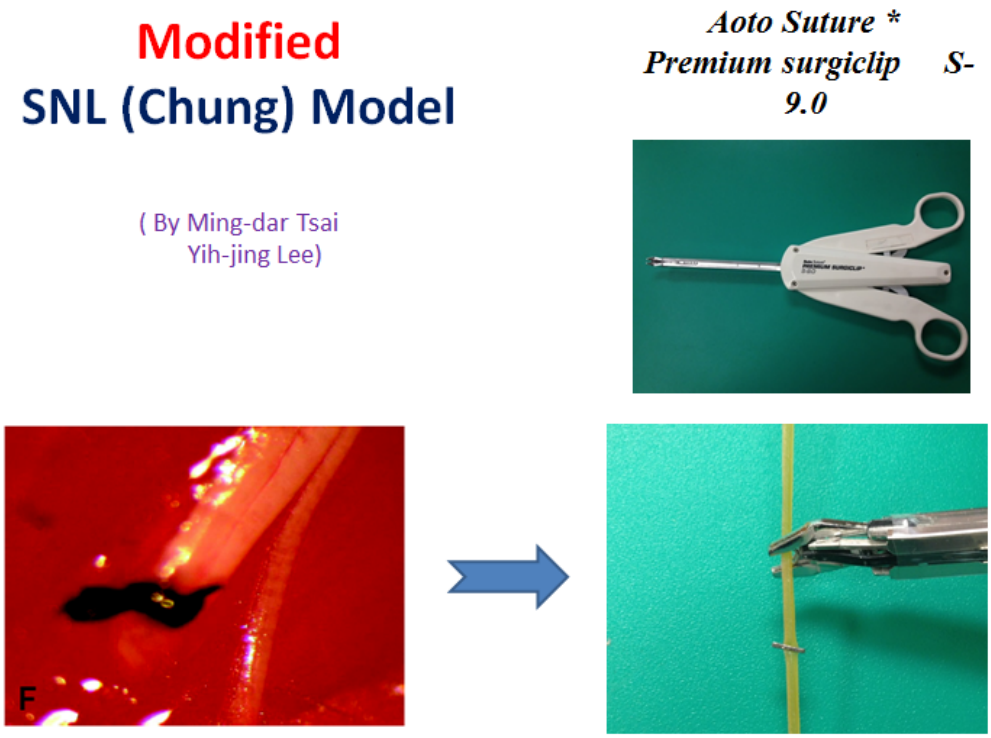


Figure 1. Modified Chung animal model for neuropathic pain study. The lesion to the left L5 root of the rat was clipped by Premium surgiclip instead of silk ligation in order to get uniform lesioning.

SEQ ID NO: 1

5′- GCACUGUGGCUGAGAUCUA-3′

SEQ ID NO: 2

5′- GAACGAGCGUGCCGUGGAU-3′

SEQ ID NO: 3

5′- GUUCCUACGUGGCCUAUAG-3′

SEQ ID NO: 4

5′- UGGUGAACACUAUGAUAUA-3′

Figure 2. The sequences SEQ ID NO: 1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4 of bradykinin B2 receptor siRNA.

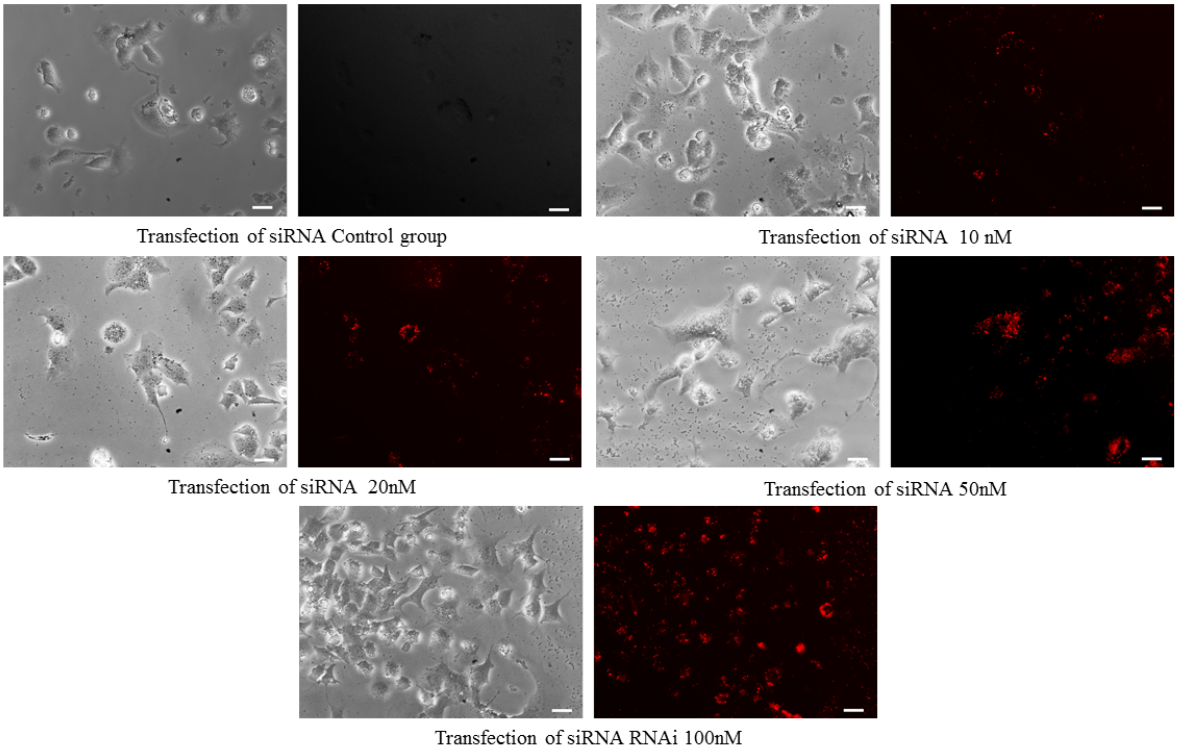


Figure 3. Microscopic images of cultured neural cells (PC12). PC12 cells are transfected with bradykinin B2 receptor siRNA during the culture. The left image shows the morphology of the cultured PC12 cells and the right image shows the bradykinin B2 receptor siRNA (in red fluorescence) was transfected into the cells. The results demonstrated a steady increase in the degree of transfection from the low concentration (10 nM) up to high concentration (100 nM) (Objective power 400x)

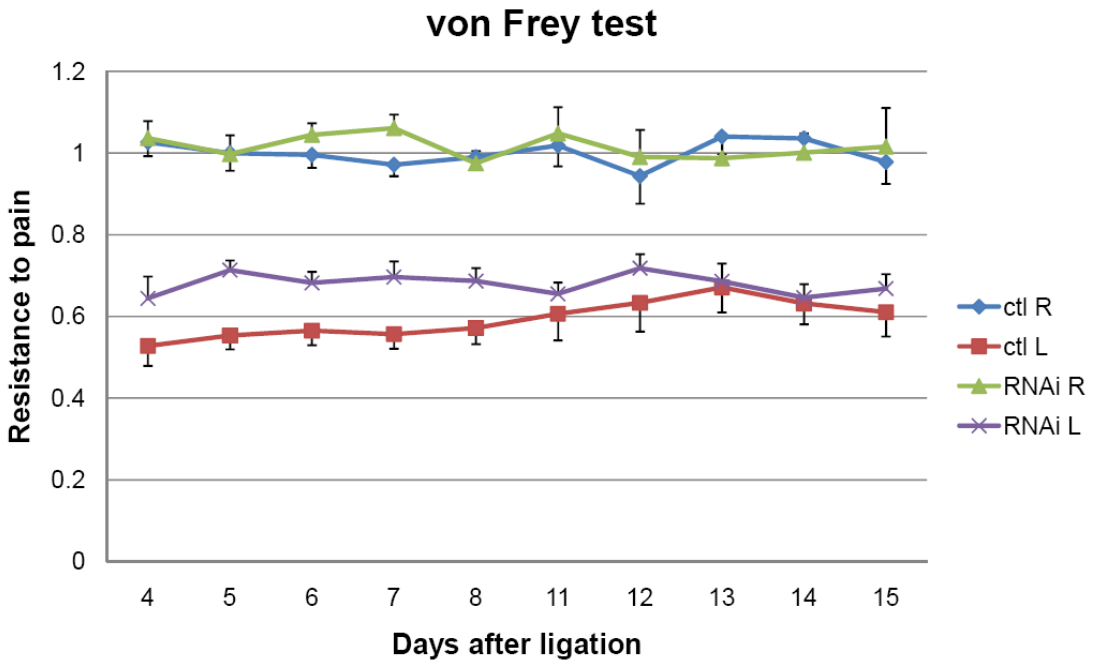


Figure 4. Mechanical pressure test (von Frey's test) on neuropathic pain models. Both right and left hind legs from RNAi treated animals and control animals were detected in this study. The X-axis refers to days after neuropathic surgery and the Y-axis refers to the power animals can resist. The results indicate that RNAi of bradykinin B2 receptor can reduce the nociceptive sensation cause by sciatic neuropathy.

