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水解磷酸脂促進 PC-3 前列腺癌細胞株之鈣網蛋白表現

Lysophosphatidic acid upregulates calreticulin expression in PC-3 human prostate cancer cells

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今之歌者黃玠有云:「每個人在實驗，願望能否實現，然後繼續實驗，然後換來紀念。」不知不覺，過著這樣尋找細胞運作秘辛的實驗生活已兩年有餘，如今正感端倪，碩班生涯卻已，謹以此論文為念，秘辛終有撥雲見日的一天。

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摘 要

鈣網蛋白 (Calreticulin, CRT) 為一種多功能之伴護蛋白 (Chaperone protein)，在許多種類的癌症組織切片中，皆發現 CRT 蛋白質的表現量相對高於正常組織。而在我們實驗室先前的研究中，已證實 CRT 會促進胃癌和膀胱癌之腫瘤生長及轉移。但在攝護腺癌中，CRT 所扮演的角色及其調控機制並不清楚。本研究中，我們首先針對早期攝護腺癌細胞株 LNCaP 與晚期攝護腺癌細胞株 PC-3 進行比較，發現 PC-3 的細胞貼附能力以及細胞增生能力皆顯著高於 LNCaP，並且 PC-3 比 LNCaP 表現更多量的 CRT 與血管內皮生長因子 A 型（Vascular endothelial growth factor A, VEGF-A）。為了進一步研究 CRT 在攝護腺癌細胞癌化中扮演的角色，我們建立了穩定降低 CRT 表現之 PC-3 細胞株，結果顯示抑制 CRT 之表現會顯著地降低 PC-3 之細胞貼附能力以及細胞增生能力，此外 VEGF-A 之表現量顯著下降。由這些結果可推論 CRT 為影響攝護腺癌細胞癌化之重要調控因子。接著我們試著找出造成 CRT 於晚期攝護腺癌細胞株增量表現的上游調控分子。水解磷酸脂 (Lysophosphatidic acid, LPA) 是一種多功能的信息傳遞分子，已知會影響攝護腺癌細胞株之增殖 (proliferation)、轉移 (migration) 以及侵入 (invasion)。先前已有研究顯示，在小鼠 D3 ES (D3 embryo stem cells) 細胞中，LPA 會經由磷脂酶 C（phospholipase C, PLC）的路徑調控內質網 (endoplasmic reticulum, ER) 的鈣離子 (Ca^{2+}) 流失。另一方面，在小鼠纖維母細胞 NIH/3T3 細胞株中，ER 的 Ca^{2+} 流失會活化 CRT 的啟動子。根據這些研究，我們假設在攝護腺癌細胞株中，LPA 會促進 CRT 的表現。實驗結果證實，在晚期攝護腺癌細胞株 PC-3 中，LPA 會促進 CRT 之 RNA 和 protein 表現。但在早期攝護腺癌細胞株 LNCaP 中，LPA 卻無促進 CRT 表現之作用。而利用 LPA 受器之拮抗劑 (antagonist) 或促進劑 (agonist) 以及基因敲落 (gene knockdown)

或基因大量表達 (gene overexpression) 的方式來對 LPA 受器進行操作的實驗中，我們發現在晚期攝護腺癌細胞株 PC-3 中，LPA 可經由細胞表面受器 LPA_1 和 LPA_3 促進 CRT 的表現; 但同時 LPA 亦可經由細胞表面受器 LPA_2 抑制 CRT 的表現。這些結果顯示，透過調整細胞表面的 LPA 受器表達模式 (LPA receptor expression pattern) ，或可有效調控 CRT 之表現並進一步控制攝護腺癌細胞之癌化。

關鍵字: 攝護腺癌，鈣網蛋白，水解磷酸脂，水解磷酸脂受器，血管內皮生長因子 A

Abstract

Calreticulin (CRT), a multifunctional Ca^{2+}-binding chaperone, has been shown to associate with poor prognosis in gastric cancer and bladder cancer. However, the roles of CRT in prostate cancer remain elusive. Prostate cancer is one of the most frequently diagnosed cancers in males and PC-3 is a popular cell model for investigating late stage prostate cancer. By comparing early stage prostate cancer cell line LNCaP with late stage prostate cancer cell line PC-3, we found that PC-3 showed higher cell adherent ability, cell proliferation ability and higher expression of vascular endothelial growth factor-A (VEGF-A), which is an important regulator for angiogenesis and tumor growth. Furthermore, knockdown of CRT in PC-3 caused lower cell adherent ability , cell proliferation ability and VEGF-A expression. These results indicate that CRT may be a poor prognosis factor in prostate cancer. Subsequently we further investigate the upstream regulation mechanism for calreticulin expression. Lysophosphatidic acid (LPA), a low molecular weight lipid, has been proved to stimulate cell migration, invasion and proliferation in prostate cancer cells. It has been demonstrated that LPA evoked Ca^{2+} mobilization from the lumen of the endoplasmic reticulum (ER) via phospholipase C (PLC) pathway. On

the other hand, depletion of Ca^{2+} from ER activated CRT promoter activity in NIH/3T3 cells. Based on these evidences, we hypothesized that LPA regulate CRT expression in prostate cancer cells. By using RT-qPCR and Western Blotting, we found that CRT expression is up-regulated both in mRNA and protein level after LPA treatment. Pharmacological blockade by LPA_1-specific antagonist AM966 or $LPA_{1/3}$-selective antagonist Ki16425 inhibits the enhancement effect of LPA on CRT expression. In addition, LPA-dependent CRT expression was abolished in LPA_1 and LPA_3 stable knockdown PC-3 cells. Furthermore, activation of LPA_3 by LPA_3-specific agonist OMPT enhances CRT expression. These results indicated that activation of LPA_1 and LPA_3 up-regulate the expression of CRT. On the contrary, activation of LPA_2 by LPA_2-selective agonist MDP and LPA_2-specific agonist GRI977143 impaired CRT expression. In conclusion, our findings suggested that, $LPA_{1/3}$ and LPA_2 inversely regulate CRT expression and subsequently regulate cell adhesion, cell proliferation and VEGF-A expression in PC-3 cells.

Keywords: Prostate cancer, Calreticulin, Lysophosphatidic acid, Vascular endothelial growth factor-A.

Contents

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Introduction

Prostate cancer

Prostate is a male-specific gland located under the bladder and surround the urethral. It is involved in urination and ejaculation. In some cases, cancer cells arise in the prostate and then develop into prostate cancer cells. Up to date, prostate cancer is one of the most frequently diagnosed cancers in males [1,2,3], so it is urgent to investigate the progression mechanism of prostate cancer. In females, Skene's glands were identified to be homologous to the prostate glands [4,5]. However, Skene's gland carcinoma is rare in females [6]. Androgen, a steroid hormone which maintains male characteristics in vertebrates by binding to androgen receptors (AR) and then activating androgen-response genes, is a key factor for prostate cancer cells to survive during the progression of prostate cancer. Therefore, androgen depletion therapy is an effective first-line therapy in the early stage of prostate cancer. However, prostate cancer cells gradually become androgen-insensitive in the late stage, which means that AR signaling pathways can be activated in an androgen-depleted environment. Several mechanisms for late stage prostate cancer cells to withstand androgen depletion therapy were elucidated. First, AR mutations allow the activation of AR by less androgen levels or other steroids. In addition,

AR overexpression or increased cytosolic synthesis of androgens keep the AR signaling pathways be activated. Besides, AR can be activated by interacting with substitutive AR cofactors. Furthermore, prostate cancer stem cells, which expressed high level of integrin α2β1 and CD133 on cell surface, can survive androgen depletion therapy then cause relapse of prostate cancer. Moreover, activation of other AR-independent bypass pathways can take over the AR signaling pathways and make the prostate cancer cells survive [7,8,9]. As in vitro experimental models, androgen-dependent LNCaP cells and androgen-independent PC-3 cells are commonly used prostate cancer cell lines to investigate the progression mechanism of prostate cancer. Previous studies have shown that PC3 cells are highly metastatic compared to LNCaP cells which is poorly metastatic [10]. In this study, we used both cell lines to compare the mechanisms in our hypothesis under different cell conditions.

Calreticulin in prostate cancer

Calreticulin (CRT) , a 46 KDa multifunctional Ca^{2+}-binding chaperone, is composed of three domains: N-domain, P-domain and C-domain. N-domain is a globular domain containing eight antiparallel β-strands. This domain interacts with P-domain to execute the chaperone function; P-domain is a proline-rich domain with a high-affinity but

low-capacity of Ca^{2+}-binding ability; C-domain is a low-affinity but high-capacity Ca^{2+}-binding domain which can interact with other chaperone proteins in endoplasmic reticulum (ER). The KDEL (lys-asp-glu-leu) sequence at the C-terminal of CRT is responsible for its localization to the ER lumen. However, CRT is also expressed in cytosol and cell membrane [11]. Interestingly, membrane CRT acts as a phagocytic signal for promoting phagocytosis of cancer cells by macrophages. Many cancers disrupt this prophagocytic function of membrane CRT by expressing CD47 on cell membrane to block the recognition of CRT by phagocyte [12,13,14]. On the other hand, previous studies have shown that CRT overexpressed and showed positive correlation to the carcinogenesis process in various cancers including bladder cancer, gastric cancer, oral cancer, esophagus cancer, breast cancer, pancreas cancer, colon cancer, vagina cancer, ovarian cancer and neuroblastoma [11]. However, in prostate cancer, the functional roles of CRT in carcinogenesis remain controversial. In prostate carcinoma specimens, *CRT* mRNA and protein expression levels are higher compared to those of benign prostatic hyperplasia specimens [15]. On the other hand, CRT is proposed to be down regulated in prostate cancer tissue with Gleason Score 5~9 and nodal metastasis prostate cancer specimens compared to adjacent benign tissue in the same sample. Therefore CRT is thought to act as a suppressive role in tumor growth and metastasis of prostate cancer

[16]. These studies suggest CRT expression levels are upregulated in prostate cancer patients compared to benign prostatic hyperplasia patients. However, in the same prostate cancer patient, CRT expression level of prostate carcinoma tissue is down-regulated compared to the adjacent benign tissue. To explore this paradoxical phenomenon, more details of the functional roles and regulatory mechanisms of CRT are needed to explain the meaning of CRT expression in prostate carcinoma specimens.

