計畫編號：DOH97-TD-G-111-018

# 行政院衛生署九十七年度科技研究計畫

# 以基因剔除小鼠爲基礎之環境荷爾蒙於肺腺癌致癌效應之研究

## 研究報告

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＊本研究報告僅供參考，不代表本署意見，依合約之規定：如對媒體發布研究成果應事先徵求本署同意＊

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**摘要：**

肺癌是台灣癌症死亡率第一位，也是全世界癌症發生率最高的疾病，因此肺癌的成因、預防、與治療是全人類的重要課題。許多環境污染物質如硝基多環芳香族化合物會造成基因突變而進行肺癌發生的起始階段，此類化合物與肺癌的致癌機制較爲了解。另有一些化學物質如多氯聯苯、戴奧辛等，被發現會干擾性腺激素的正常功能，同時也會引起肺腺癌的產生。其次，過去流行病學的研究發現肺癌在兩性之間有許多差異；男性肺癌的罹患率高於女性，但女性罹患肺腺癌的比例卻高於男性。女性罹患肺腺癌與廚房烹煮的油煙有關；且女性肺腺癌對於治療的療效較佳。但是導致這些兩性差異的確實原因卻仍有許多未知之處。進一步了解荷爾蒙受器與肺腺癌之關係對於肺腺癌的預防與治療具有重要的意義，本研究首先建立一種研究雄性荷爾蒙受器與肺腺癌癌化過程的之小鼠平台，此平台的建立對於對於了解肺腺癌的病因、預防、與治療有重要的幫助。以免疫組織化學染色研究證實雄性素受器在人類的肺腺癌有大量表現。細胞株的研究也顯示 CL1-0, CL1-5, A549, H1299 等細胞株內有雄性素受體的表現。以慢病毒爲載體抑制細胞內雄性素受體之表現時細胞之生長會受到明顯的抑制。細胞形成族群的能力會明顯下降。細胞以流式細胞儀分析則發現其較多停止於 G2/M 時期。本研究首次證實雄性素受器會調節肺腺癌細胞之生長與癌化。此一研究可進一步建議合併抑制雄性素受器可作爲肺腺癌未來治療的研究方向。

關鍵字：基因剔除鼠、慢病毒、雄性素受器

Abstract

Environmental pollutants including cooking oil fumes, cigarette smoke, and automobile emissions are the major causes of lung cancer. Some of chemical carcinogens (environmental hormones) in lung cancer such as dioxins can affect the endocrine system especially the sexual development. It has been suggested that other factors such as sex steroids may act as cocarcinogens, especially in lung adenocarcinoma, the most common histologic type among women. It was proposed that carcinogenesis induced by environmental hormones and the gender differences in lung adenocarcinoma were based on different genetic background induced by sex hormones and their receptors. Although research has clearly shown that environmental hormones can act at multiple sites via multiple mechanisms, receptor-mediated mechanisms have received the most attention. Therefore, it is very important to investigate the effects of sex steroid hormone receptors during carcinogenesis in lung adenocarcinoma induced by chemical carcinogens. In human tissue array study, we have demonstrated that AR can be detected in nuclei of lung adenocarcinoma cells. AR was expressed in lung adenocarcinoma cell line CL1-0, CL1-5, A549, and H1299 by western blotting and RT Q-PCR. The cell proliferation in CL1-5 cells was suppressed and G2/M arrest was induced by lentivirus-mediated knockdown of AR expression. We have obtained conditional androgen receptor knockout mice and crossed back with A/J mice from Jackson lab to establish NNK-induced lung adenocarcinoma mouse model. Our results at the first time demonstrate AR regulates cell growth and carcinogenesis in lung adenocarcinoma and further suggest the therapeutic potential of AR pathways in lung adenocarcinoma.

Keywords: conditional knockout, lentivirus, androgen receptor

本文：

## Introduction

Lung cancer is the leading cause of cancer death worldwide including Taiwan and adenocarcinoma is the most common histological subtype. The pathogenesis of lung adenocarcinoma is still unclear. The development of lung adenocarcinoma is usually linked with tobacco in the smokers and associated with cook hum in the nonsmokers.