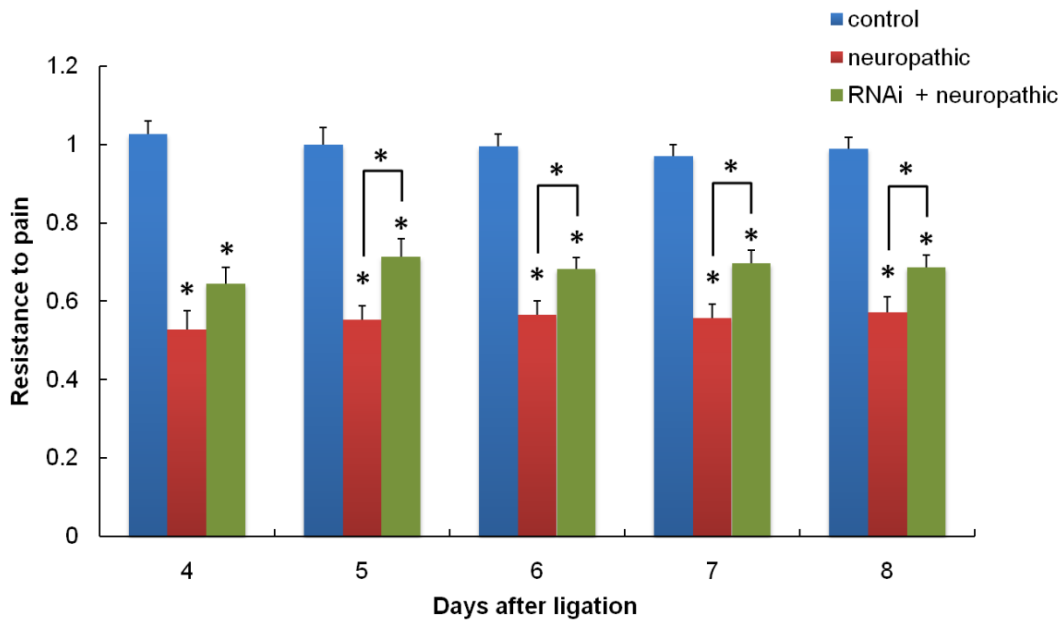


Figure 5. Results of mechanical pressure test (Von Frey's test) on neuropathic pain models. The X-axis is the day after neuropathic surgery and the Y-axis is the power that animal resists to pain. The results indicate that RNAi of bradykinin B2 receptor can reduce the nociceptive sensation cause by neuropathy. (*P<0.05, compared to the control group)

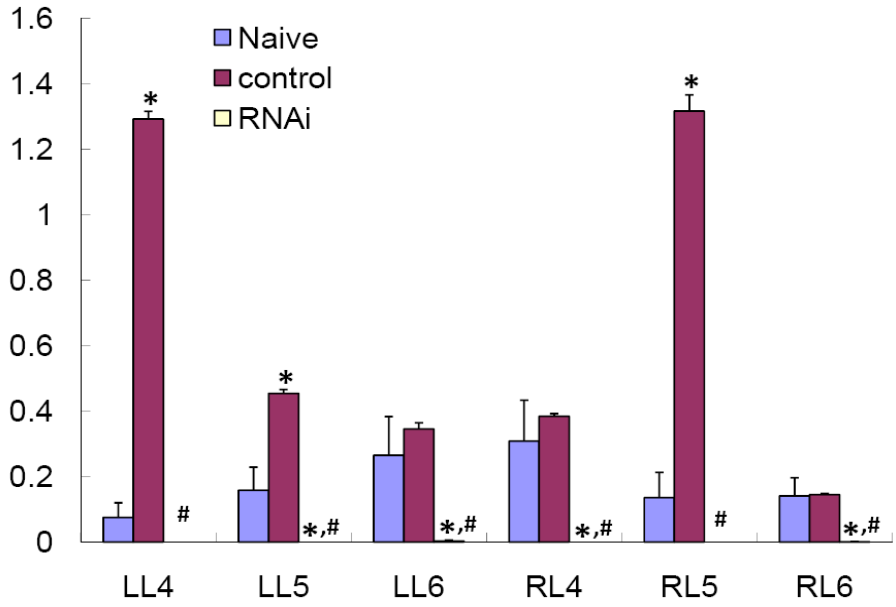


Figure 6. Quantitative evaluation of bradykinin B2 receptor expression by real-time PCR. Dorsal root ganglia from left lumbar nerves (LL4, LL5, LL6) and right lumbar nerves (RL4, RL5, RL6) are collected for real-time PCR study. Naïve: the untreated group.

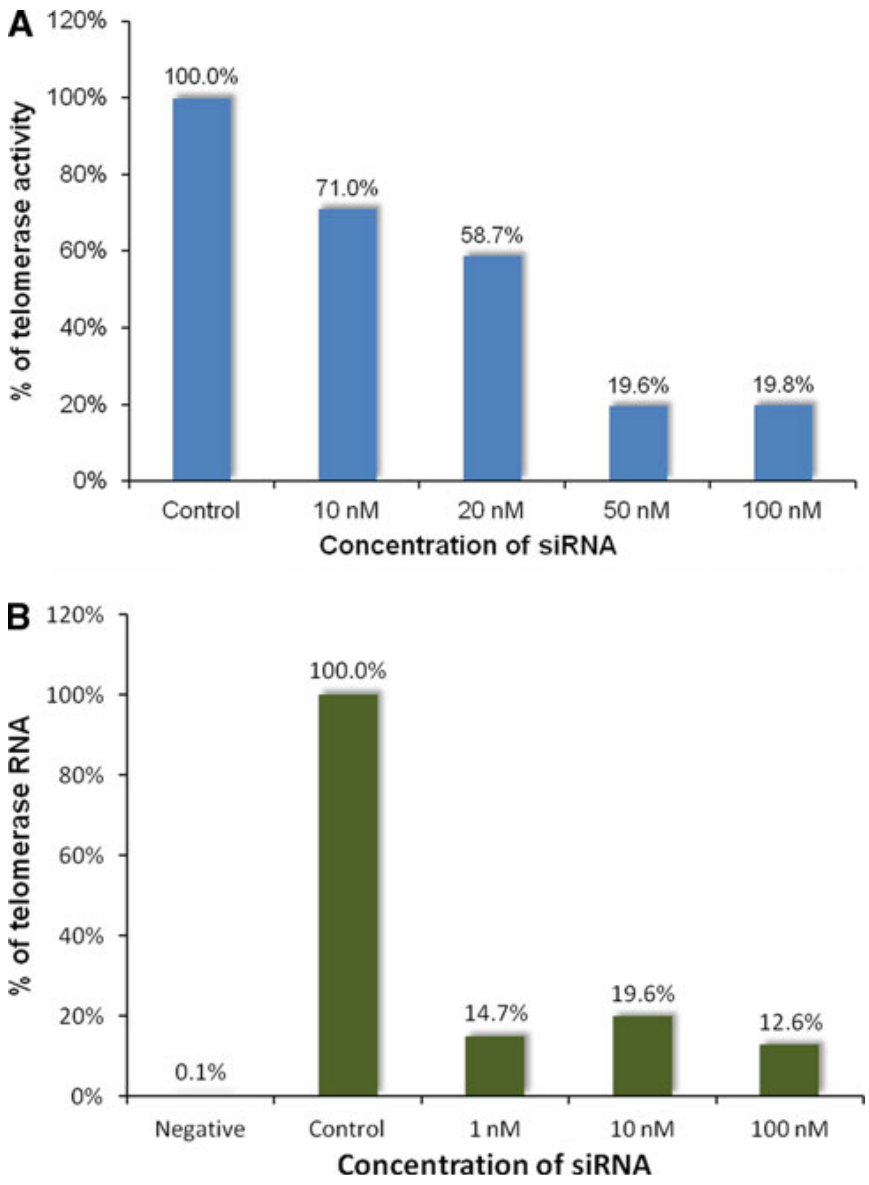


Figure 7. The effect of telomerase siRNA on telomerase activity and telomerase RNA. IMR-32 cells were transfected with various concentrations of telomerase siRNA. The telomerase activity (A) and telomerase RNA (B) were detected after 24 h incubation. The effect of siRNA was calculated as a percentage of the control (untreated) groups. The results showed that siRNA decreased the expression of telomerase RNA and activity.

