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## 由動物實驗模式探討雄性素受體的輔助因子在雄性素不依賴性生長之機轉

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Title page.

# Gene expression changes of Androgen Receptor Coactivators after hormone deprivation in LNCaP Cell Line by a multi-probe non-isotope ribonuclease protection assay

Hong-Chiang Chang, Ju-Ton Hsieh, Shyh-Chyan Chen, Jun Chen

From Department of Urology, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China

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Correspondence to: Hong-Chiang Chang,
Address: Department of Urology, College of Medicine, National Taiwan University, No.7, Chung-Shan South road, Taipei, Taiwan.
E-mail: bird@ntumc.org

Running title: multi-probe RPA for AR coactivators

**KEY WORDS:**

androgen receptor, coactivators, prostate cancer, cell culture, ribonuclease protection assay.

**Abbreviations:**

AR, androgen receptor; FBS, fetal bovine serum; ARA, AR associated coactivator; SRC-1, steroid receptor coactivator-1; Rb, retinoblastoma protein; HF, hydroxyflutamide; ER, estrogen receptor; $E_2$, 17β-estradiol; CAT, chloramphenicol acetyltransferase; Adiol, androst-5-ene-3β;

## Abstract

**Objectives.** This study investigated the expression patterns of several AR coactivators after androgen deprivation in LNCaP cell line. There are several evidences have been shown that during androgen deprivation, the androgen receptor (AR) cofactors can enhance the transcription activity of AR to activate the AR target gene. So we want to know the expression patterns of AR cofactors in androgen deprivation condition. If the expression of AR cofactors in the hormone independent status were increased, that would support the AR cofactors to be the trigger protein.

**Methods** An androgen dependent prostate cancer cell line (LNCaP) was cultured in RPMI medium with charcoal/dextran treated FBS for 28 days. Samples of total RNA collected in one-week interval were analyzed by using a non-isotope multi-probes ribonuclease protection assay system with the hAR multi-probe template set. We simultaneously analyzed the expression changes of 10 interested mRNA (AR, Rb, ara160, ara24, ara54, ara55, ara70, BRCA1, F-SRC-1 and RAC3) of AR and AR associated cofactors in one hybridization reaction.

**Results:** From the preliminary data, we found the LNCaP cell in charcoal-treated serum for 28 days clearly show more than 1.5 folds increase in AR, ara160, and ara70 expression. The expression of ara24 and ara54 show increase but the increase below 1.5 folds. Only the RAC3 and F-SRC-1decreased in RNA expression.

**Conclusions:** This preliminary results support the previous reports that AR cofactors may be the trigger key factor between androgen dependent and independent.

## INTRODUCTION

Androgen ablation has been the cornerstone of treatment for advanced prostate cancer, but the effect is often short-lived, as hormone-refractory elements continue to proliferate. There are several theories to explain why prostate cancer can transform from hormone dependent to independent status. It include, 1)AR mutations, 2)post-receptor alterations, 3)alterations in down-stream AR target genes, *et al.* But within them, the androgen receptor cofactor is an important finding since 1996. Several evidences have been shown that during androgen ablation or androgen blockage, the AR cofactors can enhance the ability of AR to activate the AR target gene. By this phenomenon, the prostate cancer cell can keep growing in androgen deprivation status[1-3].

Several cofactors were found since 1996, e.g. ARA70[4], ARA55[1], and ARA54[5] for the C-terminal AR-ligand binding domain(AR-LBD), ARA160[6] and ARA24[7] for N-terminal. Some other cofactors have also been identified to associate with the AR DNA binding domain or AR N-terminal domain, such as RB, SRC1, TFIIH, etc. These cofactors can enhance DHT-mediated AR transcriptional activity and these interactions between AR and ARA70, ARA55, or ARA54 are androgen-dependent.

Katzenellenbogen *et al.* proposed a new tripartite system (ligand-receptor-cofactor) to explain the molecular interaction of steroid receptors that may define the potency and biological character of steroid hormones[8].

Miyamoto *et al.* reported that antiandrogens (hydroxyflutamide, casodex) can activate androgen target genes in the presence of ARA70 or ARA55 in DU145 cell line [2]. AR coactivators may be the trigger key factor between androgen dependent and independent. However depending on the cell and tissue context, hydroxyflutamide can also behave as a partial agonist suggesting that its mechanism of action is complex and tissue specific. Furthermore, it is known that prostate cancer cells may develop resistance to

hydroxyflutamide or may even cycle between phases of resistance and responsiveness. This varied response and abundant other evidence, suggests that hydroxyflutamide resistance is due to a cellular adaption phenomenon rather than to permanent genetic alterations.