Environmental hormones (endocrine disruptors, endocrine-disrupting compounds, EDCs) are exogenous agents that change endocrine function and cause adverse effects at the level of the organism, its progeny, and/or subpopulations of organisms. An increased risk for all cancers combined is seen in the cohort studies of dioxins. An increased risk for lung cancer is also present in the most informative cohort studies, especially in the more highly exposed sub-cohorts. The relative risk for lung cancer in the combined highly exposed sub-cohorts was estimated to be 1.4. It is possible that lung cancer relative risks of this order could result from confounding by smoking, but only if there were a pronounced difference in smoking habits between the exposed population and the referent populations, a difference which seems unlikely. It therefore seems unlikely that confounding by smoking can explain all the excess lung cancer risk, although it could explain part of it.

Lung adenocarcinoma is associated with strong gender-difference in its prevalence, prognosis, and therapeutic response to targeted therapy. The molecular mechanism of gender difference in lung adenocarcinoma is also poorly understood. Estrogen receptors, both ERα and ERβ, have been identified in both normal lung tissue and lung tumors. Interactions between ER and EGFR pathways have been identified. A study demonstrates enhanced anti-proliferative effects with combined targeting of the estrogen receptor and EGFR using fulvestrant and gefitinib in NSCLC. However, the expression and molecular role of androgen receptor in human lung cancer is still poorly understood.

## Materials and Methods

Animals

Homozygous flox-ar/ar female mice were a gift from Chawnshang Chang in University of Rochester. Pathogen-free A/J, Mx-1-cre, Actb-cre mice were purchased from Jackson Lab. Athymic NCr-nu/nu male homozygous nude mice, 6 to 9 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA). All experiments were in compliance with the NIH Guide for the Care and Use of Laboratory Animals and according to the institutional guideline of Taipei Medical University. Homozygous flox-ar female was mated with a male A/J mouse for they F1 offspring. The heterozygous flox-ar female F1 was crossed back with male A/J mice for F2 offspring. The heterozygous flox-ar female F2 was crossed back with male A/J mice again for F3 offspring. After 7 generation, the heterozygous flox-ar F7 female offspring was mated with Actb-cre or Mx-1-cre male mice. The male offsprings contain both cre gene and recombinant ar allele was used as AR-deficiency mice for NNK-treatment. Male littermates contained the wild ar allele was the wild type AR mice for NNK-treatment.

NNK Treatment

NNK was purchased from ChemSyn Labs (San Diego, CA). Mice were injected intraperitoneally with three doses of NNK (100 mg/kg/d in 0.1 ml PBS) on three alternate days. Control animals received an equivalent volume of PBS. Mice will be sacrificed 9 weeks later.

Immunohistochemical Stain

Two sets of tissue array for human lung adenocarcinoma were purchased from Pantomics Inc. and Biomax Co. Two tissue array slides were incubated with polyclonal rabbit anti-human androgen receptor antibodies (C19, Santa Cruz, CA) for 1 hour. EnVision + system from Dako (San Francisco, CA) was used as second antibody. After washing, tissue array slides were developed with DAB. The mounted slides were scanned for analysis.

Cell Culture

The human lung adenocarcinoma cell lines CL1-0 and CL1-5were obtained from Academia Sinica and maintained in growth medium RPMI1640 and 10% fetal bovine serum (FBS, HyClone), at 37°C in 5% CO2. 293T cells were obtained from ATCC and were cultured in Iscove's modified DMEM with 10% FBS.

Western Blot Analysis

Western blot analysis was performed as described previously. Primary antibodies used were as follows: AR, PR (Santa Cruz Biotechnology Inc.), actin, tubulin (Sigma).

AR Knockdown and Overexpression

AR knockdown and overexpression were achieved by infecting lung adenocarcinoma cells with lentivirus expressing AR-specific short hairpin RNA (shRNA) and mouse AR cDNA, respectively. Lentiviral vector production and transduction were performed according to the method modified from the previous described. In lentiviral vector production, 293T cells were plated on a 10-cm dish in 10 mL of medium and, on the following day at 70% confluence, were transfected with 2 μg of VSV-G packaging plasmid, 5 μg of pΔVpR packaging plasmid, and 5 μg of pLKO vector by calcium phosphate precipitation. The produced virus was collected 48 hours later. Viral supernatants were filtered through 0.45-μm pore size filters and stored at -80°C until used for transduction. In lentiviral vector transduction, cells at six-well plates were transduced with 2 mL of the viral supernatants for 2 h in the presence of 8 μg/mL Polybrene. After 2 h, viral supernatants were removed, and 2 mL of medium was added to the cells. The cells were used for clone formation assay and growth assays 1 week after the transduction.