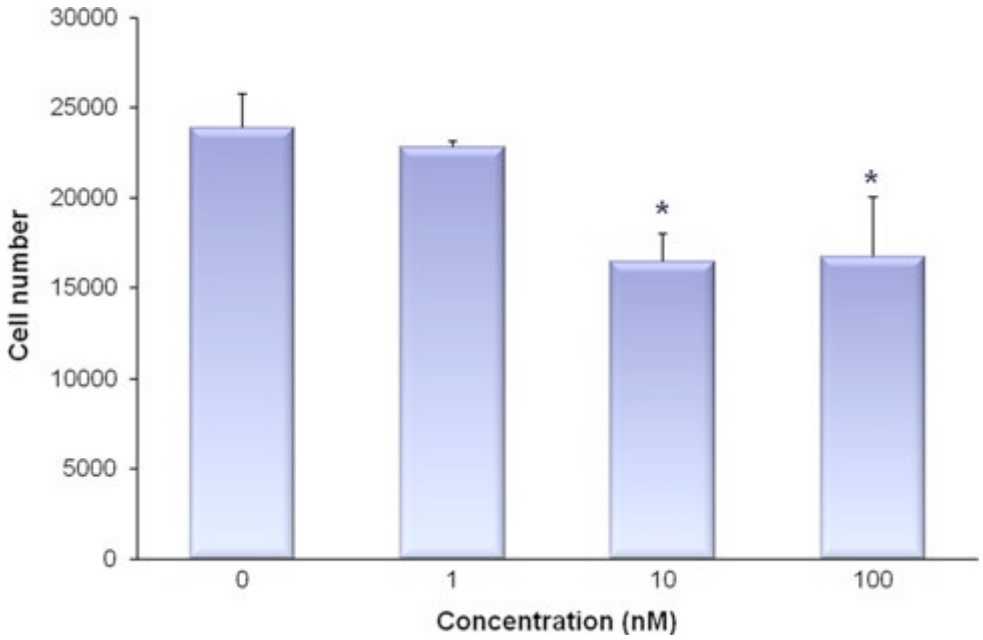
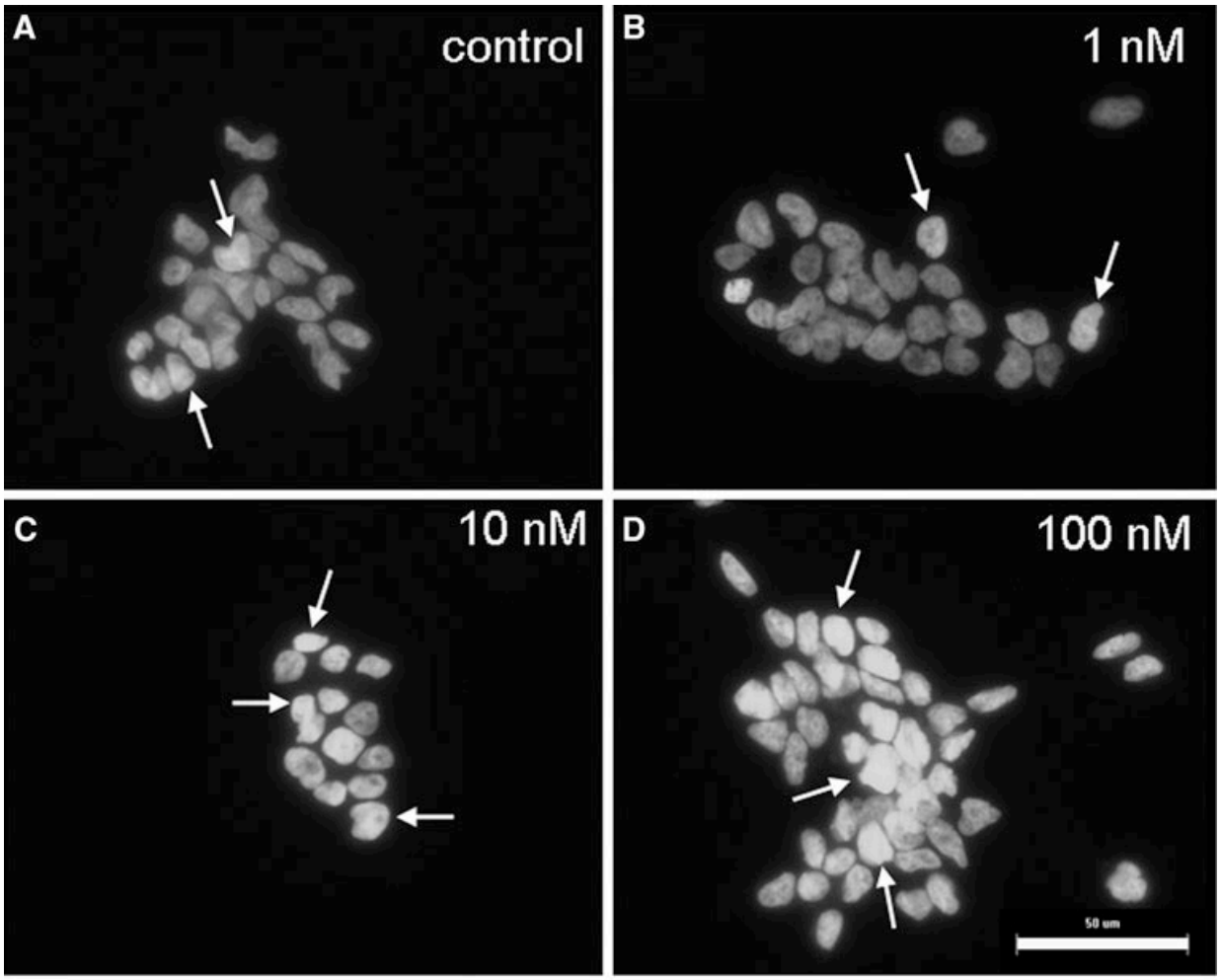


Figure 8. Cell viability of IMR-32 cells treated with various concentrations of telomerase siRNA. Cells cultured without siRNA were used as controls (concentration zero). Mean averages of cell numbers in each group are presented as bars in the figure, and error bars represent the SEM for each group. One-way ANOVA with Duncan's multiple comparisons analysis was used for statistical analyses. *p values<0.05 are considered significant.


A
control
B
1 nM
C
10 nM
D
100 nM
50 um

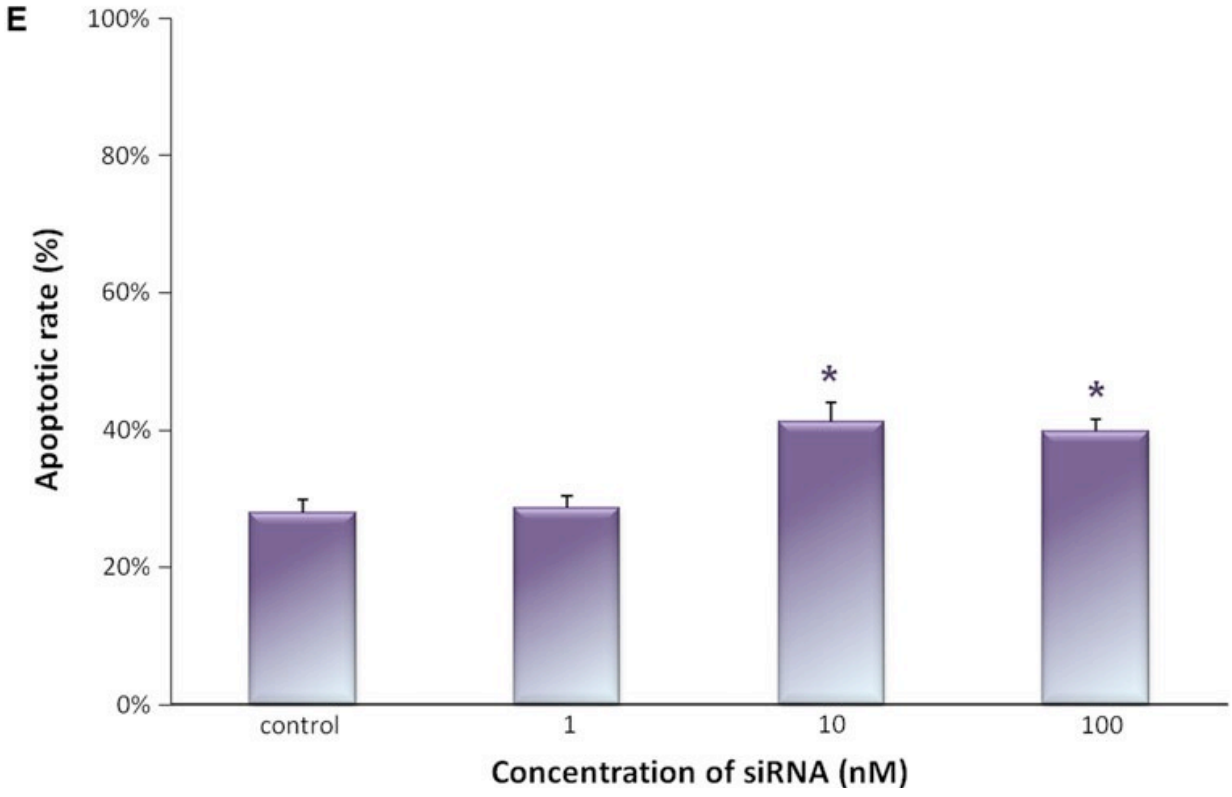

E
100%
80%
60%
40%
20%
0%
Apoptotic rate (%)
*
*
control
1
10
100
Concentration of siRNA (nM)

Figure 9. Telomerase siRNA mediated human IMR-32 cell apoptosis. DNA was labeled with DAPI (A-D). Apoptotic cells with DNA fragmentation reveal much stronger fluorescence than normal cells. DNA fragments are indicated by arrows. The numbers of apoptotic IMR-32 cells treated with or without telomerase siRNA were calculated (E). Means of cell numbers are presented in the figure, and error bars represent the SEM for each group. Scale bar = 50 μm. One-way ANOVA with Bonferroni's multiple comparison adjustment was conducted to compare the data within each experiment. *p values<0.05 are considered significant.