Yeh *et al.* reported that $E_2$-AR-ARA70 also plays an essential role for the AR function. ARA70 can induce AR transcriptional activity in the presence of $E_2$ 10nM. ARA70 is an essential factor to modulate this pathway, without ARA70, this pathway will be closed[9]. Androstenediol(Adiol), a natural estrogen, can activate AR target genes in the presence of AR, and that ARA70 can further enhance this Adiol-induced AR transcriptional activity. Hydroxyflutamide and casodex fail to block Adiol-mediated induction of AR transcriptional activity in prostate cancer cells[10]. This may raise critical questions about the possible role of Adiol and ARA70 in overcoming the effects of androgen ablation therapy for prostate cancer[10-12].

Together, it is suggested that some selective AR coactivators can modulate the specificity of sex hormones and antiandrogens. The post receptor alterations have been suggested that relative expression of coactivators can modulate androgen and antiandrogen's regulation for AR transcriptional activity. The agonist/antagonist receptor interactions alone may not be able to explain the AR function, and the interaction between ligand-receptor complex and cofactors could be essential for steroid hormone function and sensitivity.

The specific changes in the AR coactivator gene expression profile of the cells could explain the molecular mechanism that prostate cancer cell become to hormone independent. In summary, AR coactivator could be the key protein to support the prostate cancer cell growth in androgen independent mode. In our hypothesis, the expression of AR coactivators, e.g. ARA70 or ARA 55, may increase in prostate cancer cell after long-term androgen deprivation, so that AR function will be enhanced and AR antagonist will be converted to agonist.

In order to mimic hormone refractory prostate cancer cell growth, an androgen dependent

prostate cancer cell line (LNCaP) was cultured in hormone deprivated status. Serial mRNAs were collected to analyze AR cofactors expression. For the purpose of simultaneous analysis with multiple targets, we used a multiple RPA template that includes ten of the AR cofactors and apply the non-isotope multi-probe RNAse protection assay to investigate the effect of androgen deprivation on mRNA expression of AR cofactors.

This study is the first multi-probe ribonuclease protection assay (RPA) reported to measure expression of AR and AR associated cofactors mRNA influenced by androgen independent growth. This is also the first report of non-isotope labeling in multi-probe RPA. From this study, it may help us to compare the expression change of several AR cofactors proteins in hormone deprivated condition. This would provide the evidence whether the increase expression of AR cofactor is compatible with transition from androgen dependence to androgen independence. This data can further clarify our preliminary molecular biology finding. Furthermore, these finding can help us to forecast the responsiveness of androgen-deprivation therapy in prostate cancer.

## Materials and Methods

### Materials

RPMI1640 medium, penicillin/streptomycin, FBS and trypsin/EDTA were purchased from Life Technologies, Inc. (Grand Island, NY). Charcoal/dextran-treated certified FBS (Lot No. AKD11642, C-FBS) was from Hyclone (Logan, UT), with a certificate that the concentration of testosterone was below 3.0 ng/dl.

### Cell culture and RNA isolation

The prostate cancer cell line, LNCaP, was purchased from the American Type Culture Collection (Rockville, MD). They were grown in phenol red-positive RPMI 1640 medium supplemented with 10% FBS, 1% glutamine and penicillin/streptomycin at 37℃ in an atmosphere of 5% $CO_2$ in air. We developed a subline of LNCaP cells grown in phenol red-free RPMI1640 medium supplemented with 10% charcoal/Dextran treated FBS (C-FBS). Cell were fed twice per week and split once per week with trypsinization. We developed this subline of LNCaP cell in the condition mimics the androgen deprivated growth, and RNA of this LNCaP subline were collected in serial time course: 7th, 14th, 21st, 28th days.

Total RNA was extracted from the prostate cell line using TRIzol (Life technologies) according to the manufacturer's specifications. Total RNA was extracted in chloroform, ethanol precipitated and stored at -80 ℃. Total 5 RNA samples were collected for time course analysis in week interval, one before and 4 after androgen deprivation.