Lysophosphatidic acid in prostate cancer

Lysophosphatidic acid (LPA) is a lipid signaling molecule which has been shown to stimulate the development of prostate cancer by inhibiting autophagy and promoting cell survival, migration and invasion [17,18,19,20,21]. LPA was also found to regulate vascular endothelial growth factor-A (VEGF-A) expression for enhancing angiogenesis and vascular endothelial growth factor-C (VEGF-C) expression for enhancing lymphangiogenesis in prostate cancer cells [22,23]. Up to date, six G-protein couple receptors have been identified on the cell membrane. Among these receptors, LPA_1 (EDG-2) , LPA_2 (EDG-4) and LPA_3 (EDG-7) belong to the endothelial differentiation gene (Edg) family G protein-coupled receptors; LPA_4 (P2Y9) and LPA_6 (P2Y5) belong to purinergic G protein-coupled receptors [24,25,26]. By activating these receptors, LPA

induces various cell signaling. It is known that LPA activates an increase of intracellular Ca^{2+} concentration and causes a lot of calcium sensitive cellular events [27]. For instance, LPA-induced c-myc expression is mediated by Ca^{2+} mobilization via the phospholipase C (PLC) pathway [28]. Ca^{2+} is also involved in cell cycle progression [29], cell migration [30], angiogenesis [31], and apoptosis [32]. In addition, Ca^{2+} mobilization is involved in CRT expression [33]. Previous studies suggested that *LPAR* mRNA expression pattern depends on the stage of prostate cancer. *LPAR3* mRNA is predominantly expressed in early stage prostate cancer cells, LNCaP; *LPAR1* mRNA is predominantly expressed in late stage prostate cancer cells, PC-3 [34]. However, the metastasis-suppressive roles of *LPAR1* is proposed in PC-3 cells [35].

VEGF-A in prostate cancer

There are two distinct mechanisms for the formation of blood vasculature: formation of vessels from endothelial progenitors (vasculogenesis) or developing new branching vessels from existing vasculature (angiogenesis) [36,37]. Angiogenesis is observed in fetal growth, endometrial hyperplasia associated with the menstrual cycle, wound healing and cancer progression [38]. Vascular endothelial growth factor-A (VEGF-A) is a dimeric, disulfide-bound glycoprotein which plays important roles in angiogenesis, vasculogenesis,

lymphangiogenesis and tumor growth [39,40,41]. The human VEGF-A gene comprises eight exons separated by seven introns. To date, eighteen VEGF-A isoforms from alternative exon splicing have been documented (database: TopFIND$^{3.0\ beta}$). Interestingly, VEGF165b (isoform 8) plays the antiangiogenic roles compared to the other isoforms, which are almost proangiogenic molecules [42]. During tumorigenesis, hypoxia-inducible factors (HIFs) were upregulated in prostate cancer [43]. Furthermore, HIFs bind to the promoter region of VEGF-A to promote VEGF-A expression under hypoxia conditions [44,45,46]. In addition, VEGF-A is associated with developing of prostate cancer. Metastasis of prostate cancer cells is controlled by VEGF-A [47]. Plasma levels of VEGF-A are increased in patients with metastatic prostate cancer [48]. On the other hand, single nucleotide polymorphisms (SNPs) in angiogenesis genes, including HIF1-α, have been shown to associate with progression of prostate cancer [49]. All the previous studies suggested the malignant roles of VEGF-A in prostate cancer. However, the underlying regulatory mechanisms of VEGF-A are still to be clarified.

Rationale

In this study, we aim to investigate the functional roles and regulatory mechanisms of CRT in human prostate cancer cells. It has been demonstrated that LPA evoked the Ca^{2+} mobilization from the lumen of the endoplasmic reticulum (ER) via phospholipase C (PLC) pathway in Mouse D3 ES cells [28]. On the other hand, depletion of Ca^{2+} from the ER activated the CRT promoter in NIH/3T3 cells [33]. Furthermore, LPA was found to stimulate axonal *CRT* mRNA translation through 5'UTR (Untranslated Region) with LPA-dependent phosphorylated eIF2α [50]. Based on these evidences, we hypothesized that LPA regulate CRT expression in prostate cancer cells.

Material and Methods

Cell culture

PC-3 and LNCaP human prostate cancer cell lines were obtained from American Type Culture Collection (ATCC; Manassas, Virginia, USA) and cultured in RPMI 1640 (GE Healthcare Life Sciences, Hyclone Laboratories, Logan, Utah, USA) supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences, USA), penicillin (100U/mL) and streptomycin (100μg /mL) (by adding Penicillin-Streptomycin, 100x liquid stock, Life Technologies, Gibco) in a humidified atmosphere containing 5% CO_2 at 37℃. One unit of penicillin equals to 0.6μg of penicillin. For the subcultures, cells were trypsinized with 0.05% EDTA-trypsin (Life Technologies).

Puromycin lethal dose test of PC-3 cells

Human prostate cancer cells PC-3 were seeded on polystyrene 6 well plate (Corning) and cultured in RPMI 1640 medium (supplemented with 10% fetal bovine serum, 100U/mL penicillin and 100μg /mL streptomycin) with different concentration of puromycin for 4 days. The concentration 0.5μg/mL was chosen for the drug selection of stable transfected PC-3 cells in this study.

Transfection and selection of stable cell lines

CRT-shRNA plasmid was purchased from the National RNAi Core Facility Platform, Academia Sinica (Taipei, Taiwan). The CRT-shRNA target sequence was 5′-CCAGTATCTATGCCTATGATA-3′ (shCRTa, TRCN0000019989). pLKO.1 was the control vector of the CRT-shRNA plasmid. *LPAR1*-shRNA plasmid (TR313300, pGFP-V-RS) and *LPAR3*-shRNA plasmid (TG313295, pGFP-V-RS) were purchase from Origene (MD, USA). pGFP-V-RS was the control vector of the *LPAR1*-shRNA or *LPAR3*-shRNA plasmids. *LPAR2* overexpression plasmid was purchased from Genecopoeia (MD, USA), catalog number: EX-Z0788-M03. pEZ-M03 was the control vector of the *LPAR2* overexpression plasmid.

Pharmacological treatment

18:1 LPA (1-oleoyl LPA) (L7260, Sigma-Aldrich) was dissolved in methanol and chloroform (9:1) and stored at -20℃ before used. EC_{50} values of 18:1 LPA were 200 nM for LPA_1, 10 nM for LPA_2 and 75 nM for LPA_3 [51]. For each experiment, 3×10^5 cells/well were cultured in 10 cm-diameter plates. After 24 h of normal condition culture with serum-containing medium and 16 h of starvation culture with serum-free medium, the medium was replaced with LPA added serum-free medium, coupled with 0.005%

fatty acid-free bovine serum albumin (BSA) as a LPA carrier.

LPA_1-specific antagonist AM966 (ApexBio, USA) was dissolved in DMSO and stored at -20℃ before used. IC_{50} values of AM966 were 17 and 19 nM for human and mouse LPA_1 respectively; 1.7 and 25 μM for human and mouse LPA_2; 1.6 and 0.17μM for human and mouse LPA_3 respectively [52].

LPA_2-selective agonist mono-n-dodecyl phosphate (MDP) (Sigma-Aldrich, USA) was dissolved in ethanol and stored at -20℃ before used. EC_{50} value of MDP for LPA_2 was 700 nM and Ki value of MDP for LPA_3 was 90 nM [53]. LPA_2-specific agonist GRI977143 (Sigma-Aldrich, USA) was dissolved in DMSO before used. EC_{50} value of GRI977143 for LPA_2 was 3.3 μM. IC_{50} value of GRI977143 for LPA_3 was 6.6 μM. GRI977143 has no effect on LPA_1 even the concentration is up to 10 μM [54].

LPA_3-specific agonist (2S)-OMPT (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in DMSO and stored at -20℃ before used. According to previous study, at low concentrations (<100 nm), OMPT selectively activates LPA_3. At concentrations of 1 μm or higher, OMPT starts to activate LPA_2. However, even at concentrations exceeding 10 μm, OMPT may not be able to activate LPA_1 [55].

$LPA_{1/3}$-selective antagonist Ki16425 (provided as a racemate, Cayman Chemical, Ann Arbor, MI, USA) was dissolved in DMSO and stored at -20℃ before used. Ki values

of Ki16425 were 0.34 μM for LPA_1, 6.5 μM for LPA_2 and 0.93 μM for LPA_3 [56].

Reverse transcription (RT) and Real-time PCR

400 μL/ well of Trizol (invitrogen) or TriPure isolation reagent (Roche, Indianapolis, IN, USA) was used to extract total RNA from the cells seeded on polystyrene 6 well plate (Corning) . Complementary DNA (cDNA) was synthesized from 1 μg total RNA using the Toyobo RT-PCR kit (Toyobo, Osaka, Japan). The condition of reverse transcription was 42℃ for 50 min, 70℃ for 15 min then cool down to 4℃. SYBR Green supermix (Bio-rad, made in Singapore) as a fluorescent dye was used to perform real-time PCR in the Mini Opticon Real-time PCR system (Bio-Rad). Gene-specific primers were used and the specificity for each primer was checked by melting curve analysis following real-time PCR. Cycling condition was 95℃ for 3 min, followed by 55 cycles of 95℃ for 30 s, 60℃ for 30 s, and 72℃ for 30 s. To quantify the target gene expression, each gene was normalized using GAPDH as an internal standard. Primers for real-time PCR were as follows: GAPDH (F-5'- AAG GTG AAG GTC GGA GTC 3' and R-5'- TGT AGT TGA GGT CAA TGA AGG -3'), *CRT* (F-5'- CCT CCT CTT TGC GTT TCT TG 3' and R-5'- CAG ACT CCA AGC CTG AGG AC -3') , *VEGF-A* (F-5'- GCA GAA GGA GGA GGG CAG AAT C 3' and R-5'- GGC ACA CAG GAT GGC TTG AAG -3') , *LPA1* (F-5'- GTC TTC TGG GCC ATT TTC AA 3' and R-5'- TCA TAG TCC TCT GGC GAA CA -3') ,

LPA2 (F-5’- CGC ACA GCC CGA CTT TCA CTT 3’ and R-5’- CAC AAT GAG CAT GAC CAC GC -3’) , *LPA3* (F-5’- GAA GCT AAT GAA GAC GGT GAT GA 3’ and R-5’- AGC AGG AAC CAC CTT TTC AC -3’) , *LPA4* (F-5’- TCT GGA TCC TAG TCC TCA GTG G 3’ and R-5’- CCA GAC ACG TTT GGA GAA GC -3’) , *LPA5* (F-5’- CTC GGT GGT GAG CGT GTA CAT G 3’ and R-5’- GCG TAG CGG TCC ACG TTG AT -3’) , *LPA6* (F-5’- TCT GGC AAT TGT CTA CCC ATT 3’ and R-5’- TCA AAG CAG GCT TCT GAG G -3’).