Statistical Analysis

Data are shown as mean ± SE. In the cell growth assays, differences between means of the control and the treated group were analyzed by Dunnett's test or unpaired Student's *t* test with Holm's correction for repeated tests. A value of $P \leq 0.05$ was considered significant.

## Results

Strong Immunohistochemical stain of androgen receptors in human lung adenocarcinoma

Androgen receptor is expressed in human testis, prostate, and liver; however, expression of AR in lung adenocarcinoma is unclear. We performed immunohistochemical stain of androgen receptor on lung cancer tissue array slides. Our results demonstrate strong DAB staining in lung adenocarcinoma

cells with both nuclear and cytoplasmic localization (Fig. 1). In some stromal cells, androgen receptor can be localized in their nuclei, too. These results clearly showed elevated AR expression in human lung adenocarcinoma cells in vivo. By western blot analysis and real-time Q-PCR, androgen receptor expression is abundant in lung adenocarcinoma cell lines CL1-0, CL1-5, and A549 cells.

Role of androgen receptor in NNK-induced lung cancer

In our study, the genetic background of cre-loxP conditional AR knockout mice was changed to A/J mice after seven generation of cross back. In order to understand the role of androgen receptor in NNK-induced lung cancer formation, offsprings from F7 cross with actb-cre mice were treated with NNK intraperitoneally and the formation of lung tumor will be defined as small red nodules on the lung pleura surface after 9 weeks. This result will available soon, because the seven back-cross generation spent more than 70 weeks.

AR knockdown and overexpression in lung adenocarcinoma cells

We have successfully knockdown and over-express androgen receptor in CL1-5 cells. The mRNA of AR was suppressed to less than 30% in scramble control. In western blot analysis, AR protein expression was markedly reduced (Fig. 2).

Reduced cell proliferation after knockdown androgen receptor in lung adenocarcinoma cells

Because AR expression is increased in CL1-5 cells, we asked whether cell growth is affected by or dependent on AR activity. We performed lentivirus-mediated knockdown of AR to clarify the effects of AR in cell growth. In cell growth assays, CL1-5 cells were significantly decreased in its clone formation capability (Fig. 3). We confirmed that reduced AR level achieved by knockdown significantly decreased the growth rate of CL1-5 cells (Fig. 4). In flow cytometry analysis, cell proportion in G2/M increased from 12.74% to 17.95% (Fig. 5), it imply a G2/M arrest in CL1-5 cells with knockdown in AR expression.

## Discussion

Lung adenocarcinoma is a malignant disorders associated with chemical carcinogens including tobacco and environmental hormones. The incidence and mortality of lung cancer is approximately twice in men than women. Female patients have a better prognosis and therapeutic response than male. Female patients in Asian population have higher incidence of EGFR mutation, and EGFR mutation was proposed to be one of the molecular mechanism for their better therapeutic response for targeted therapy to EGFR pathway. EGFR is a member of ERBB receptor family. Activation of EGFR stimulates cell proliferation, anti-apoptosis, angiogenesis, invasion and metastasis. Several EGFR inhibitors, small molecular tyrosine kinase inhibitors (TKI), have been developed in recent years. The clinical response of NSCLC to EGFR-TKI, such as gefitinib and erlotinib, are dramatic among Asian female non-smokers of adenocarcinoma cell type. Studies have identified mutation of the EGFR catalytic domain that predicts the response to TKIs of NSCLC. In East Asia, around 30-40% of NSCLC patients have EGFR mutations and have good responses to EGFR-TKIs. Estrogen receptors, both ERα and ERβ, have been identified in both normal lung tissue and lung tumors. Interactions between ER and EGFR pathways have been identified. A study demonstrates enhanced anti-proliferative effects with combined targeting of the estrogen receptor and EGFR using fulvestrant and gefitinib in NSCLC.