### Non-isotope Multi-probe ribonuclease protection assay (RPA)

For the purpose of non-isotope condition, our multi-probe RPA was succeeded by combining the hAR Multiple-Probe Template set (BD Pharmingen), MAXIscript T7™ in vitro transcription Kit (Ambion), Biotin-14-CTP (GIBCOBRL®) for nucleotide labeling, RPAIII kit (Ambion), and BrightStar™ BioDect™ Kit (Ambion). This novel non-radioisotope

method can simultaneously and rapidly measure mRNA change of 10 AR associated coactivaors.

The hAR Human Androgen Receptor Multi-Probe template set was purchased from BD PharMingen Co., containing DNA templates which can be used for the T7 RNA polymerase-directed synthesis of biotin-labeled, anti-sense RNA probe that can hybridize with target human mRNAs encoding androgen receptor[13], RB[14], ara160[6], ara24[7], ara54[5], ara55[1], ara70[4], BRCA1[15], F-SRC-1[16], RAC3[17] as well as the two housekeeping gene products, L32 and GAPDH. The multiple hAR RNA probes was synthesis using the MAXIscript T7™ in vitro transcription Kit (Ambion). 0.4µl Biotin-14-CTP (10 mM, GIBCOBRL®) (in the ratio of 40% biotin-CTP: 60%CTP) was used as label nucleotide in each 20 µl reaction in vitro transcription. NucAway™ Spin Columns (Ambion, Inc) was used for the removal of unincorporated nucleotides and salts after probe synthesis reactions.

Multi-probe RNase protection assay was performed by using RPAIII kit (Ambion, Inc). For the purpose of non-isotope design, biotin-labeled anti-sense RNA probe set was used as manufacturer's protocol. The probe set is hybridized in excess to target RNA in solution, after which free probe and other single-stranded RNA are digested with RNase. The remaining RNase-protected probes are purified and analyzed by resolving on denaturing polyacrylamide gels.

Briefly, after denature in 90℃~95℃ for 2~3 minutes, dried total RNA (10 µg) with 1 µl biotin-labeled multi-probe sets was hybridized (56℃; 12 h) in 9µl RPAIII hybridization buffer, Reaction mixtures were then treated with 100µl (500x diluted) RNase A/RNase T1 Mix (Ambion) and incubated 45 minutes at 30℃. Digestion and precipitation were easily performed by adding 150µl RPAIII Inactivation/Precipitation III solution (Ambion). RNA was precipitated from each reaction and pellets were washed with ethanol, air dried, resuspended in Gel loading Buffer II (Ambion) , heated (95℃, 3 min), and resolved on a denaturing 5%

polyacrylamide gel at 250V for 3.5 h. The RNase-protected probes on the polyacrylamide gel was then electroblotted onto a positively charged nylon membrane. The biotinylated RNA probe on the nylon membrane was then detected by BrightStar™ BioDect™ Kit (Ambion, Inc.) and the film was exposed by the light emission for one to two hours. Protected bands on the x-ray film were quantitated by densitometry. Relative level of protected probe were calculated by normalizing the specific band to the GAPDH band included in the multi-probe kits.

For each multi-probe RPA assay, negative controls included 5 μl of probe mixture incubated with 10 μg of yeast tRNA (Ambion). The probes-only control was treated with 1x RNase Digestion buffer in the absence of RNase A/RNase T1 Mix.

## Results

### Cell-growth properties of LNCaP cells in Charcoal-treated serum

We examined the in vitro growth properties of LNCaP cells under the steroid-reduced culture conditions. By continuous passage of LNCaP parental cells in the medium supplemented with 10% charcoal-treated FBS, we found the cell morphologic feature and growth rate changed gradually (Fig. 1A-C). The LNCaP cell is epithelial cancer cell and the doubling time is 2 days. After 2 weeks in charcoal-treated serum, the cell growth rate became slower and the size of cell body became smaller. At the 4th week, the morphology changes of LNCaP are so obvious. The attachment to cell culture dish is loosening and cell became neuron-like shape with elongated cytoplasmic process.