Table 1. Primers used for real time-PCR

Gene name	Orientation	Primer sequence (5′–3′)	Species
GAPDH	Forward	AAG GTG AAG GTC GGA GTC	Human
GAPDH	Reverse	TGT AGT TGA GGT CAA TGA AGG	Human
CRT	Forward	CCT CCT CTT TGC GTT TCT TG	Human
CRT	Reverse	CAG ACT CCA AGC CTG AGG AC	Human
VEGF-A	Forward	GCA GAA GGA GGA GGG CAG AAT C	Human
VEGF-A	Reverse	GGC ACA CAG GAT GGC TTG AAG	Human
LPA1	Forward	GTC TTC TGG GCC ATT TTC AA	Human
LPA1	Reverse	TCA TAG TCC TCT GGC GAA CA	Human
LPA2	Forward	CGC ACA GCC CGA CTT TCA CTT	Human

LPA2	Reverse	CAC AAT GAG CAT GAC CAC GC	Human
LPA3	Forward	GAA GCT AAT GAA GAC GGT GAT GA	Human
LPA3	Reverse	AGC AGG AAC CAC CTT TTC AC	Human
LPA4	Forward	TCT GGA TCC TAG TCC TCA GTG G	Human
LPA4	Reverse	CCA GAC ACG TTT GGA GAA GC	Human
LPA5	Forward	CTC GGT GGT GAG CGT GTA CAT G	Human
LPA5	Reverse	GCG TAG CGG TCC ACG TTG AT	Human
LPA6	Forward	TCT GGC AAT TGT CTA CCC ATT	Human
LPA6	Reverse	TCA AAG CAG GCT TCT GAG G	Human

Mycoplasma test

Mycoplasma test is used to examine whether cultured cells are contaminated with mycoplasma or not. 1 mL culture media were centrifuged at 2,000 rpm for 5 min and clarified supernatants were transferred to new eppendorf tubes then centrifuged at 14,000 rpm for 10 min. After discarding the supernatants and dissolving the pellets with 50μL ddH_2O, the samples were examined by PCR. Primers for the mycoplasma test were as follows: *16S rRNA* (F-5'- G GGG AGC AAA CAG GAT TAG ATA CCC T 3' and R-5'- GAG GTT AAC AGA GTG ACA GAT GGT GCA -3') [57]. Cycling conditions were

94℃ for 1 min, followed by 35 cycles of 94℃ for 30 s, 62℃ for 60 s, and 72℃ for 60 s. PCR ends with 72℃ for 5 min. The PCR product samples are checked by agar gel electrophoresis.

Western blot analysis

Cells seeding on 6 well plate were washed with 1mL/well of ice-cold phosphate buffered saline (PBS) three times after removing the medium. Then the cells were lysed on ice with 80 μL/well of lysis buffer (40 mM Tris (tris(hydroxymethyl)aminomethane, THAM), 274 mM NaCl, 1% NP40, 10% glycerol and 0.2M Na_3VO_4) containing 1% of protease inhibitor cocktail (Merck Millipore, Billerica, MA, USA). After scraping and collecting, lysates were centrifuged at 4℃ and 14,000 rpm for 15 min and clarified supernatants were collected. BSA protein assay was used to quantify the protein concentrations. Equal amounts and volumes of protein samples were separated by polyacrylamide gel electrophoresis (PAGE) with 4% stacking gel and 12% running gel then transferred to 0.45 μm pore size polyvinylidene fluoride (PVDF) transfer membranes (Millipore, Billerica, MA, U.S.A. or GE Healthcare, Amersham Hybond-P). The following antibodies were used for blotting: goat polyclonal anti-humanVEGF-A antibody (GTX102643, GeneTex; 1:2000), rabbit polyclonal anti-CRT antibody (Upstate Biotechnology, Lake Placid, NY; 1:4000), rabbit polyclonal anti-GAPDH antibody

(GTX100118, GeneTex; 1:8000), goat-anti-rabbit IgG-HRP (horseradish peroxidase) (E1012, Santa Cruz Biotechnology; 1:8000). ECL reagent (Advansta, Menlo Park, CA, USA) was used as Western Blotting detection reagents.

Adhesion assay

Cells (5×10^4 cells/300μL) were seeded on 96-well collagen-coated (10 μg/ml) plate then incubated for 15min, followed by 3 times of PBS wash. 100 μL of 0.5 % crystal violet dissolved in 5% alcohol was added to stain the cells. After staining, destain the cells by PBS wash until the supernatant became transparent. Crystal violet sticking to the cells was suspended in 100 μL of 10% acetic acid then loaded to 96-wll plate for quantification by detecting OD550 values.

Proliferation assay

Cells (5×10^4 cells/2mL) were seeded on 6-well plate then incubated for 3 days, followed by 3 times of PBS wash. 100 μL of 0.5 % crystal violet dissolved in 5% alcohol was added to stain the cells. After staining, destain the cells by PBS wash until the supernatant became transparent. Crystal violet sticking to the cells was suspended in 100 μL of 10% acetic acid then loaded to 96-wll plate for quantification by detecting OD550 values.

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Statistical analysis

Data were statistically analyzed using student's t test (TWO-Sample Assuming Unequal Variances, one tail) or one-way analysis of variance (ANOVA), followed by Fisher's protected least-significant difference (LSD) test (StatView, Abacus Concept, Berkeley, CA, USA). Each result was obtained from at least three independent experiments and a value of $p<0.05$ was considered statistically significant.

Results

PC-3 cells show higher levels of adhesion, proliferation, CRT/VEGF-A expression compared with LNCaP prostate cancer cells.

Previous studies have shown that PC3 cells are highly metastatic compared to LNCaP cells [10]. In order to confirm this, we used crystal violet assay to analyze the adherent ability and proliferation ability of LNCaP and PC-3. The results indicated that PC-3 shows higher levels of cell adherent ability and proliferation ability compared with LNCaP (Fig. 1A & 1B), which are consistent with previous study. More importantly, PC-3 cells showed higher expression levels of CRT (Fig. 1C & 1D) and angiogenesis factor VEGF-A (Fig. 1E & 1F) both in mRNA level and cytosolic protein level compared to LNCaP cells. These results suggested that CRT and VEGF-A are up-regulated in late stage prostate cancer cells PC-3.

Knockdown of CRT decreases VEGF-A expression, cell adherent ability and cell proliferation ability in PC-3 cells.

In order to clarify the functional roles of CRT in late stage prostate cancer cells, we select stable CRT knockdown cell lines. PC-3 cells were transfected with CRT-shRNA plasmids and selected by 0.5 μg/mL of puromycin for 4 months. CRT knockdown

efficiency was analyzed with real-time PCR and Western blotting in the generated CRT-shRNA PC-3 cells compared to cells which were transfected with control vectors. CRT-shRNA PC-3 cells showed lower VEGF-A expression level compared to control cells (Fig. 2A & 2B). Furthermore, cell adherent ability and proliferation ability of CRT-shRNA PC-3 cells were significantly lower than control cells (Fig. 2C & 2D). These results indicated that knockdown of CRT impaired the VEGF-A expression, cell adherent ability and cell proliferation ability in PC-3 cells.

LPA induces calreticulin expression in late stage prostate cancer cell line PC-3 , but not in early stage prostate cancer cell line LNCaP.

In order to explore the regulatory mechanisms involved in high CRT expression in late stage prostate cancer, PC-3 cells were cultured with different concentrations of LPA, which is a candidate molecule to regulate the CRT expression. The results suggested that LPA induces CRT expression both in mRNA and cytosolic protein level in PC-3 cells (Fig. 3C & 3D), but not in LNCaP cells (Fig. 3A & 3B). From these results, we concluded that in the late stage of prostate cancer, up-regulated CRT expression is taken over through a LPA-dependent mechanism, compared to early stage prostate cancer whose CRT expression mechanism is androgen dependent.

LPA receptor mRNA profile comparison between high metastatic

potential PC-3 and low metastatic potential LNCaP prostate cancer cells

We hypothesized the LPA-dependent CRT expression in late stage prostate cancer is due to change of LPA receptor expression pattern. To confirm the hypothesis, we made the mRNA profile of 6 different LPA receptors (Fig. 4A). The results suggested that, *LPAR1* was predominantly expressed in PC-3 cells; *LPAR3* was predominantly expressed in LNCaP cells. Both PC-3 cells and LNCaP cells showed high expression level of *LPAR6*. In addition, the LPA receptor expression patterns under starvation condition (Fig. 4A) or normal condition (Fig. S4A) are similar both in LNCaP cells and PC-3 cells.

LPA_1 and LPA_3 are involved in up-regulation of CRT expression in PC-3 cells

In order to confirm the roles of each LPA receptor in the LPA-dependent CRT expression, we used $LPA_{1/3}$ selective antagonist: Ki16425, to block the LPA-activation of both LPA_1 and LPA_3 receptors (Fig. 5A). The results showed that the LPA induction effect on *CRT* mRNA expression was counteracted under Ki16425 treatment. We further used LPA_1 specific antagonist: AM966, to block the LPA-activation of LPA1 receptors (Fig. 5B), LPA induction effect on *CRT* mRNA expression was also counteracted. On the other hand, *CRT* mRNA expression was up-regulated when the LPA_3 was activated by the LPA_3 specific agonist OMPT (Fig. 5C). These results indicated that LPA_1 and LPA_3 are

involved in the up-regulation of *CRT* mRNA expression in PC-3 cells. To further confirm the effects of LPA_1 and LPA_3 on CRT, we selected *LPAR1* and *LPAR3* stable-KD PC-3 cells respectively (Fig. 5D & 5E). Both of them showed lower *CRT* mRNA expressions (Fig. 5F), and the LPA induction effects on CRT protein expression in both cells were compromised (Fig. 5G).

LPA_2 is involved in down-regulation of *CRT* mRNA expression in PC-3 cells

We also use LPA_2 selective agonist: MDP and LPA_2 specific agonist: GRI977143, to study the effects of LPA_2 on CRT expression. Surprisingly, *CRT* mRNA expression was inhibited under treatments of both LPA2 agonists (Fig. 6A & 6B). Furthermore, we overexpressed LPAR2 transiently in PC-3 cells (Fig. 6C) and it also showed that *CRT* mRNA level was compromised (Fig. 6D). These results suggested that LPA_2 is a negative regulator of CRT in PC3 cells.

Discussion

Activation of the androgen receptors (ARs) in the cytoplasm by androgens is required for the development of early stage prostate cancer. Activated ARs translocate into cell nucleus and regulate androgen-responsive genes which implicated in prostate cancer [58]. Therefore, Androgen ablation therapy is effective in inhibiting early stage prostate cancer progression. However, after a period of time, the prostate tumor would become androgen-insensitive, which means that ARs can still localizes to the nucleus and functions in androgen-depleted conditions [59,60], or AR-independent pathways emerge to take over the roles of ARs [9]. Androgen-insensitive prostate tumor becomes more aggressive (Fig. 1A & 1B), ultimately leading to death of the patients [61]. Previous study has showed that CRT is one of the AR regulated genes [62]. On the other hand, early stage prostate cancer LNCaP cells expressed ARs, while late stage prostate cancer PC3 cells did not express ARs [63,64]. However, in the present study we found that AR–negative PC3 cells shows higher expression level of CRT than AR–positive LNCaP cells (Fig. 1C & 1D). This observation suggested there are alternative mechanisms to take over high CRT expression in late stage prostate cancer.