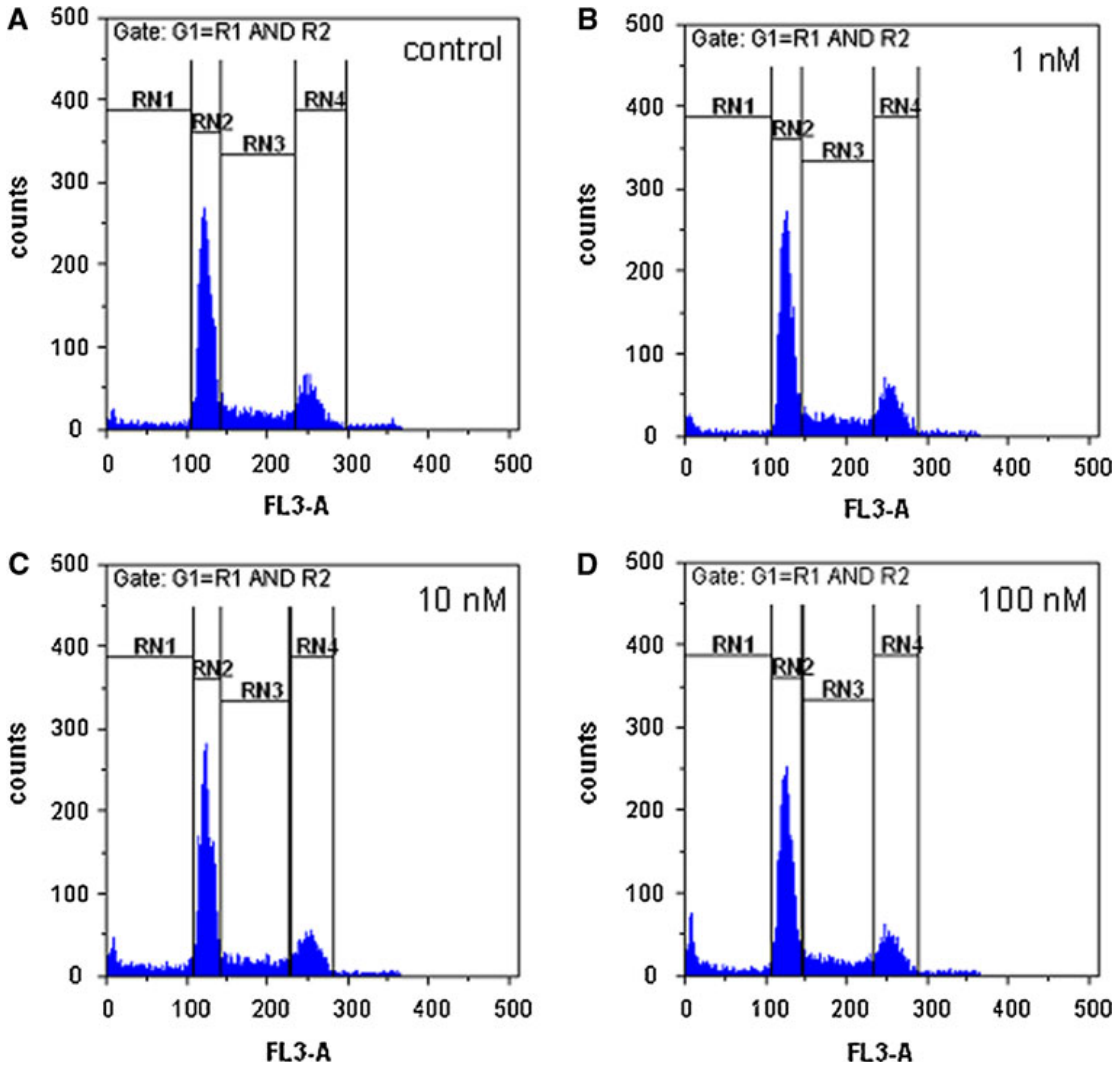


Figure 10. Change of phases in cell cycle induced by telomerase siRNA. Human neuroblastoma cells were collected after treatment with various concentrations of siRNA, and stained with PI. In each preparation, 1 x 10^4 cells were detected by flow cytometry. RN1, RN2, RN3, and RN4 represent the subG1, G1, S, and G2/M phases of the cell cycle respectively.

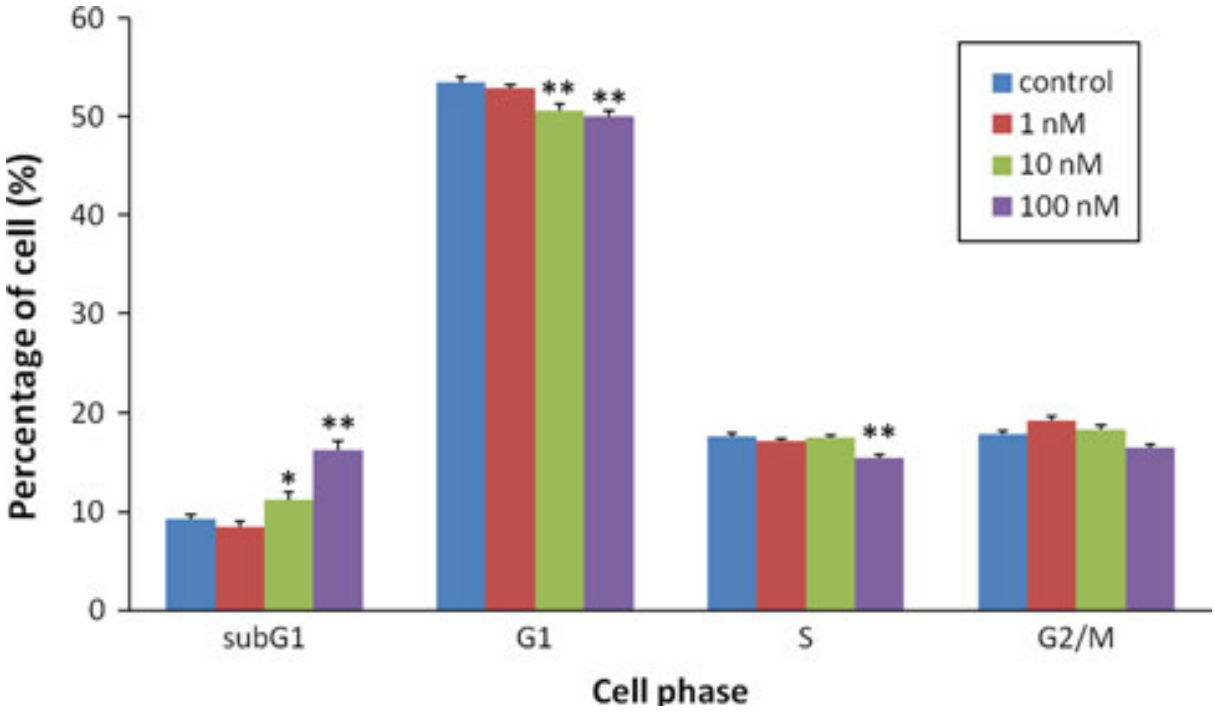


Figure 11. The cell cycle was determined by detection of DNA degradation. The IMR-32 cells were treated with various concentrations of telomerase siRNA and stained with PI. Bars represent mean percentages, error bars represent SEM. One-way ANOVA with Bonferroni's multiple comparison adjustment was conducted to compare data within each experiment. *p values<0.05 are considered significant.