In our current results, we demonstrated AR overexpression in human lung adenocarcinoma cells both in vitro and in vivo. We found AR knockdown suppressing cell proliferation in human lung adenocarcinoma CL1-5 cells. CL1-5 cells have low clone formation rate and showed increased cell proportion in G2/M arrest. In RT Q-PCR analysis, different NSCLC cells (CL1-0, CL1-5, A549, and H1299) have different levels of AR mRNA. This is the first report about the AR's role in lung adenocarcinoma. The molecular role of AR can partially explain the reasons of gender diversity in human lung adenocarcinoma and also the different effects of environmental hormones on lung carcinogenesis.

## Conclusions and suggestions

Lung cancer is the leading cause of cancer mortality in Taiwan. This is the first study to demonstrate that AR regulates the chemical carcinogenesis and cell proliferation in lung adenocarcinoma. We suggest to further study the effects of AR in invasion and metastasis of lung adenocarcinoma.

Combination therapy targeting to EGFR, receptor tyrosine kinases, and AR pathways may have the potential to improve the therapy in lung adenocarcinoma.

97 年度計畫重要研究成果及對本署之具體建議

1.本計畫之新發現或新發明：雄性素受器會促進肺腺癌細胞的生長與癌化。

2.本計畫對民眾具教育宣導之成果：環境荷爾蒙或外生性之荷爾蒙有可能與某些化學致癌物產生協同作用使癌細胞生長不受控制、預防癌症需要避免這些可能有害物質。

3.本計畫對醫藥衛生政策之具體建議：建議進一步評估合併表皮生長因子受器阻斷與雄性素受器合併阻斷對於肺腺癌治療的療效。

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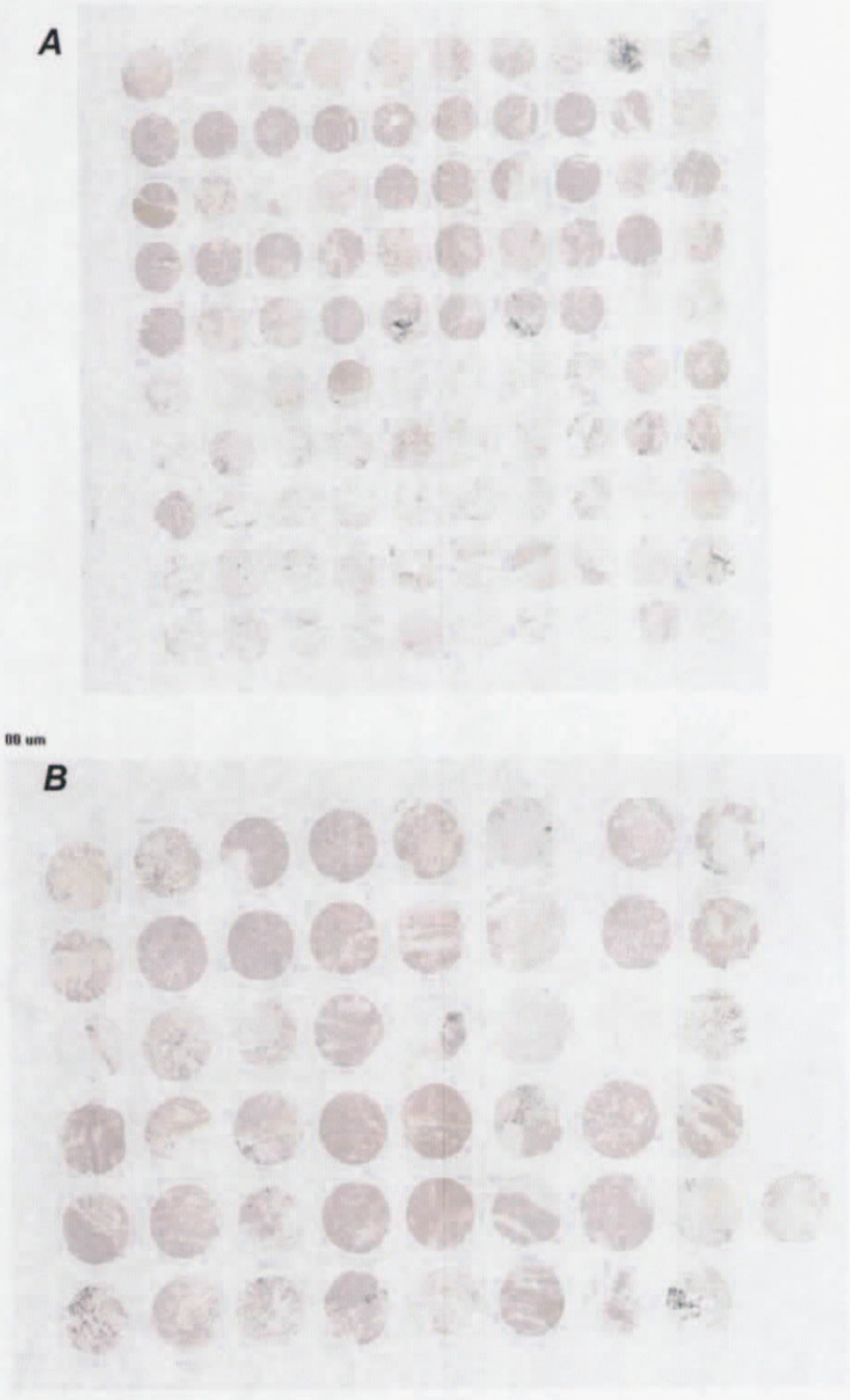