It has been demonstrated that LPA evoked the Ca^{2+} mobilization from the endoplasmic reticulum (ER) lumen via the phospholipase C (PLC) pathway in Mouse D3

embryonic stem (ES) cells [28]. On the other hand, depletion of Ca^{2+} from the ER activated the CRT promoter in mouse embryonic fibroblast cell line NIH/3T3 [33]. Furthermore, LPA was found to stimulate axonal *CRT* mRNA translation through 5'UTR (Untranslated Region) by activating LPA-dependent phosphorylated eIF2α [50]. Based on these studies, we hypothesized that there might be a correlation between LPA and CRT in prostate cancer. Here we demonstrate that LPA positively regulates CRT expression in PC-3 cells, but not in LNCaP cells (Fig. 3). These results suggest that LPA-dependent mechanism is one of the ways to take over CRT expression in androgen-insensitive late stage prostate cancer. We further provided a detailed regulatory mechanism by which LPA promotes CRT expression through LPA_1 and LPA_3 (Fig. 5), but LPA also inhibits CRT expression through LPA_2 activation (Fig. 6). The mRNA expression pattern of LPA receptors might explain the effect of LPA on CRT. *LPAR1* and *LPAR3* mRNA expression levels are relative higher compared to *LPAR2* both in PC-3 cells and LNCaP cells (Fig. 4). However, how the LPA receptors contribute to different effects of LPA on CRT between PC-3 and LNCaP remains unknown. The protein expression pattern of LPA receptors is required for further investigation.

Vascular endothelial growth factor-A (VEGF-A) expression is reported to be positively regulated by androgens in the early stage androgen-responsive prostate cancer

cells [65]. However, in late stage androgen-insensitive cells, VEGF-A expression levels are still increased (Fig. 1E & 1F). The regulatory mechanism of this androgen–independent upregulation of VEGF-A is unknown [66]. Our previous study has demonstrated that CRT has a positive effect on VEGF-A expression in bladder cancer cells and neuroblastoma cells [11,67]. In this study, we used CRT knockdown / androgen–insensitive PC-3 cells to investigate how CRT regulates VEGF-A expression in prostate cancer cells. Our results suggested that knockdown of CRT impaired VEGF-A expression (Fig. 2A & 2B). In addition, knockdown of CRT impaired cell adherent ability and cell proliferation ability in PC-3 cells (Fig. 2C & 2D). These results indicate that CRT is a potential poor prognosis factor for prostate cancer patients.

In conclusion, this is the first study demonstrating that LPA promotes CRT expression in PC-3 human prostate cancer cells, which provides evidence that increased CRT expression in late stage prostate cancer is taken over by a LPA-dependent mechanisms compared to early stage prostate cancer, whose CRT expression mechanism is androgen dependent. Our study also clarifies that upregulation of CRT is mediated through LPA_1 and LPA_3 and that inhibition of CRT is mediated through LPA_2. These findings suggest that manipulating LPA receptor expression pattern on the cell membrane might be a feasible way to control the expression of CRT and the progression of prostate

cancer.

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Figures

Figure 1

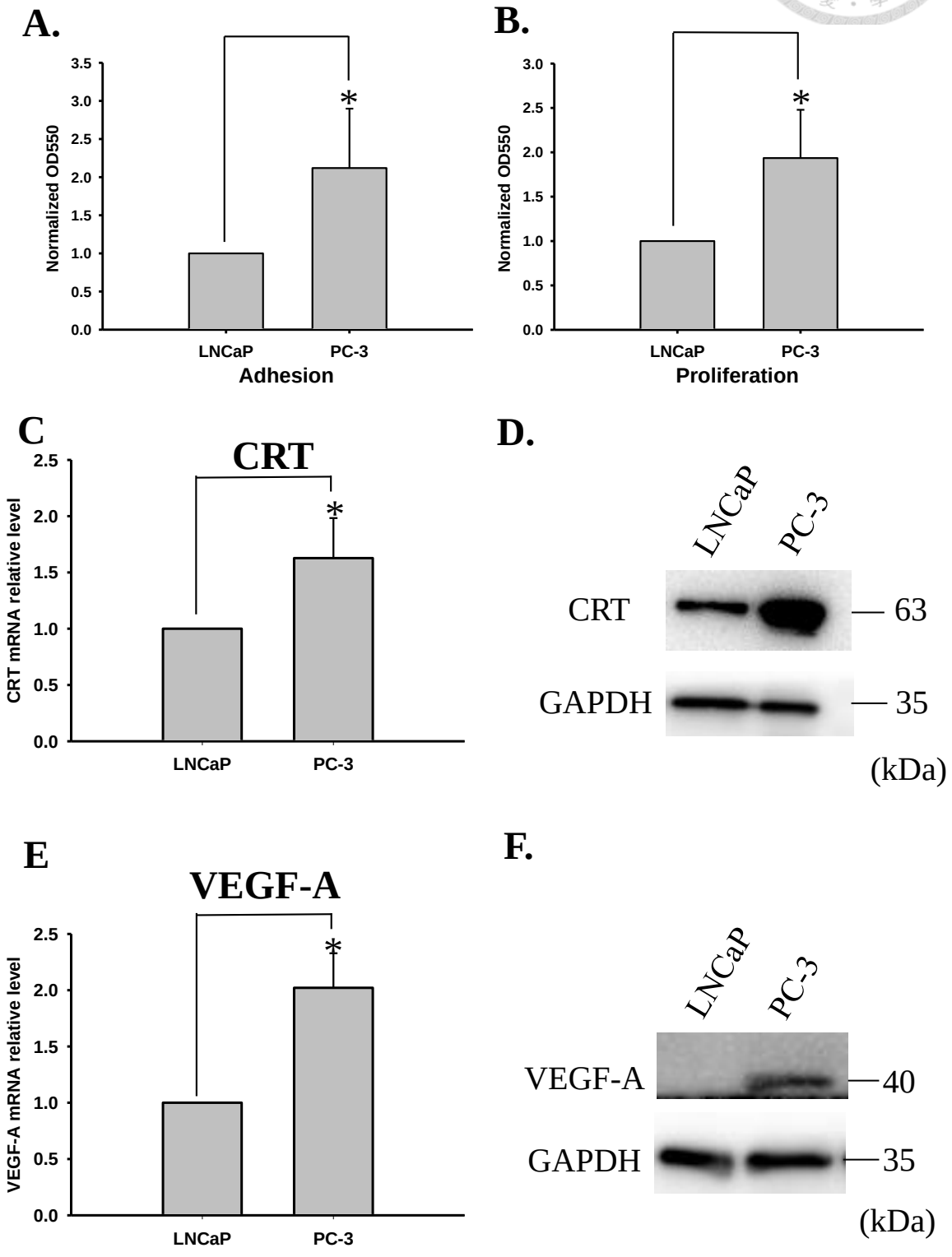
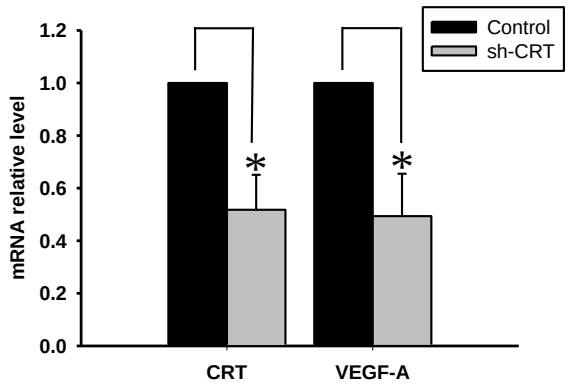
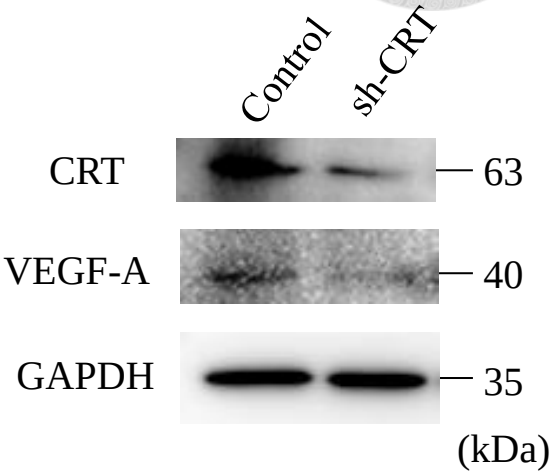


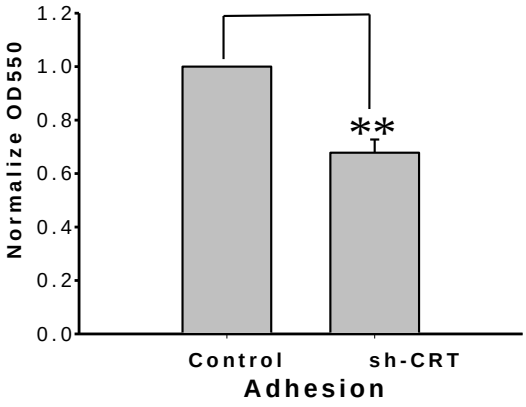
Fig.1 PC-3 cells show higher levels of cell adhesion, proliferation, CRT/ VEGF-A expression compared with LNCaP prostate cancer cells. (A) Cells (5×10^4 cells/300 μL) were seeded on 96-well collagen-coated (10 μg/ml) plate then incubated for 15min. Adherent ability of LNCaP cells and PC-3 cells were analyzed by crystal violet assay. N=4, t-test. (B) Cells (5×10^4 cells/2 mL) were seeded on 6-well plate then incubated for 3 days. Proliferation ability of LNCaP cells and PC-3 cells were analyzed by crystal violet assay. N=3, t-test. (C & E) CRT and *VEGF-A* mRNA levels were analyzed by qPCR. N=4, t-test. (D & F) CRT and VEGF-A protein levels were analyzed by Western blot. N=3. (* $P<0.05$; ** $P<0.01$; ***$P<0.001$)