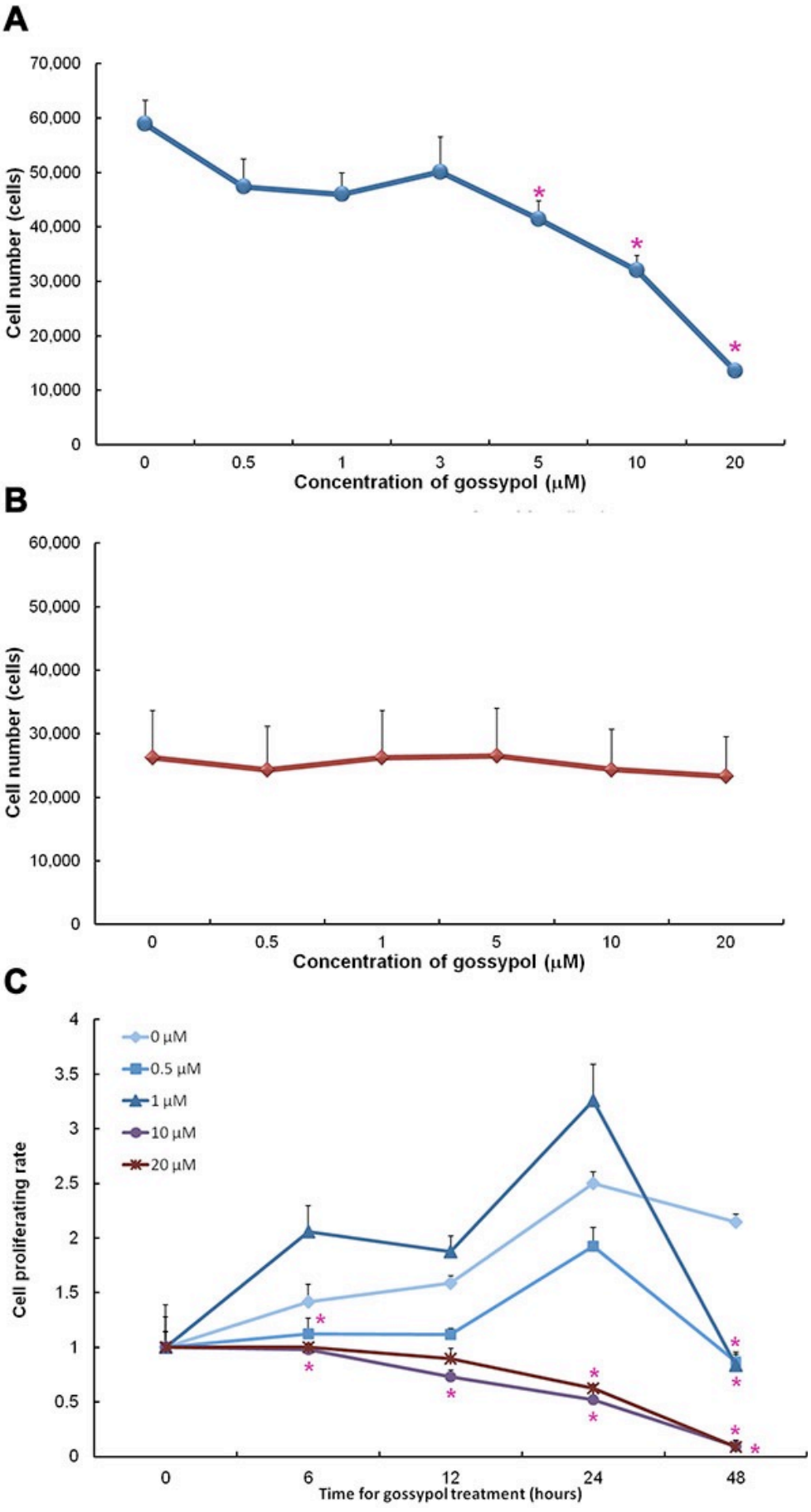

A
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
Cell number (cells)
0
0.5
1
3
5
10
20
Concentration of gossypol (μM)
B
60,000
50,000
40,000
30,000
20,000
10,000
0
Cell number (cells)
0
0.5
1
5
10
20
Concentration of gossypol (μM)
C
0 μM
0.5 μM
1 μM
10 μM
20 μM
Cell proliferating rate
4
3.5
3
2.5
2
1.5
1
0.5
0
0
6
12
24
48
Time for gossypol treatment (hours)

Figure 12. Cell viability of retinoblastoma cells detected with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The effects of gossypol in different concentrations on human retinoblastoma (Y79) cells (A) and human RPE (ARPE) cells (B) are shown. Cells were cultured at different concentrations of gossypol for 24 h. Gossypol was found to decrease Y79 cell numbers at concentrations of 5, 10, and 20 μM on retinoblastoma cells but had no effect on RPE cells. Time courses of different concentrations of gossypol are shown in (C). Data are presented as mean±SEM. One-way ANOVA with Bonferroni multiple comparisons was used for statistical analysis. n=12–32 in (A), 8–9 in (B), and 6–12 in (C). * $p<0.05$, compared to the control group.

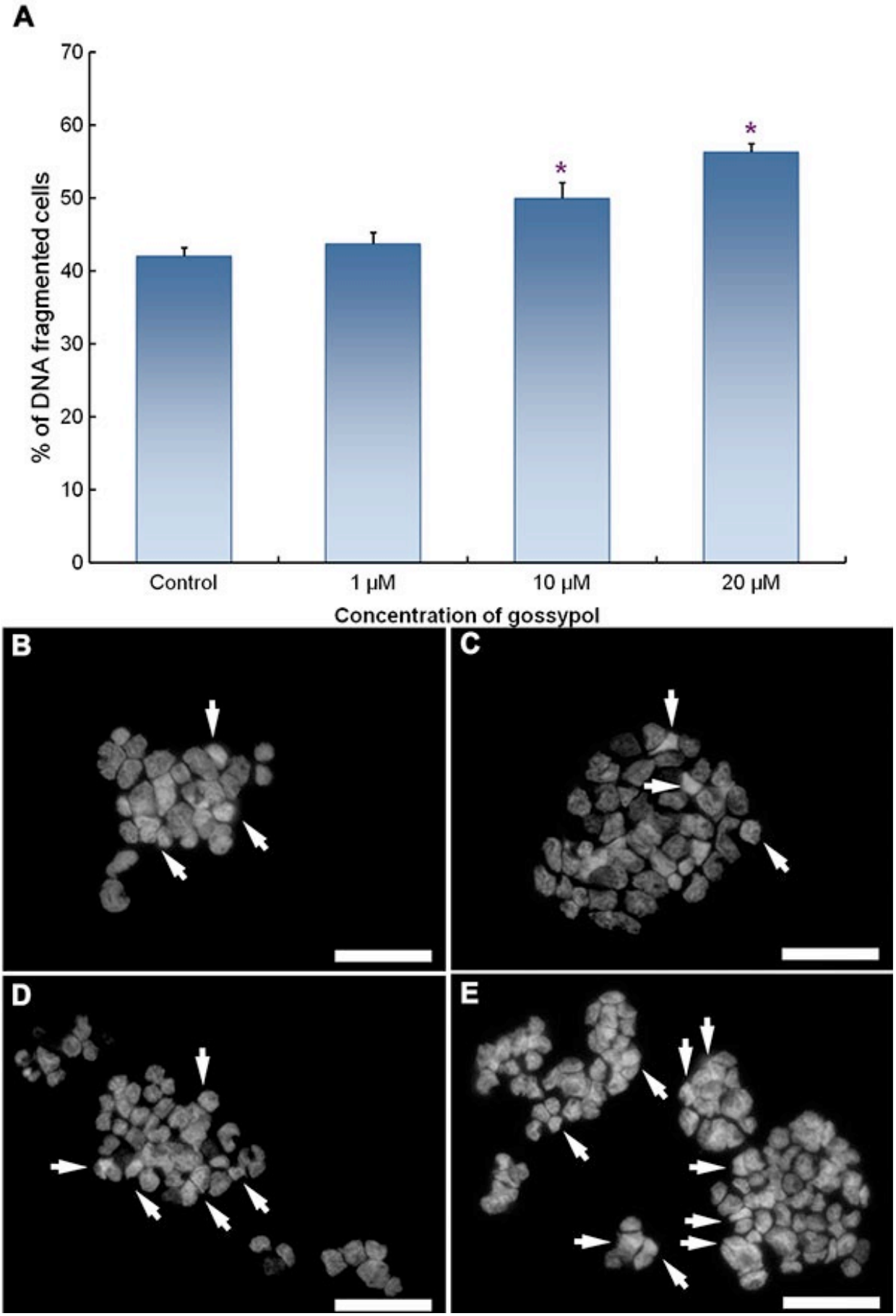

A
70
60
50
40
30
20
10
0
% of DNA fragmented cells
*
*
Control
1 μM
10 μM
20 μM
Concentration of gossypol
B
C
D
E

Figure 13. Nuclear condensation of apoptotic human retinoblastoma (Y79) cells was detected with 4,6- diamidino-2-phenylindole (DAPI) labeling. Gossypol-treated Y79 cells containing condensed nuclei were counted under a fluorescence microscope. Panel A shows the percentages of condensed-nucleus (apoptotic) cells. Data are represented as the mean±SEM n=14, and * $p<0.05$, compared to the control group. The remaining figures showing microimages show apoptotic (arrows) and normal cells in (B) control and in (C) 1 μM, (D) 10 μM and (E) 20 μM gossypol treatments. Scale bar is 50 μm.