### Multi-probe RPA study

We collected 4 samples of RNA from LNCaP cell cultured in charcoal-treated FBS for 1, 2, 3 and 4 weeks and one sample of RNA from LNCaP in regular FBS. The chemiluminescent exposure on film (figure 2A) shows the lane of the hAR multi-probe not treated with RNases

(lane 1). Also shown are the corresponding RNase-protected probe following hybridization with RNA from LNCaP(10 μg, Lane 4-8) and BrightStar™ Biotinylated RNA century ™ size markers (1 μg, Lane 3). Each probe band (Lane 1) migrates slower than its protected band (Lane 4-8). For each multi-probe RPA assay, lane 2 is negative controls. The expressions of mRNA are indicated in relative light unit of CDP-Star™ on chemiluminescent exposure on film. The band of GAPDH was used to correct the loading RNA amount in each lane. The data were collected from three sets of independent experiments and show as mean ± S.D. As shown in figure 2B, we found that LNCaP cell cultured in charcoal-treated serum for 28 days would induce more than 1.5 folds expression in AR, ara160, and ara70. Another two mRNA, ara24 and ara54, also had increase pattern but the ratio less than 1.5 folds. Only the RAC3 and F-SRC-1decreased in RNA expression. The three bands of Rb, ara55 and BRCA1 are too low to be detected.

## Discussion

To date, prostate cancer has become the most frequently diagnosed neoplasm in the United States, and the 9th to 10th leading cause of cancer-related death in Taiwan. Androgen ablation has been the cornerstone of treatment for advanced prostate cancer, but the mechanisms responsible for androgen-independence remain uncharacterized. In recent ten years, the knowledge of AR associated protein has provided a new direction to study the mechanism of androgen independent prostate cancer cell growth. There are several evidences in the molecular biology to support the linkage between AR cofactors and androgen independent growth[2;3;9;18]. AR cofactors might be the critical proteins to enhance the effect of minimal androgen, turn on the androgen receptor by estrogen, or promote agonist activity of antiandrogens. Although the evidence is well demonstrated in transcriptional level, we need more data on cell biology to support our hypothesis. We investigate the expression of androgen receptor coactivators in the human prostate for a better understanding of androgen

deprivation action in prostate cancer. In this study, some of those coactivators are up regulated by the androgen deprivation, this evidence supports the critical role of coactivators in androgen independent growth.

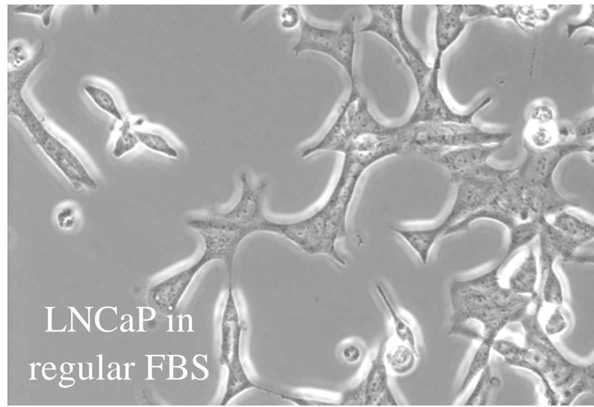
The multi-probe RPA is a highly sensitive and specific method for the detection and quantitation of mRNA species, and this can simultaneously evaluate several interesting mRNA in different time points. So we can study the multiple mRNA expression patterns of cofactors in androgen deprivation growth and compare them in one hybridization experiment. Only as little as the amount of RNA for one northern blotting, the multi-probe RPA can evaluate ten targets to the most in once procedure.

## Conclusions

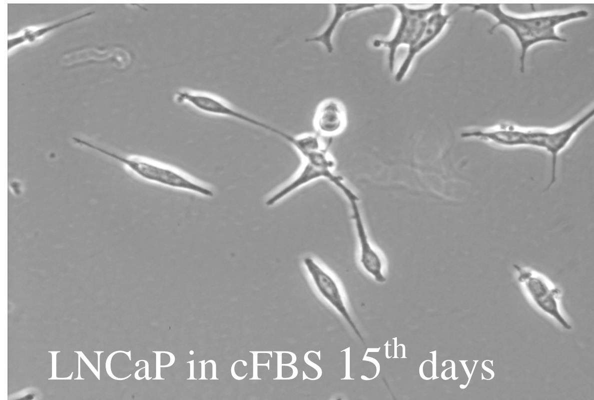
This is the first report to study AR coactivators expression pattern related to steroid-reduce environment by using a novel non-isotope multi-probe RPA assay method. This preliminary results support AR coactivators to be the key protein in androgen-deprivated growth of prostate cancer cell. From previous reports, it is known that AR cofactors may amplify or change the AR transcription pathway[2;3;9]. When the androgen dependent LNCaP cell culture in charcoal treated serum, the expression of AR cofactors (e.g. ara160, ara24, ara54, ara70) revealed increase production. From this expression change, these AR coactivators may not only enhance the ordinary AR transcription pathway by the increase amount of AR coactivators protein, but also can activate AR through different pathway or activate by other ligand e.g. estradiol or antiandrogen[2;9]. This is the first cell biological data to support the hypothesis of AR cofactors to be the trigger key factors between androgen dependent and independent. Our result can encourage us put more force to further clarify the mechanism of AR coactivators in the prostate cancel growth.