Figure 1. Positive immunohistochemical stain of AR (brownish color) was detected in nuclei of lung cancer cells. Slides of human lung cancer with adjacent paired tissue array were obtained from Pantomics Inc.(A) and Biomax Co.(B). Immunohistochemical staining was performed according to supplier's protocol. Slides were developed in DAB-$H_2O_2$.

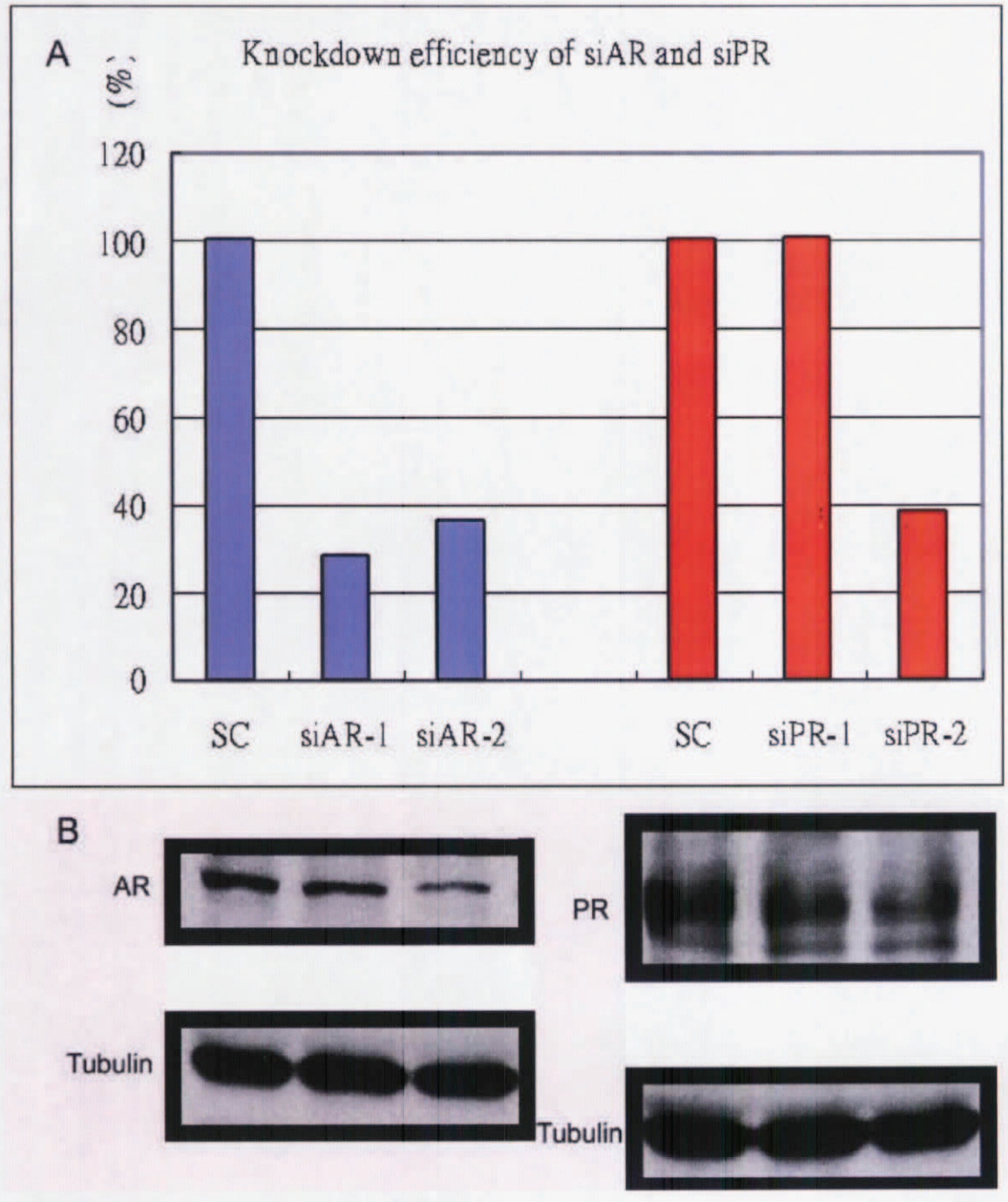