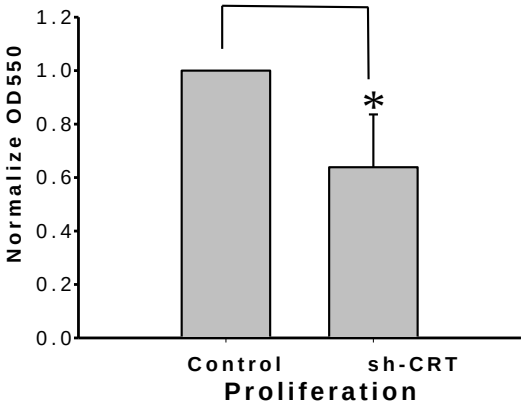
Figure 2





PC-3 **PC-3**

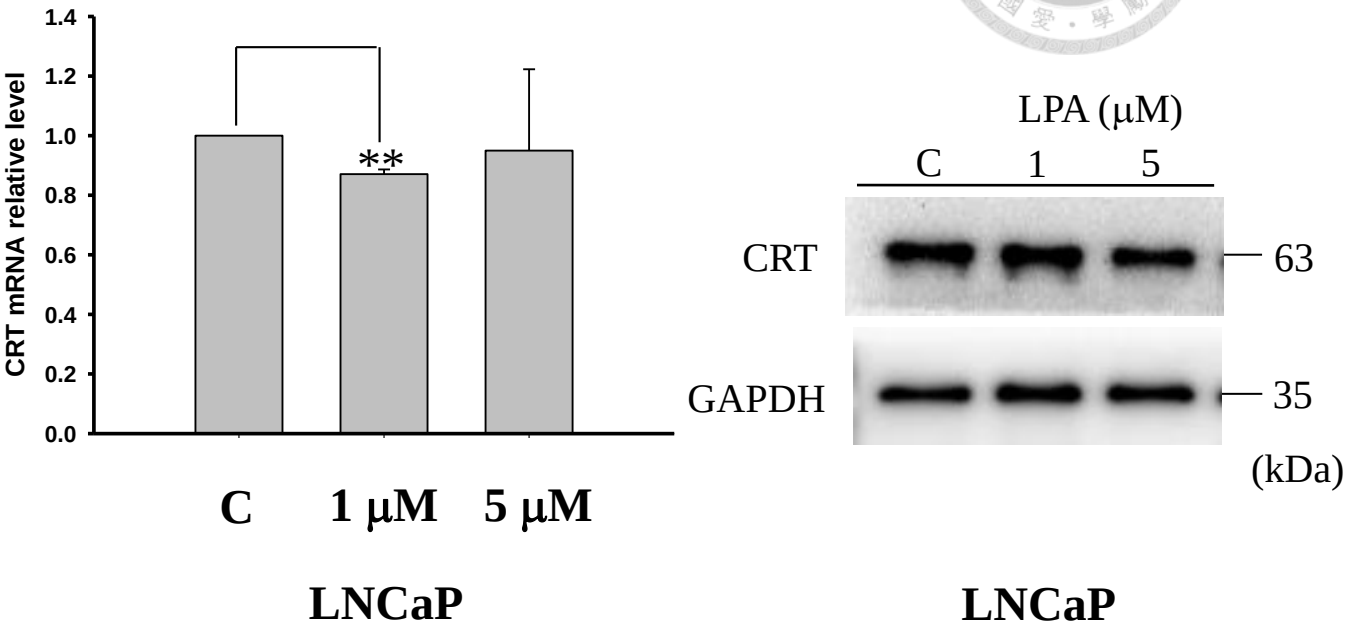




PC-3 **PC-3**

Fig.2 Knockdown of CRT decreases VEGF-A expression, cell adherent ability and cell proliferation ability in PC-3 cells. (A) CRT and VEGF-A protein levels were analyzed by Western blot. N=3. (B) CRT and *VEGF-A* mRNA levels were analyzed by qPCR. N=4, t-test. (C) Cells (5×10^4 cells/300 μL) were seeded on 96-well collagen-coated (10 μg/ml) plate then incubated for 15min. Adherent ability of CRT knockdown PC-3 cells were analyzed by crystal violet assay. N=3, t-test. (D) Cells (5×10^4 cells/2mL) were seeded on 6-well plate then incubated for 3 days. Proliferation ability of CRT knockdown PC-3 cells was analyzed by crystal violet assay. N=3, t-test. (* $P<0.05$; ** $P<0.01$; ***$P<0.001$)

Figure 3


A.
B.
CRT mRNA relative level
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
C
1 μM
5 μM
LNCaP
LPA (μM)
C
1
5
CRT
63
GAPDH
35
(kDa)
LNCaP

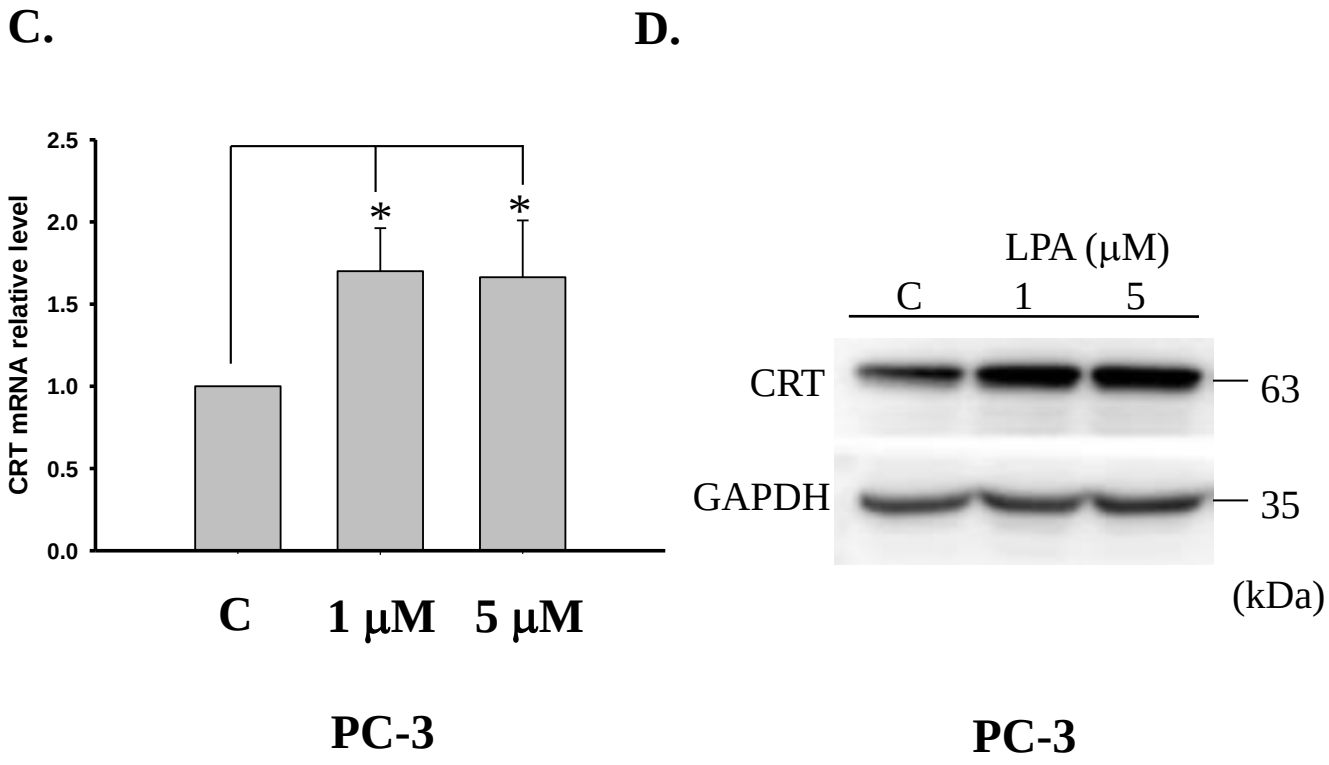
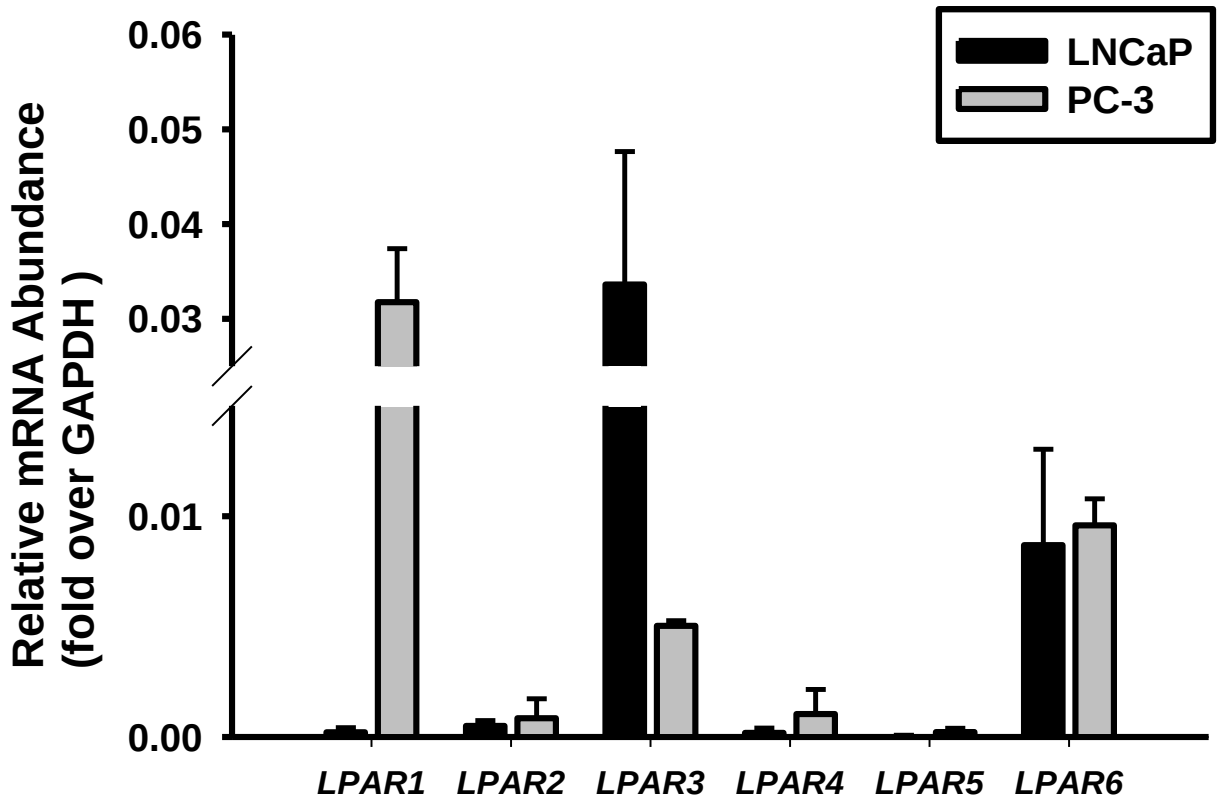

C.
D.
CRT mRNA relative level
2.5
2.0
1.5
1.0
0.5
0.0
*
*
C
1 μM
5 μM
PC-3
LPA (μM)
C
1
5
CRT
63
GAPDH
35
(kDa)
PC-3

Fig.3 LPA induces calreticulin expression in late stage prostate cancer cell line PC-3, but not in early stage prostate cancer cell line LNCaP. Starved LNCaP cells and PC-3 cells were treated with methanol/chloroform control or LPA (1 or 5 μM). (A & C) *CRT* mRNA levels after 90 minutes of LPA treatment were analyzed by qPCR. N=3, ANOVA. (B & D) CRT cytosolic protein levels after 4 hours of LPA treatment were analyzed by western blotting. N=3. (* $P<0.05$; ** $P<0.01$; ***$P<0.001$)

Figure 4

A.