**Figure 1.**

**A**




**B**




**C**

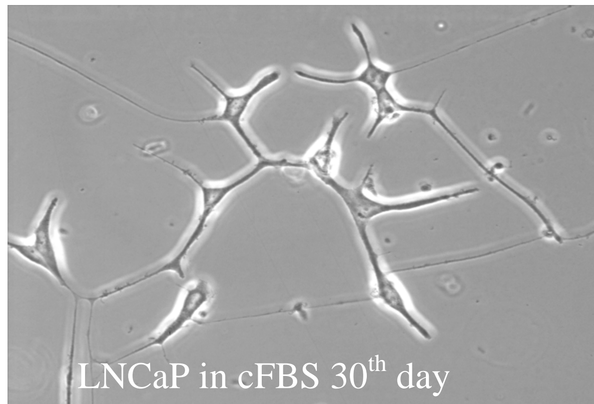
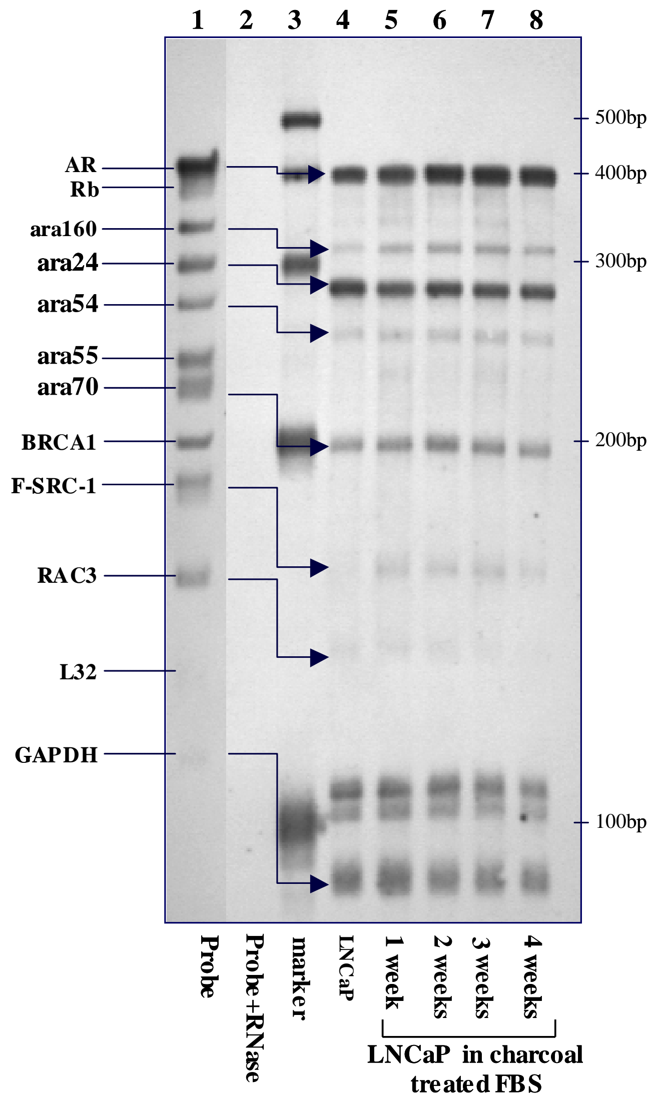