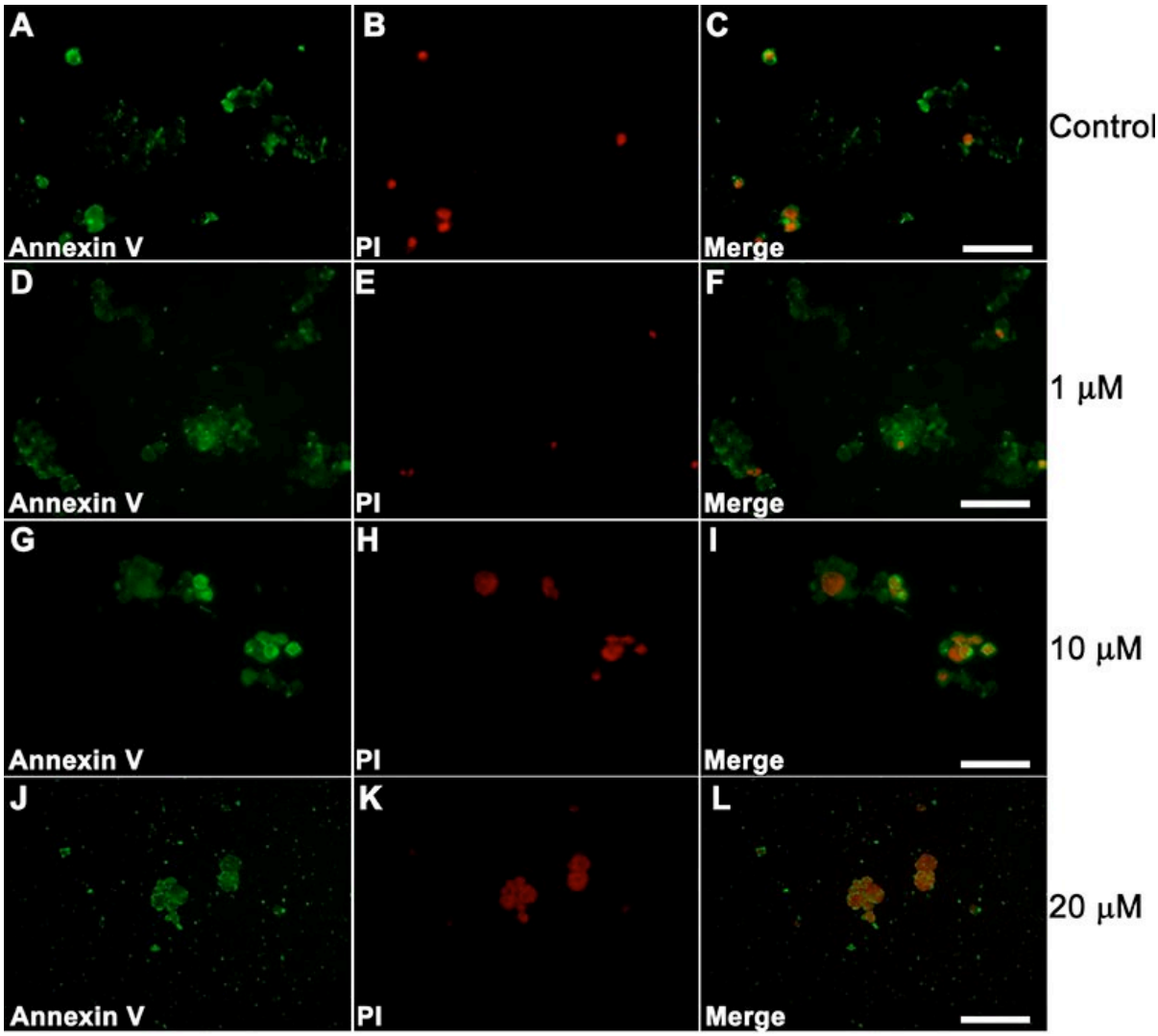


Figure 14. Human retinoblastoma (Y79) cells stained with annexin V and propidium iodide (PI) showing the different stages of apoptosis. A, B, and C are control groups. D, E, and F are 1 μM gossypol. G, H, and I are 10 μM gossypol. J, K, and L are 20 μM gossypol cultures. Scale bar is 50 μM.

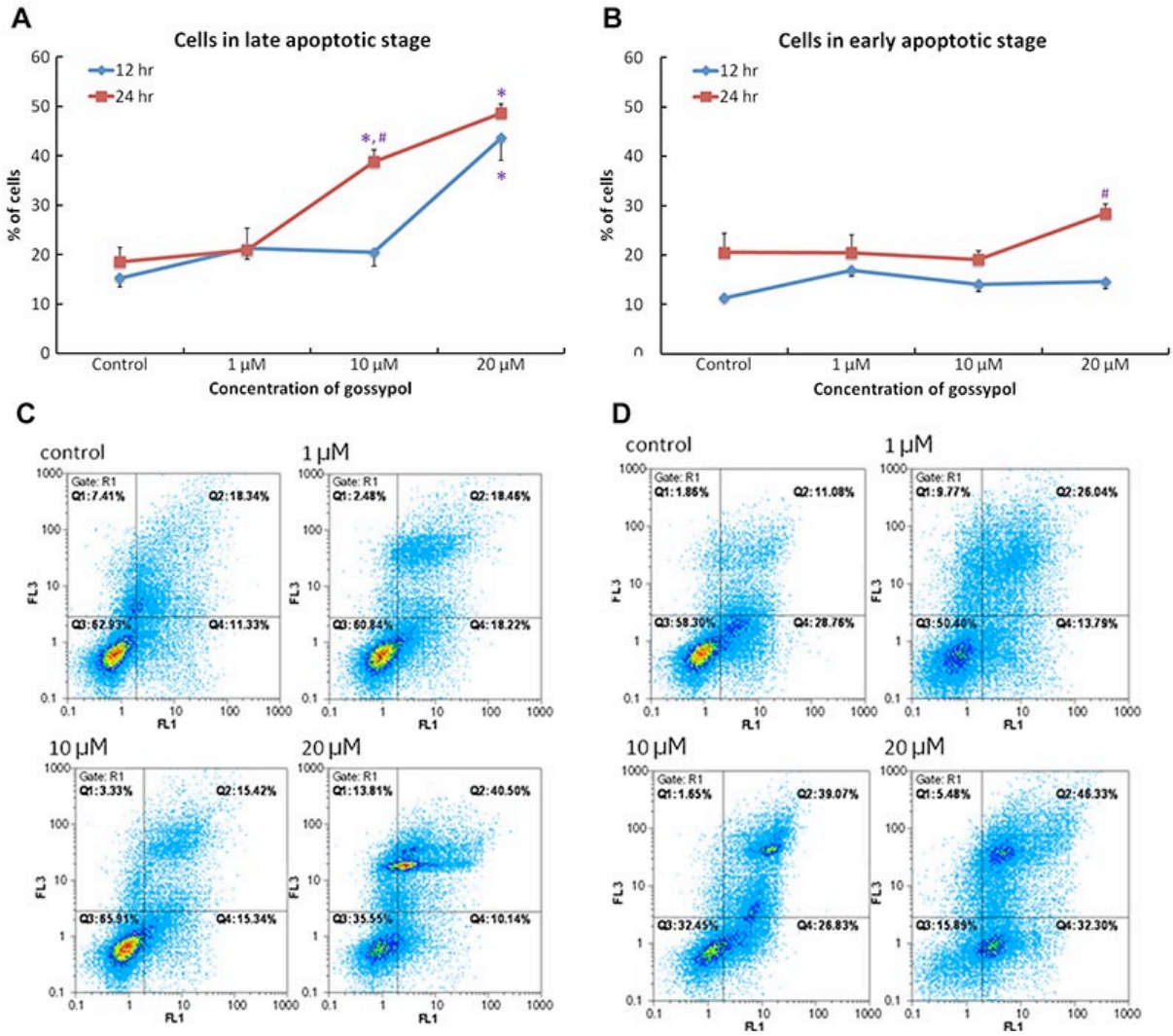


Figure 15. Flow cytometry demonstrated the stages of cell apoptosis in human retinoblastoma (Y79) cells at different time points and for different concentrations of gossypol. Panels A and B show the percentages of different apoptotic stages in cells incubated with different concentrations of gossypol for 12 and 24 h. Data are presented as the mean ±SEM. One-way ANOVA with a Bonferroni multiple comparison was used for statistical analysis. * $p<0.05$, compared to the control group. # $p<0.05$, compared to the 12-h group at the same dose. Images (C) and (D) show the distribution of cells in

different apoptotic stages with gossypol treatment for (C) 12 h and (D) 24 h, by collecting the annexin V signals as FL1 and propidium iodide (PI) signals as FL3. n=6 in each group. Q3 shows the normal cell population; Q4 presents the early apoptosis stage and Q2 the late apoptosis stage.