LNCaP
PC-3
Relative mRNA Abundance
(fold over GAPDH)
0.06
0.05
0.04
0.03
0.01
0.00
LPAR1
LPAR2
LPAR3
LPAR4
LPAR5
LPAR6

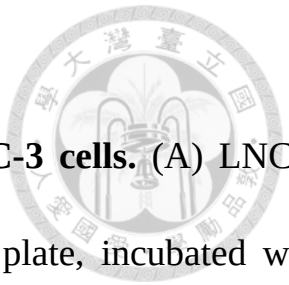
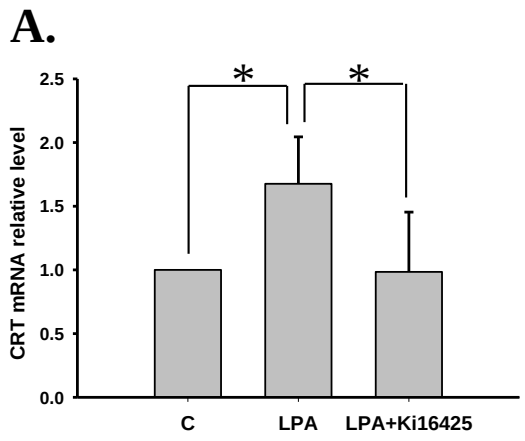
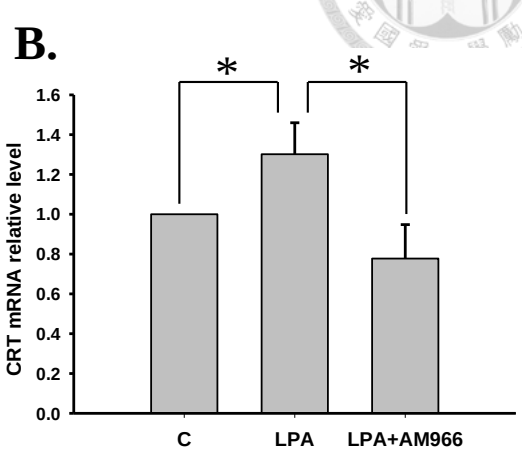


Fig.4 LPA receptor mRNA profile of LNCaP cells and PC-3 cells. (A) LNCaP cells or PC-3 cells (3×10^5 cells/2mL) were seeded on 6-well plate, incubated with RPMI-1640 medium containing fetal bovine serum (FBS) for 32 h then replaced the medium with RPMI-1640 medium without FBS for 16 h before harvest. $LPAR_{1\text{-}6}$ mRNA expression levels were analyzed by qPCR. N=3.

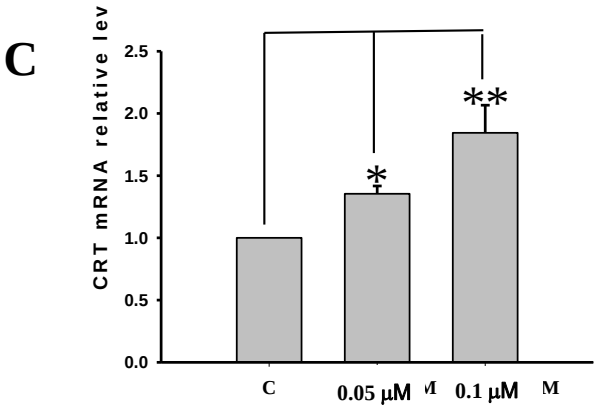
Figure 5



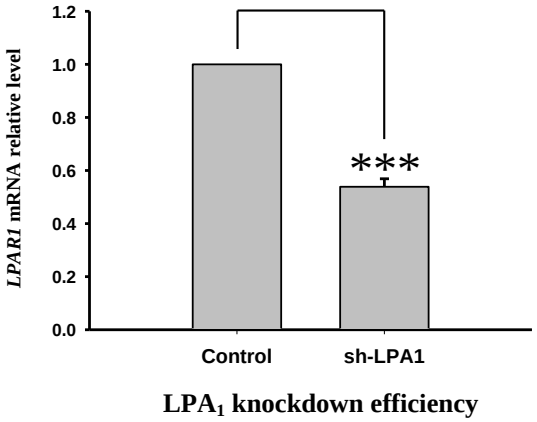
$LPA_{1/3}$ antagonist: Ki16425



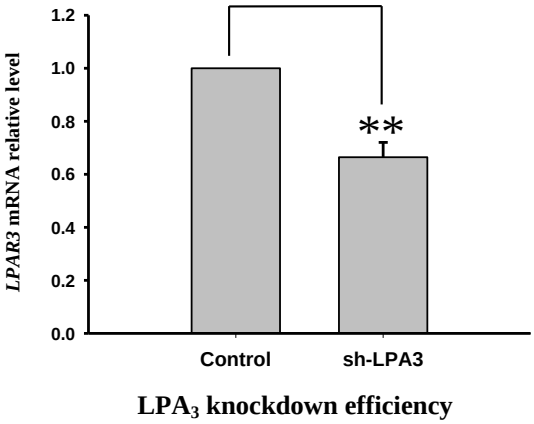
LPA_1 antagonist: AM966



LPA_3 agonist: (2S) OMPT

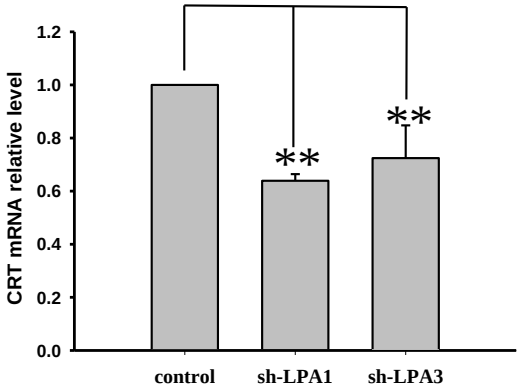


LPA_1 knockdown efficiency



LPA_3 knockdown efficiency

F.


CRT mRNA relative level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
control
sh-LPA1
sh-LPA3

G.

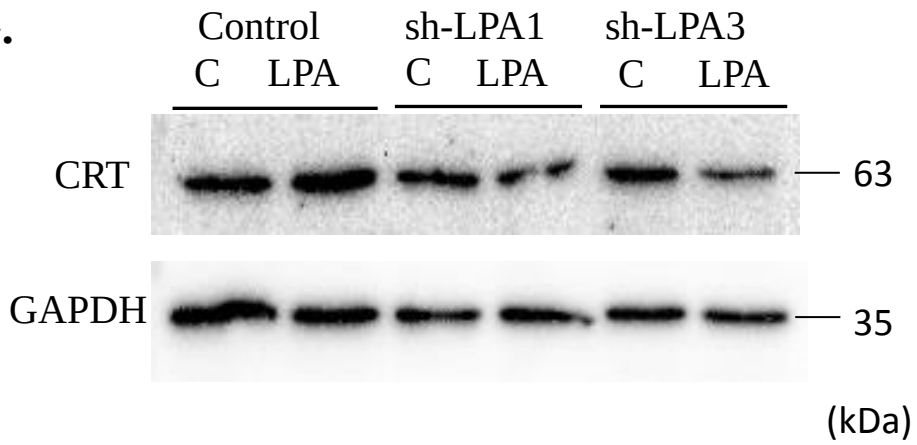
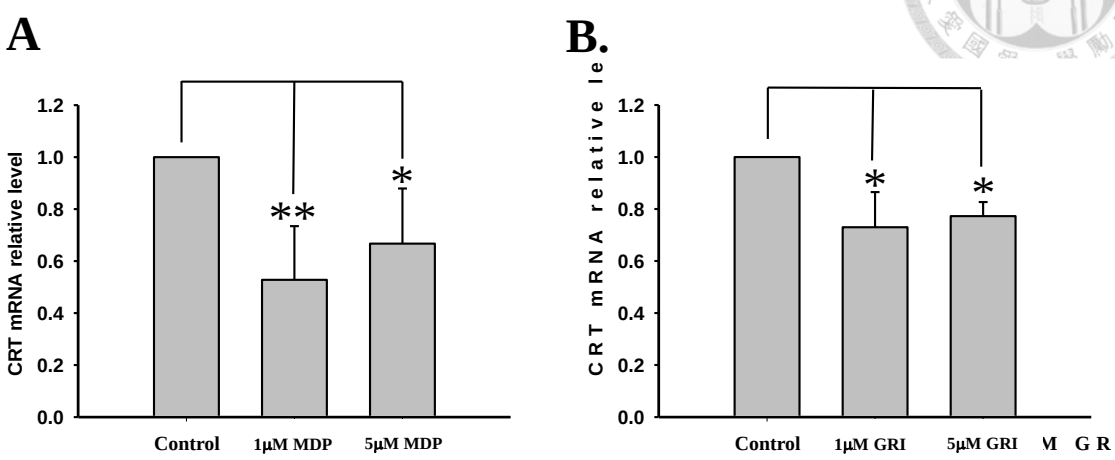

Control
sh-LPA1
sh-LPA3
C
LPA
C
LPA
C
LPA
CRT
63
GAPDH
35
(kDa)

Fig.5 LPA_1 and LPA_3 are involved in up-regulation of CRT expression in PC-3 cells. Starved PC-3 cells were separately treated with methanol/chloroform/ DMSO control, LPA (5 μM), **(A)** LPA+Ki16425 (1 μM) or **(B)** LPA+AM966 **(**1 μM**)** for 90 minutes. *CRT* mRNA levels were analyzed by qPCR. **(C)** Starved PC-3 cells were treated with DMSO control, (2S) OMPT (0.05 or 0.1 μM) for 90 minutes. *CRT* mRNA levels were analyzed by qPCR. (A) N=4 ; (B) N=3, (C) N=3, ANOVA. (D)(E) *LPAR1* or *LPAR3* mRNA knockdown efficiency were analyzed by qPCR. Control and *LPAR1* or *LPAR3* shRNA transfected cells were starved for 12-16 hours before being harvested. N=3, t-test. (F) *CRT* mRNA levels were analyzed by qPCR, Both sh-LPA1 and sh-LPA3 show lower *CRT* mRNA. N=3, ANOVA. (G) protein levels after 4 hours of methanol/chloroform control or 5 μM LPA treatment were analyzed by Western blotting. GAPDH was used as loading control. Both sh-LPA1 and sh-LPA3 compromise induction effect of LPA on CRT. N=3. (* $P<0.05$; ** $P<0.01$; ***$P<0.001$)

Figure 6



LPA_2 agonist: MDP

LPA_2 agonist: GRI977143

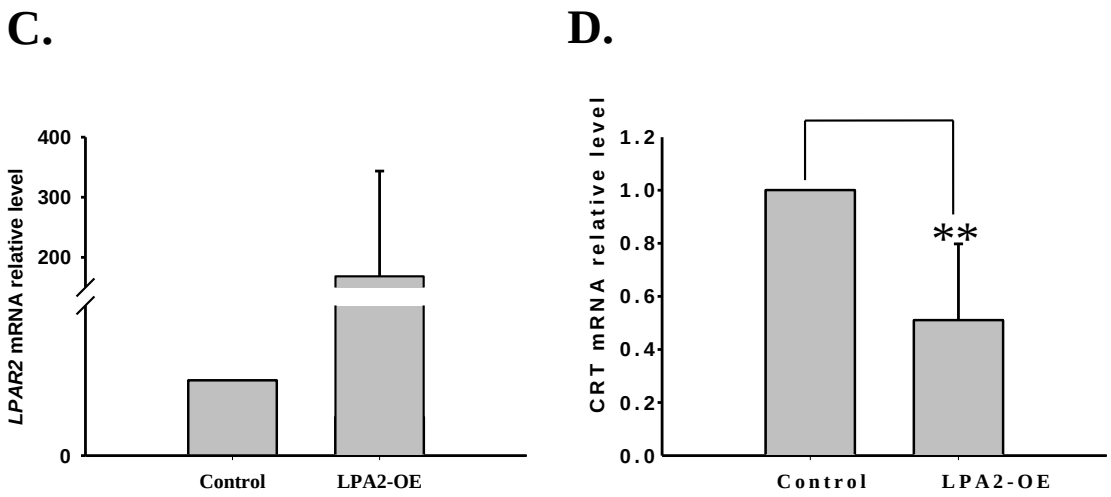


Fig.6 LPA_2 is involved in down-regulation of *CRT* mRNA expression in PC-3 cells. (A) Starved PC-3 cells were treated with methanol control, MDP (1 or 5 μM) for 90 minutes. *CRT* mRNA levels were analyzed by qPCR. N=4, ANOVA. (B) Starved PC-3 cells were treated with DMSO control, GRI (1 or 5 μM) for 90 minutes. *CRT* mRNA levels were analyzed by qPCR. N=4, ANOVA. (C) LPA transient overexpression efficiency and (D) relative CRT expression level were analyzed by qPCR. N=3, t test. (* $P<0.05$; ** $P<0.01$; ***$P<0.001$)