Figure 2 **A**




B

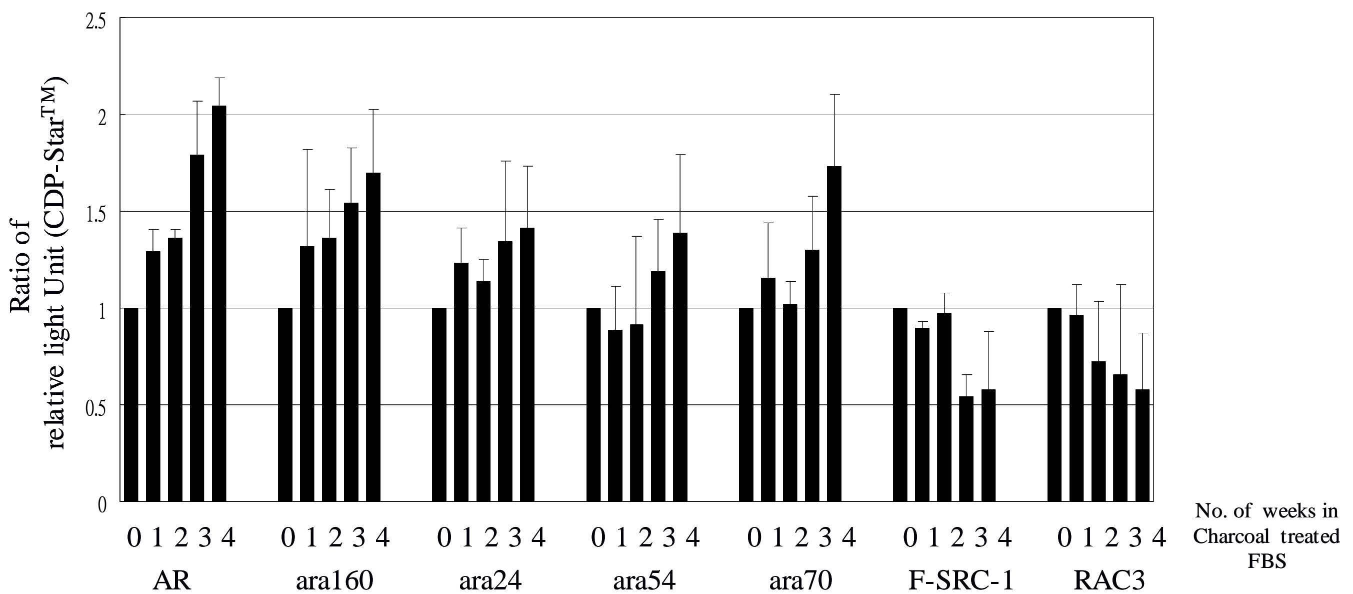


**Table 1**

AR, AR associated cofactors and internal control genes included in the twelve templates in the hAR multi-Probe template set with expected sizes (bp) of full-length probes and protected fragments.

| Template | Size (bp) | |
|---|---|---|
| | Full-length probe | Protected probe |
| AR | 429 | 400 |
| Rb | 381 | 352 |
| ara160 | 349 | 320 |
| ara24 | 315 | 286 |
| ara54 | 284 | 255 |
| ara55 | 256 | 227 |
| ara70 | 231 | 202 |
| BRCA1 | 211 | 182 |
| F-SRC-1 | 190 | 161 |
| RAC3 | 170 | 141 |
| L32 | 141 | 112 |
| GAPDH | 126 | 97 |

**Legend**

**Figure 1**. Phase-contrast micrographs of different LNCaP cells (200X). A: LNCaP cells were cultured in RPMI-1640 medium supplemented with 10% FBS. B: LNCaP cells were cultured in RPMI-1640 medium supplemented with 10% charcoal-treated FBS for 2 weeks, C: LNCaP cells were cultured in the same condition as B for 4 weeks.

**Figure 2.** Multi-probe RPA study for AR and its associated cofactors of LNCaP cell influenced by steroid reduced culture medium. (A) The LNCaP are cultured in RPMI 1642 with charcoal treated FBS for 4 weeks. Samples of total RNA (10 $\mu$ g, Lane 5-8) were isolated weekly. A GAPDH probe was used as a control for equivalent mRNA loading. The expressions of mRNA are indicated in relative light unit of CDP-Star$^{TM}$ on chemiluminescent exposure on film. The data were collected from three sets of independent experiments.
(B) Bands from figure 1A were quantified by densitometry. Results are reported as relative CDP activity. The expression of GAPDH gene was analyzed for standardization purposes and the expression level in lane 4 was set as 100% to compare with Lane 5-8. The data represent the mean ± SD from three independent experiments.

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