Figure 2. Knockdown androgen receptor and progesterone receptor by lentivirus-mediated RNA interference. (A) RT Q-PCR of AR expression in CL1-5 cells. GAPDH was used as loading control. (B) Western blot analysis in CL1-5 cells. Tubulin was used as loading control.

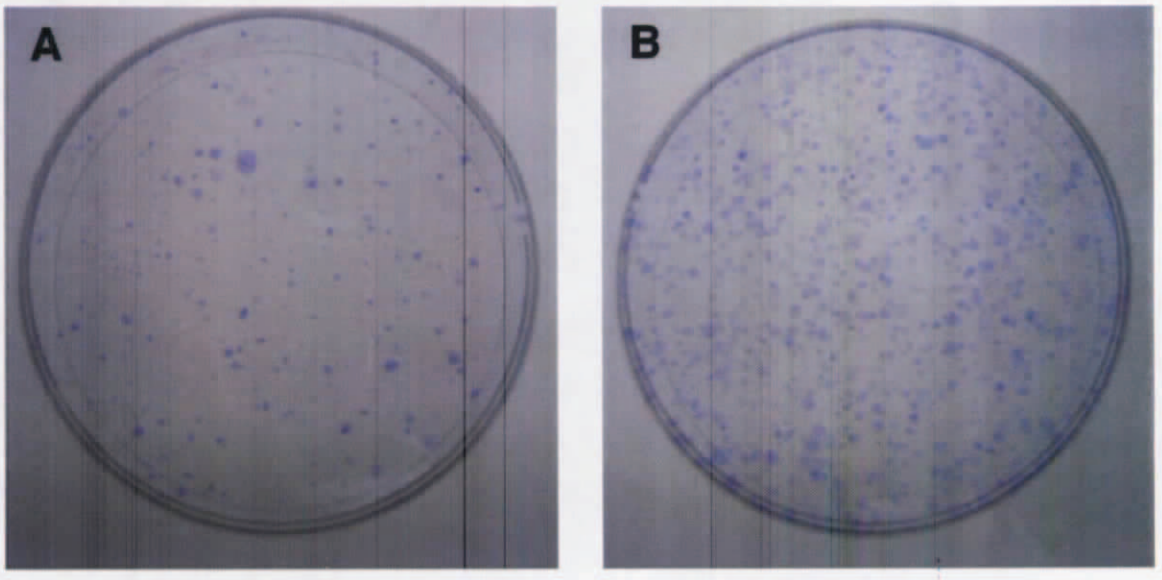



Figure 3. Decreased cloning formation after knockdown androgen receptor in CL1-5 lung adenocarcinoma cells.