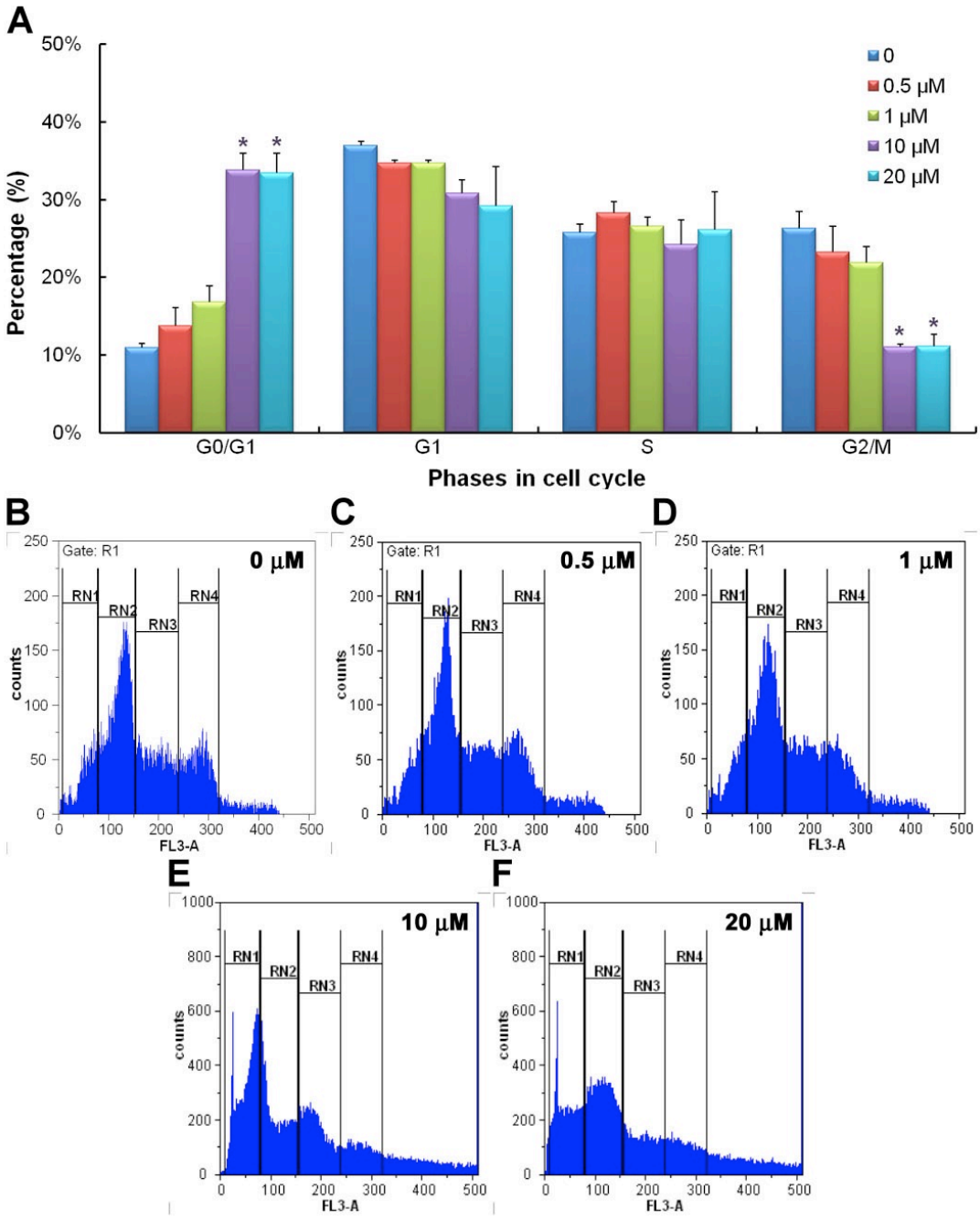


Figure 16. Flow cytometry demonstrated the stages of cell cycle in human retinoblastoma (Y79) cells at different concentrations of gossypol. Panel A shows the percentages of cells distributed in different cell cycle stages incubated with different concentrations of gossypol for 24 h. Data are presented as the mean±SEM. One-way

ANOVA with a Bonferroni multiple comparison was used for statistical analysis. * $p<0.05$, compared to the control group. Panels B to F show the distribution of cells in different cell cycle stages in different concentrations of gossypol. n=3–4. RN1 represents the G0/G1 phase, RN2 the G1 phase, RN3 the S phase, and RN4 the G2/M phase.

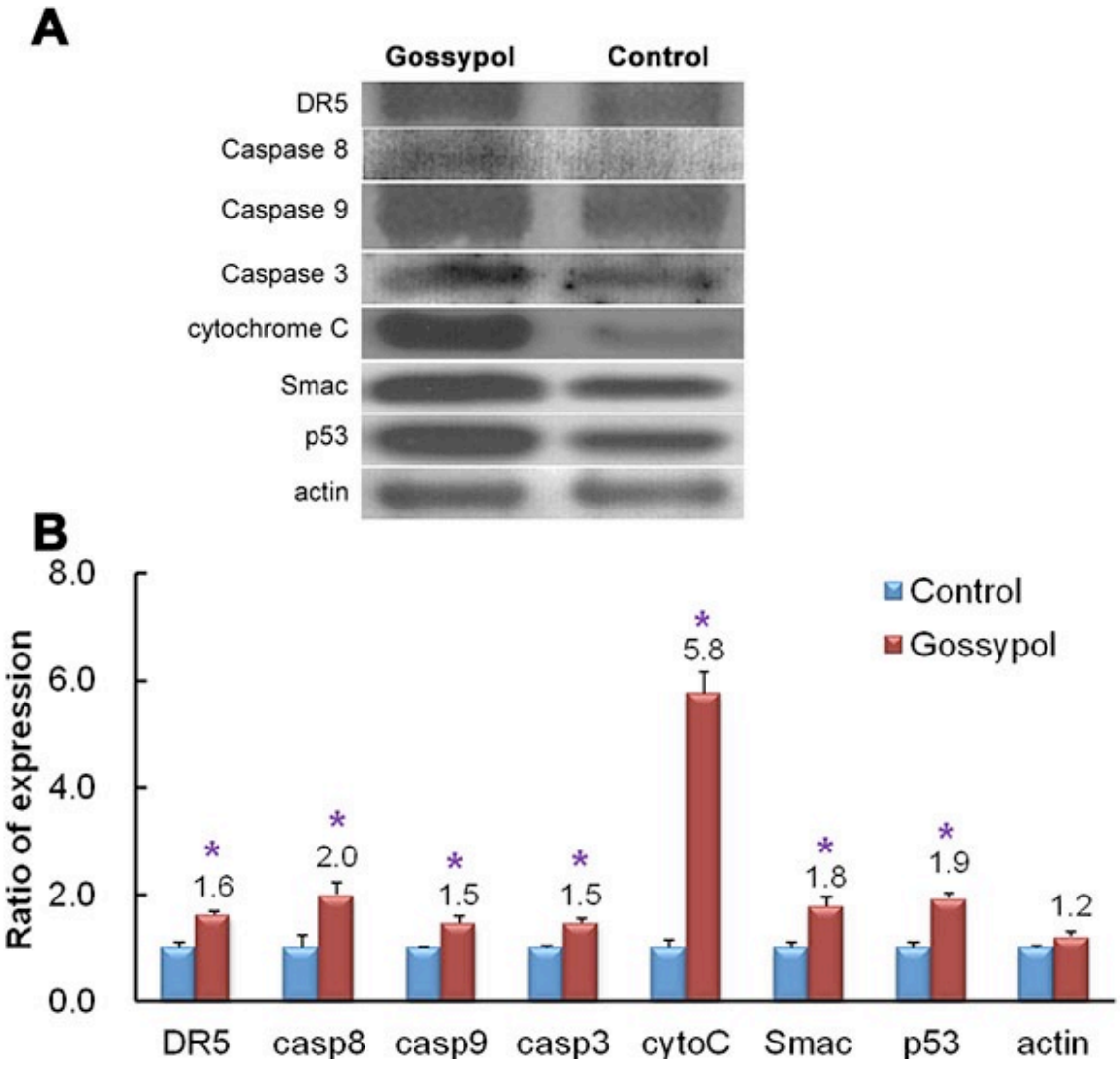


Figure 17. Protein expressions of extracts from control and 20 μM gossypol-treated human retinoblastoma (Y79) cell cultures were detected with western blotting. Panel A shows the target signal transduction proteins from the control and gossypol-treated groups. Panel B shows the ratio of increased expression in these proteins. The results indicate that the expressions of cytochrome C, caspase 8, caspase 9, caspase 3, DR5, p53, and Smac were upregulated by gossypol treatment. The Student t-test was used for

statistical analysis. * $p<0.05$, compared to the control group, and n=3 in each group in (B).