Figure 7

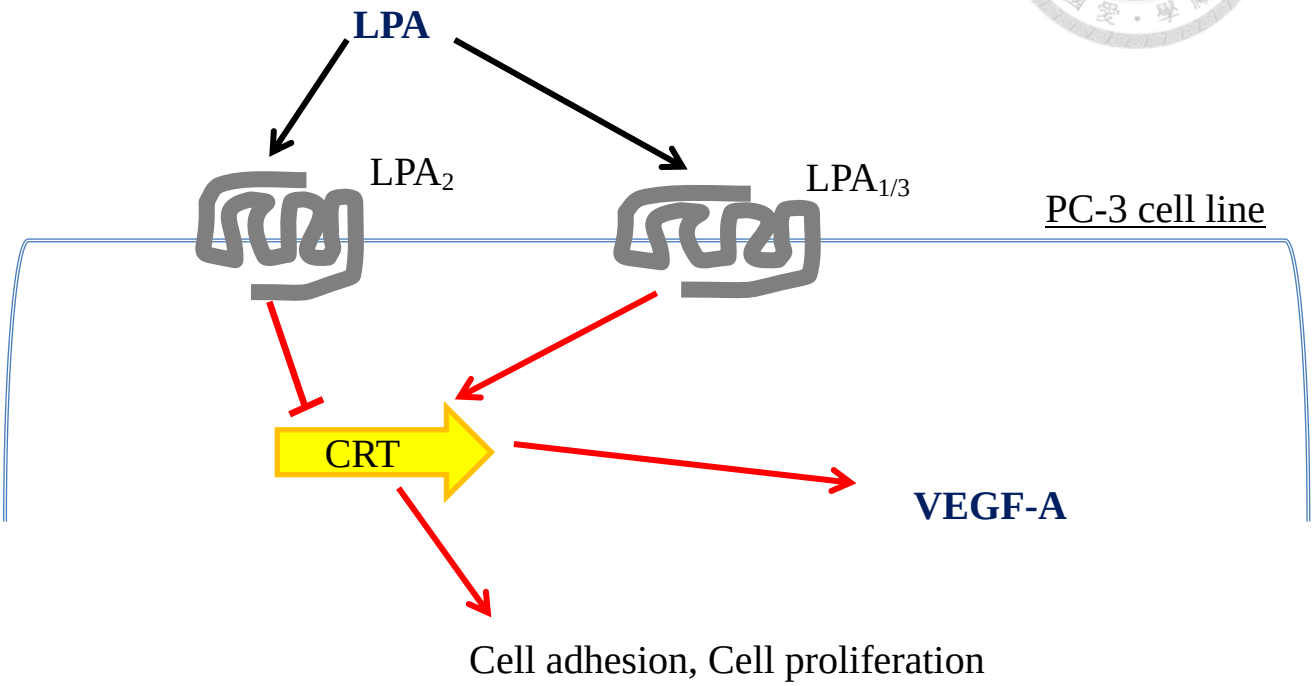
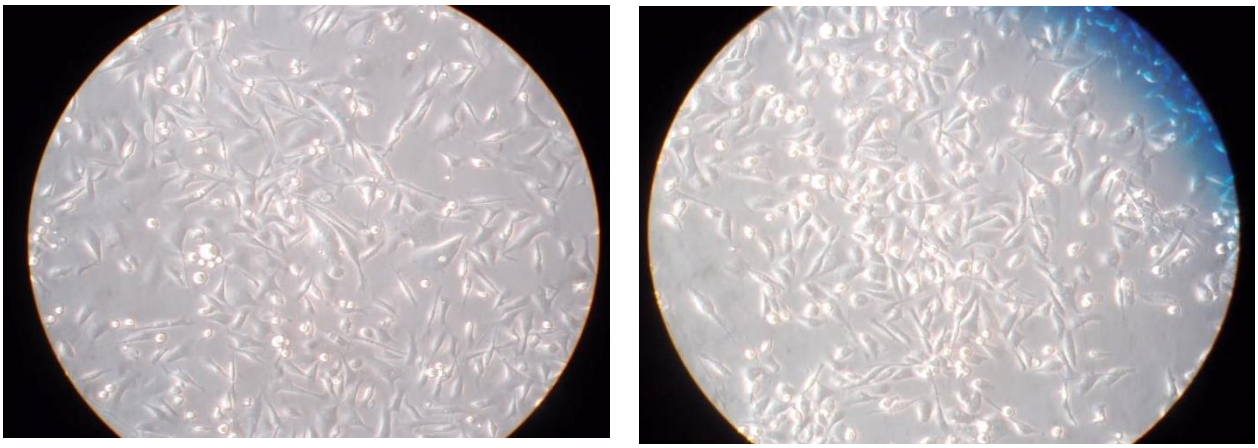


Fig.7 Schematic illustration summarizing the mechanism of LPA-dependent CRT expression in late stage prostate cancer.

Supplemental Figures

Figure S1

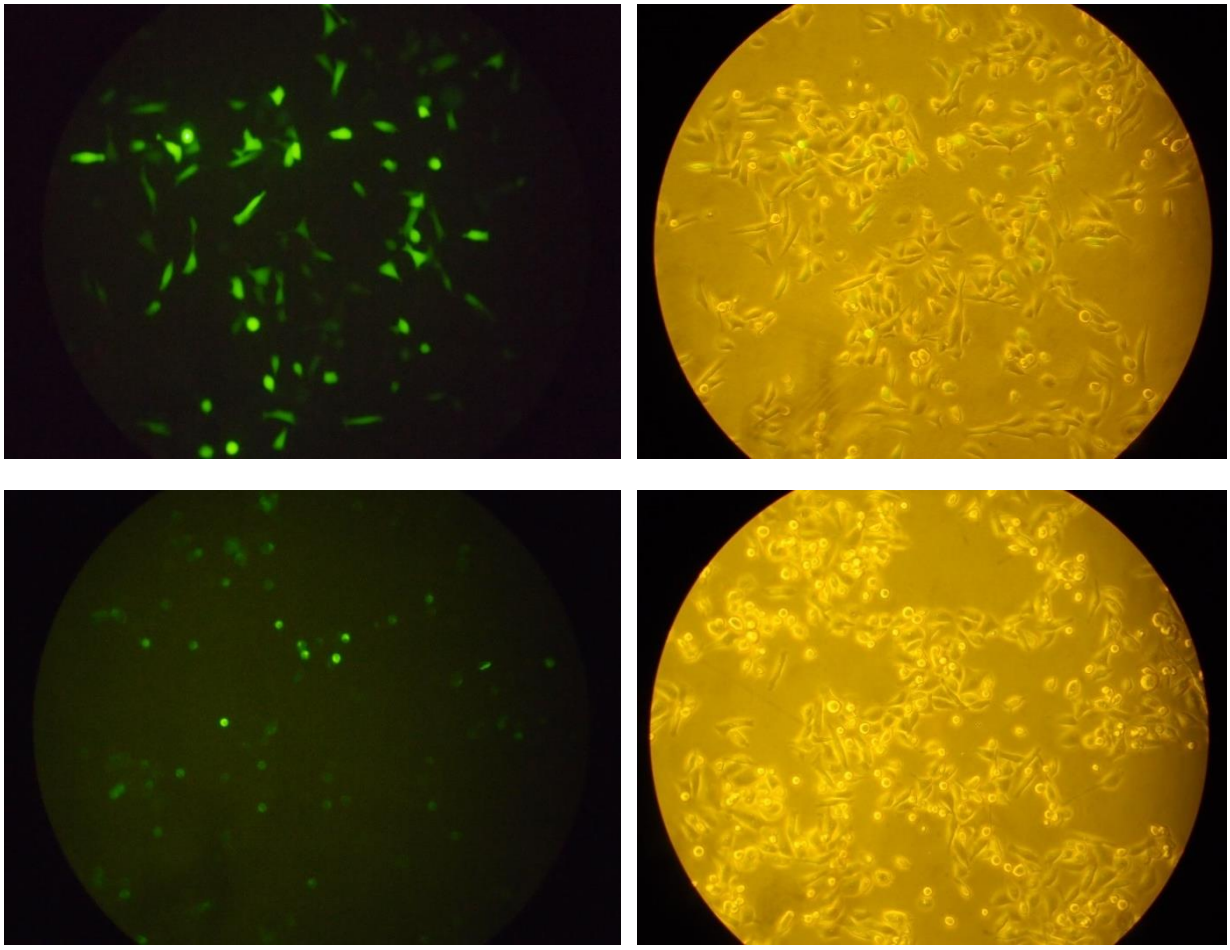


Control CRT-KD

Fig. S1 Morphology comparison between CRT knockdown and Control PC-3 cells.