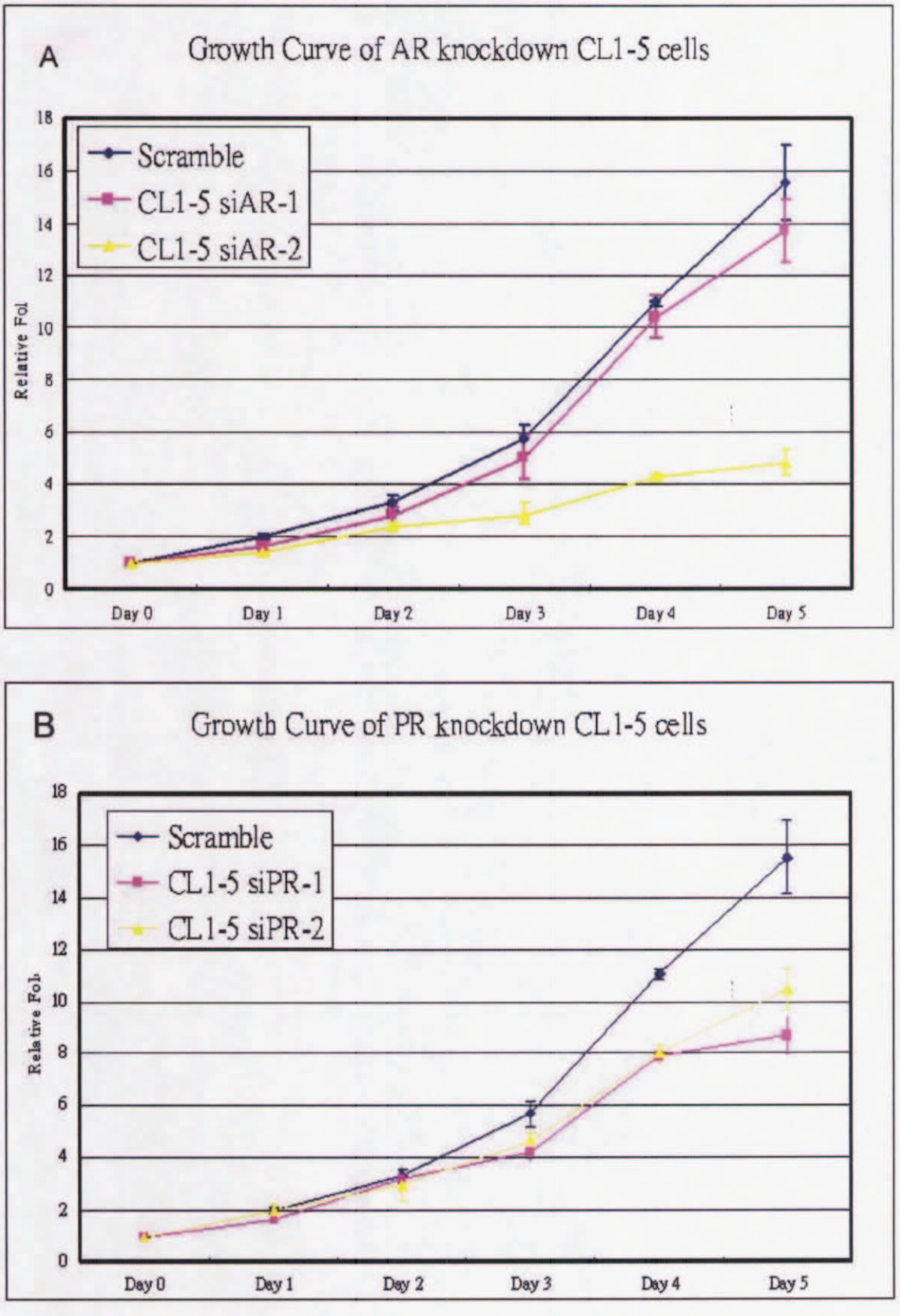
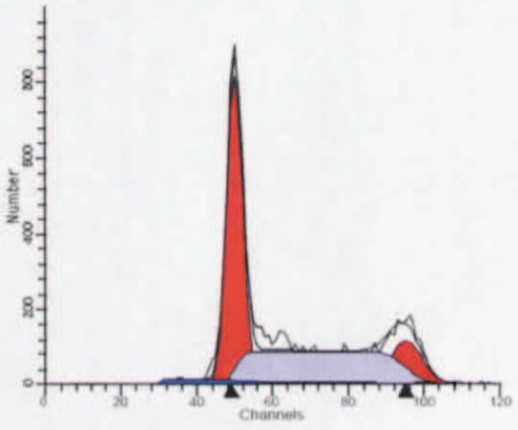
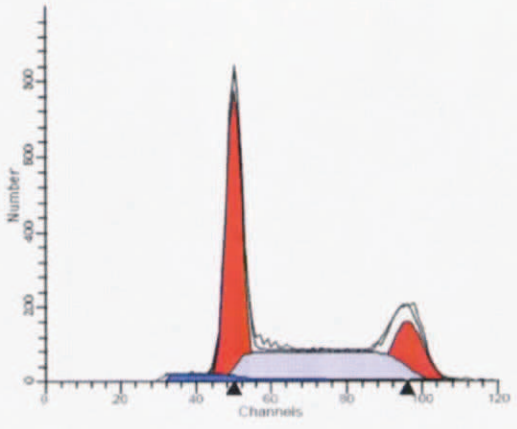