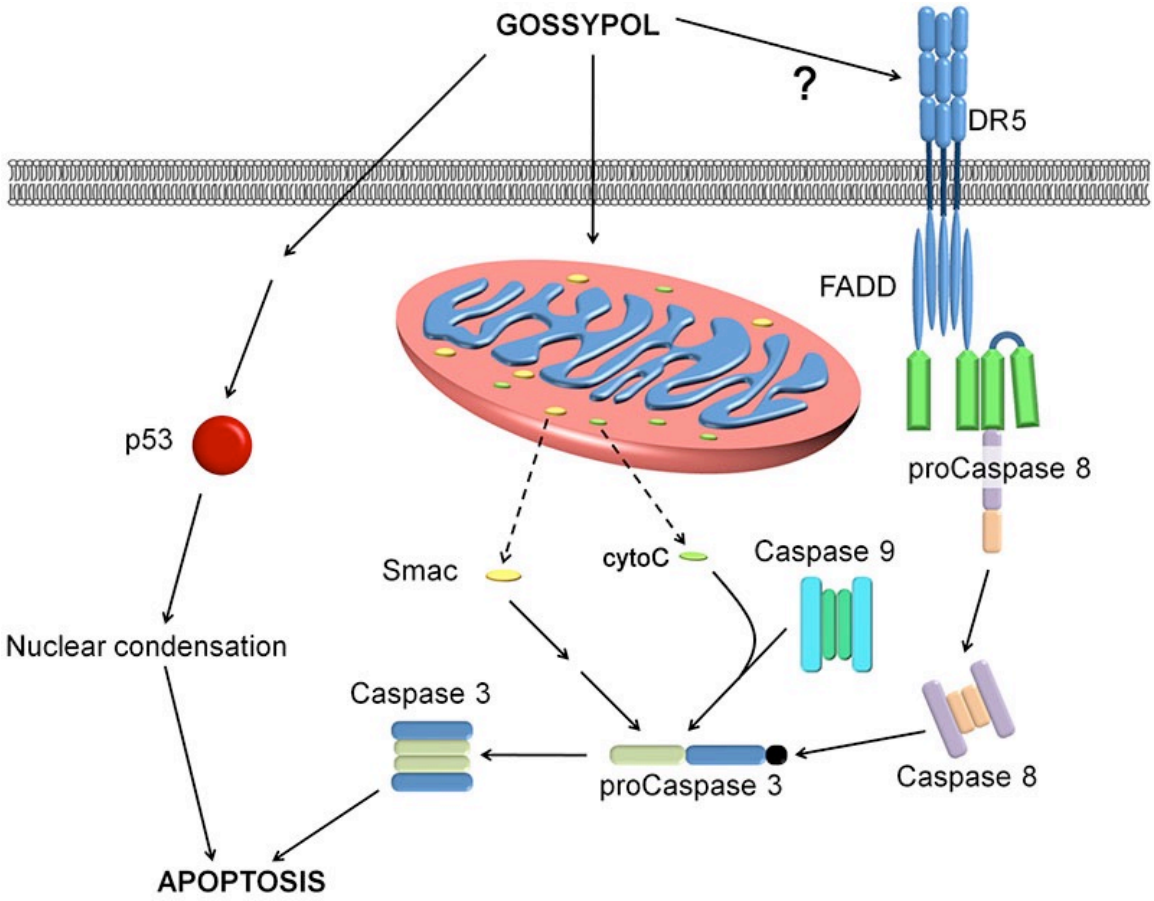


Figure 18. Possible mechanisms of gossypol-induced cell apoptosis in human retinoblastoma (Y79) cells. Gossypol may use several pathways leading to the death of Y79 cells, including activating death receptors (DR5), caspase 8, and caspase 3; upregulating mitochondria permeability, thereby causing the release of cytochrome C; and activating the Smac pathway. In addition, p53 was found upregulated after gossypol treatment, suggesting that DNA degradation is another effect of gossypol. Solid lines with arrows in the figure represent simulative effects, and the dotted lines represent translocation.

公開號	201244723 公告 I407964 審查公開資訊
專利名稱	干擾性核糖核酸用於治療或減緩疼痛之用途 A USE OF A RNA INTERFERENCE FOR TREATING OR REDUCING PAIN
公開日	2012/11/16
申請日	2011/05/10
申請號	100116272 Espacenet
國際分類號 IPC	***A61K-031/7088***(2006.01);***A61P-029/00***(2006.01)
公報卷期	10-22
發明人	蔡明達 TSAI, MING DAR; 李憶菁 LEE, YIH JING
申請人	私立輔仁大學 FU JEN CATHOLIC UNIVERSITY 新北市新莊區中正路510號　輔仁大學 TW; 新光醫療財團法人新光吳火獅紀念醫院 SHIN KONG WU HO SU MEMORIAL HOSPITAL 臺北市士林區文昌路95號之新光吳火獅紀念醫院 TW
代理人	陳昭誠
摘要	干擾性核糖核酸用於治療或減緩疼痛之用途，該干擾性核糖核酸具有序列辨識編號SEQ ID NO:1、SEQ ID NO:2、SEQ ID NO:3或SEQ ID NO:4之核酸序列，能夠有效抑制緩激肽B2受體表現，可用於治療或減緩神經病變性疼痛，或應用於製作減緩神經病變性疼痛之藥物。

Figure 19. The ROC patent of bradykinin B2 receptor siRNA for treating or reducing pain.

The United States of America

The Director of the United States Patent and Trademark Office

Has received an application for a patent for a new and useful invention. The title and description of the invention are enclosed. The requirements of law have been complied with, and it has been determined that a patent on the invention shall be granted under the law.

Therefore, this

United States Patent

Grants to the person(s) having title to this patent the right to exclude others from making, using, offering for sale, or selling the invention throughout the United States of America or importing the invention into the United States of America, and if the invention is a process, of the right to exclude others from using, offering for sale or selling throughout the United States of America, or importing into the United States of America, products made by that process, for the term set forth in 35 U.S.C. 154(a)(2) or (c)(1), subject to the payment of maintenance fees as provided by 35 U.S.C. 41(b). See the Maintenance Fee Notice on the inside of the cover.

Acting Director of the United States Patent and Trademark Office

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If the application for this patent was filed on or after December 12, 1980, maintenance fees are due three years and six months, seven years and six months, and eleven years and six months after the date of this grant, or within a grace period of six months thereafter upon payment of a surcharge as provided by law. The amount, number and timing of the maintenance fees required may be changed by law or regulation. Unless payment of the applicable maintenance fee is received in the United States Patent and Trademark Office on or before the date the fee is due or within a grace period of six months thereafter, the patent will expire as of the end of such grace period.

PATENT TERM NOTICE

If the application for this patent was filed on or after June 8, 1995, the term of this patent begins on the date on which this patent issues and ends twenty years from the filing date of the application or, if the application contains a specific reference to an earlier filed application or applications under 35 U.S.C. 120, 121, or 365(c), twenty years from the filing date of the earliest such application ("the twenty-year term"), subject to the payment of maintenance fees as provided by 35 U.S.C. 41(b), and any extension as provided by 35 U.S.C. 154(b) or 156 or any disclaimer under 35 U.S.C. 253.

If this application was filed prior to June 8, 1995, the term of this patent begins on the date on which this patent issues and ends on the later of seventeen years from the date of the grant of this patent or the twenty-year term set forth above for patents resulting from applications filed on or after June 8, 1995, subject to the payment of maintenance fees as provided by 35 U.S.C. 41(b) and any extension as provided by 35 U.S.C. 156 or any disclaimer under 35 U.S.C. 253.

US008445456B2

(12) **United States Patent**
Tsai et al.

(10) **Patent No.: US 8,445,456 B2**
(45) **Date of Patent: May 21, 2013**

(54) **USE OF RNA INTERFERENCE FOR TREATING OR REDUCING PAIN**

(75) Inventors: **Ming-Dar Tsai**, Taipei (TW); **Yih-Jing Lee**, New Taipei (TW)

(73) Assignees: **Fu-Jen Catholic University**, New Taipei (TW); **Shin Kong Wu Ho Su Memorial Hospital**, Taipei (TW)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **13/182,707**

(22) Filed: **Jul. 14, 2011**

(65) **Prior Publication Data**

US 2012/0289578 A1 Nov. 15, 2012

(51) **Int. Cl.**
A61K 31/70 (2006.01)
C07H 21/04 (2006.01)

(52) **U.S. Cl.**
USPC ..... **514/44 A**; 536/24.5

(58) **Field of Classification Search**
None
See application file for complete search history.

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Fan et al., "RNA interference of bradykinin receptor reduces nociception on neuropathic pain models", Published Nov. 27-28, 2010.

* cited by examiner

Primary Examiner — Tracy Vivlemore
(74) *Attorney, Agent, or Firm* — Edwards Wildman Palmer LLP; Steven M. Jensen; Richard B. Emmons

(57) **ABSTRACT**

A use of a nucleic acid molecule mediating RNA interference for treating or reducing pain is disclosed. The nucleic acid molecule has a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4, and is used for effectively inhibiting expression of bradykinin B2 receptor, treating or reducing pain and preparing a pharmaceutical composition for reducing neuropathic pain.

6 Claims, 1 Drawing Sheet

Figure 20. The USA patent of bradykinin B2 receptor siRNA for treating or reducing pain.

博士班修業期間所發表之相關論文

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2. **Tsai MD and Lee YJ (2013, May). Use of RNA interference for treating or reducing pain. (USA patent: US 8,445,456 B2)**

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