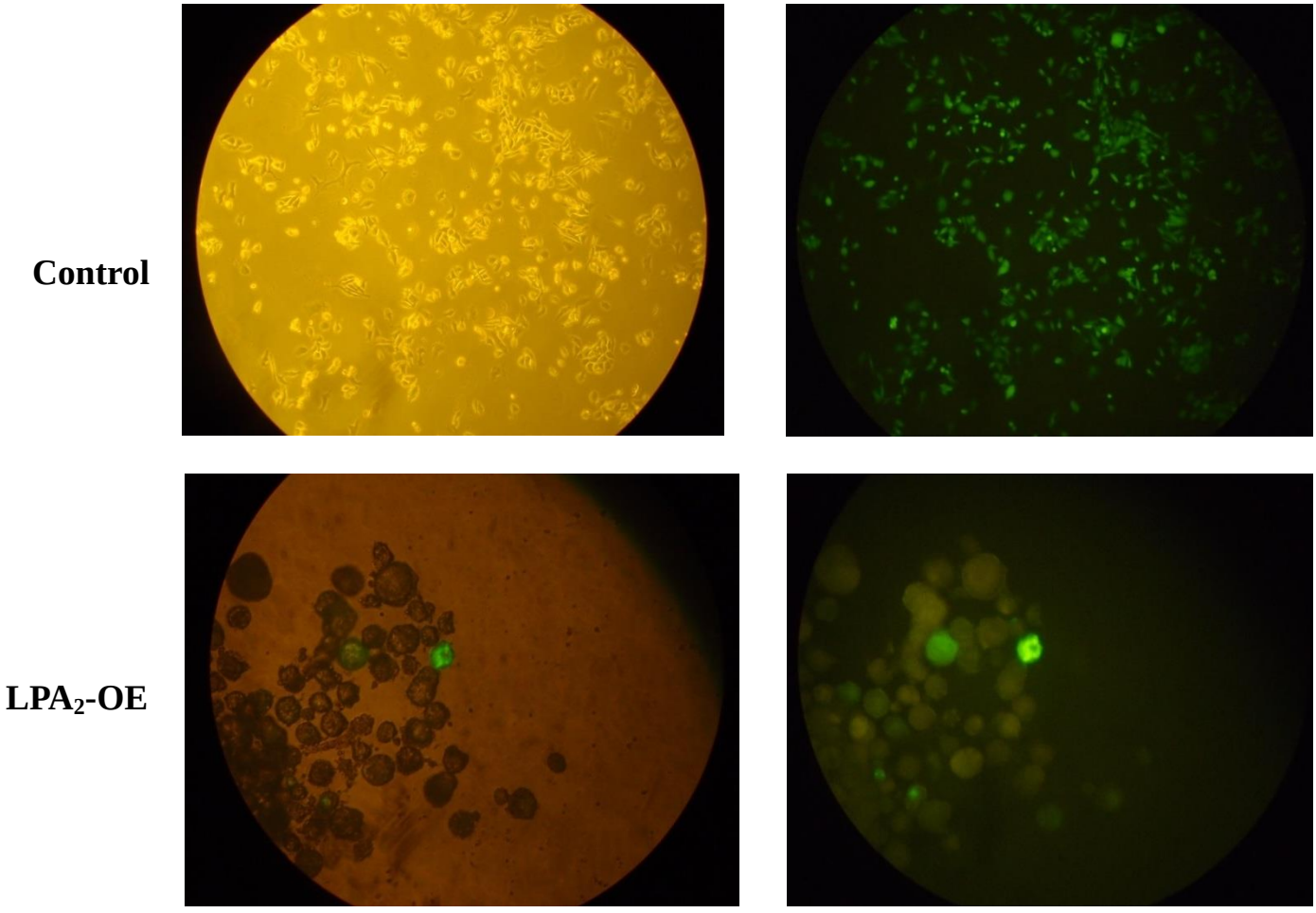
Figure S2

A



30 hours after transfection

B



2 months after transfection

Fig. S2 Transient LPA_2 overexpression PC-3 cells show more round shape cells than control PC-3 cells. (A) 30 hours after transfection. (B) 2 months after transfection.

Figure S3

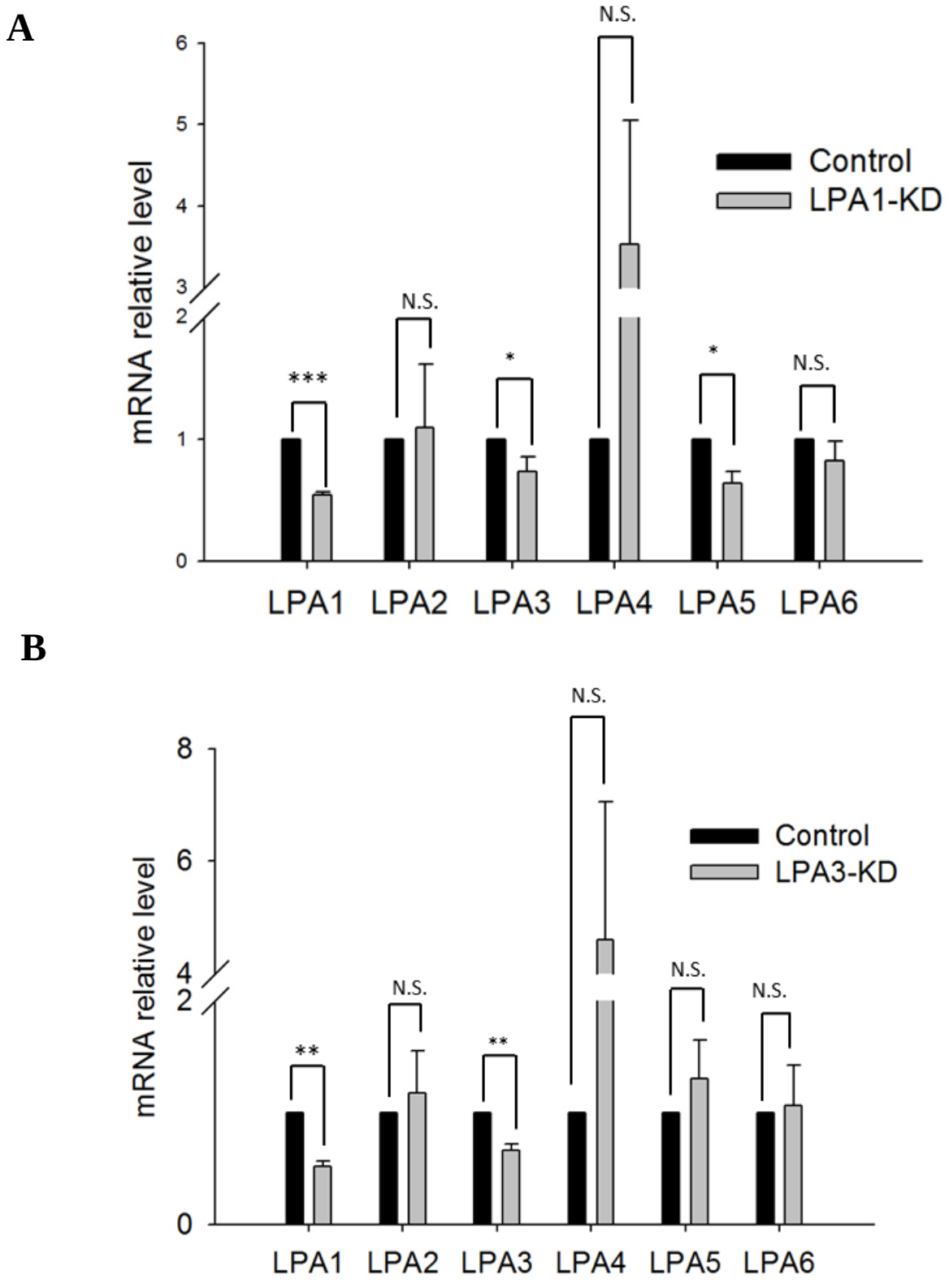

A
mRNA relative level
Control
LPA1-KD
N.S.
N.S.
N.S.
N.S.
***
*
*
LPA1
LPA2
LPA3
LPA4
LPA5
LPA6
B
mRNA relative level
Control
LPA3-KD
N.S.
N.S.
N.S.
N.S.
**
**
LPA1
LPA2
LPA3
LPA4
LPA5
LPA6

Fig. S3 Knockdown efficiencies of stable sh-*LPAR1* PC-3 cells and stable sh-*LPAR3* PC-3 cells. (A) LPA1-KD: stable sh-*LPAR1* PC-3, N=3, t-test. (B) LPA3-KD: stable sh-*LPAR3* PC-3, N=3, t-test.

Figure S4

A

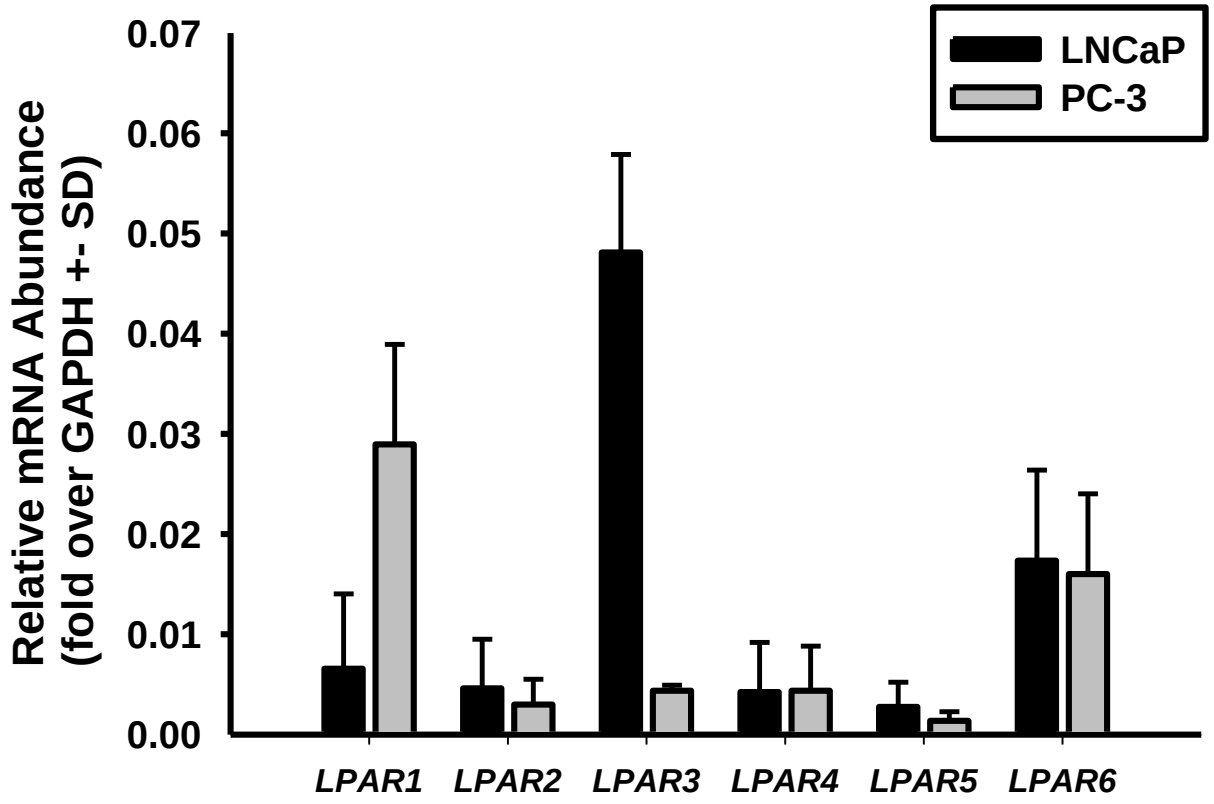


Fig.S4 LPA receptor mRNA profile of LNCaP cells and PC-3 cells under the incubation condition without starvation. (A) LNCaP cells or PC-3 cells (3×10^5 cells/2mL) were seeded on 6-well plate then incubated with RPMI-1640 medium containing fetal bovine serum (FBS) for 48 h before harvest. *LPAR1-6* mRNA expression levels were analyzed by qPCR. N=3.