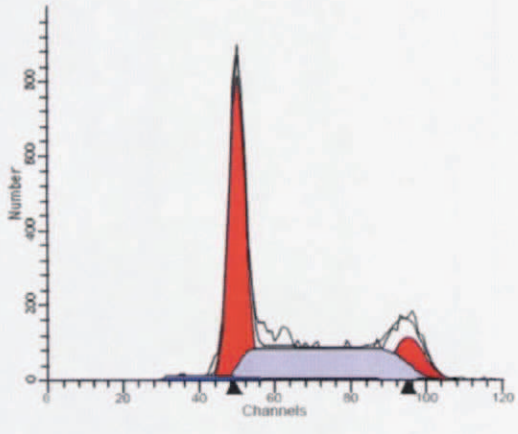

Figure 4. Knockdown androgen receptor or progesterone receptor inhibits cell proliferation in CL1-5 cells.



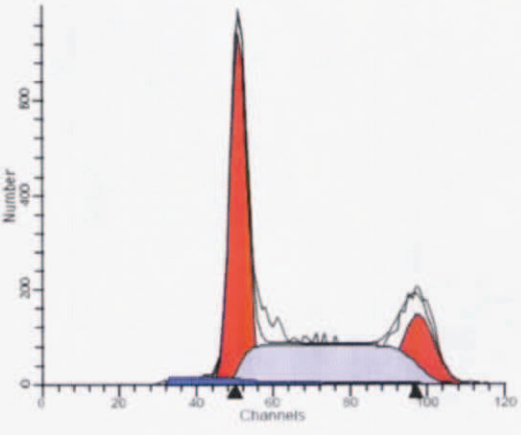

**CL1-5 Scramble**
Dip G0-G1: 45.44 % at 49.99
Dip **G2-M: 12.74 %** at 95.26
Dip S: 41.83 % G2/G1: 1.91
Dip %CV: 4.09
Total S-Phase: 41.83 %




**CL1-5 siAR-2**
Dip G0-G1: 43.35 % at 50.08
Dip **G2-M: 17.95 %** at 96.02
Dip S: 38.70 % G2/G1: 1.92
Dip %CV: 4.05
Total S-Phase: 38.70 %




**CL1-5 Scramble**
Dip G0-G1: 45.44 % at 49.99
Dip **G2-M: 12.74 %** at 95.26
Dip S: 41.83 % G2/G1: 1.91
Dip %CV: 4.09
Total S-Phase: 41.83 %




**CL1-5 siPR-2**
Dip G0-G1: 42.37 % at 51.05
Dip **G2-M: 16.45 %** at 97.56
Dip S: 41.18 % G2/G1: 1.91
Dip %CV: 4.08
Total S-Phase: 41.18 %

Figure 5. Cell cycle profile of CL1-5 cells after AR or PR knockdown.