行政院及所屬各機關出國報告

（出國類別：研　究）

男性荷爾蒙接受器在婦產科的應用

服務機關：台北榮民總醫

出 國 人：職　　稱：主 治 醫 師

姓　　名：王　鵬　惠

出國地區：美　國

出國日期：91.07.17 至 92.07.01

報告日期：92 年 07 月 21 日

系統識別號:C09101909

公 務 出 國 報 告 提 要

頁數: 40　含附件: 是

報告名稱:

男性賀爾蒙接受器在婦產科的應用

主辦機關:

行政院輔導會臺北榮民總醫院

聯絡人／電話:

／

出國人員:

王鵬惠　行政院輔導會臺北榮民總醫院　婦產部　主治醫師

出國類別: 進修　研究

出國地區: 美國

出國期間: 民國 91 年 07 月 17 日 -民國 92 年 07 月 01 日

報告日期: 民國 92 年 07 月 21 日

分類號/目: J3／醫療　J3／醫療

關鍵詞:　男性賀爾蒙接受器在婦產科的應用

內容摘要: 基於部門及國家的需要，於2002年6月份於國立陽明大學臨床醫學研究所獲得博士學位後，隨即奉派出國，主攻男性荷爾蒙接受器在女性生育器宮所扮演的角色。研究單位是由男性荷爾蒙接受器的發現者及專利所有權擁有者張傳祥教授所帶領的團隊，位於美國紐約州的羅徹斯特大學，在張教授的指導下，從事男性荷爾蒙接受器剔除雌鼠的生育能力研究，回國前，男性素荷爾蒙接受器剔除雌鼠的生育力，已做一番整理以及投稿出去，期望有一個好的結果。 而對國外實驗室擁有大量的資源及延攬世界一流學者共同創作，心想國內研究環境萬萬不及，唯有更加倍的努力，以智力工作努力來填補外在硬體及軟體設備的不足。 總之，在這一年之中，對於國外實驗室的運作方式有很深的感觸，欲取人之長截己之短，期能再創個人高峰，併爲國內研究貢獻一己之力。

本文電子檔已上傳至出國報告資訊網

目　　次

摘要

基於部門及國家的需要，於2002年6月份於國立陽明大學臨床醫學研究所獲得博士學位後，隨即奉派出國，主攻男性荷爾蒙接受器在女性生育器官所扮演的角色。研究單位是由男性荷爾蒙接受器的發現者及專利所有權擁有者張傳祥教授所帶領的團隊，位於美國紐約州的羅徹斯特大學，在張教授的指導下，從事男性荷爾蒙接受器剔除雌鼠的生育能力研究，回國前，男性素荷爾蒙接受器剔除雌鼠的生育力，已做一番整理以及投稿出去，期望有一個好的結果。

而對國外實驗室擁有大量的資源及延攬世界一流學者共同創作，心想國內研究環境萬萬不及，唯有更加倍的努力，以智力工作努力來填補外在硬體及軟體設備的不足。

總之，在這一年之中，對於國外實驗室的運作方式有很深的感觸，欲取人之長截己之短，期能再創個人高峰，併為國內研究貢獻一己之力。

正文

目的

鑑於生物科技的日行千里，深覺有進一步在分子生物上的研讀，故於民國 88 年進入國立陽明大學臨床醫學研究所就讀，歷經三年時間，於民國 91 年 6 月獲得博士學位。部門為進一步使得部內研究乃以與國外接軌，就在男性素接受器的發現者及專利權所有者美國羅徹斯特大學張傳祥教授的支持下，赴美為期一年的博士後研究，期能將國內所學與國外一流學府做進一步學術交流，以截長補短為部門及國內研究盡一份心力。

過程

91 年 7 月 17 日看完最後一天的門診，從早上、下午到晚上直到 10 點才結束，後 7 月 18 日搭乘上午 9 點的飛機飛往美國，第一站抵達美國底特律市，因為中途遇暴風圈導致飛機嚴重誤時，到底特律市已無飛機可搭，只好花了 70 塊美金在底特律住了一晚，隔日才轉機到羅徹斯特去，接機者是一位長庚醫院的腫瘤科醫師—徐醫師，其升上主治醫師後，赴美接受博士訓練，目前為四年級，徐醫師非常地親切熱誠，安排住宿、家俱及身份證明，然後進行我的實驗室生涯。我有二個主要的題目，一為男性素接受器在卵巢癌細胞的角色，另外題目為男性素接受器剔除後對雌鼠生育力的影響。

美國的實驗室環境比起國內真的是奢侈多

了，像買試劑、買材料、二次水均輕易拿到，且效率極佳，實驗室規模頗大，有十多位研究生，超過二十位以上的博士後研究，有四個助理教授，工作上大多數同事都非常認真，老師也非常的認真，每天均到實驗室，也督促大家的實驗進步，每週兩次的研究報告，因為人數眾多，所以每兩週需上台做報告，上台做報告的震憾教育，直覺國內的老師實在太好，在美國學生有時上台報告十分狼狽，電得很凶，有時所有實驗結果、假設會被批評的一文不值，對老師們能在國外環境生存下去且做得非常突出，真是打從心裡萬分敬佩。

剛開始，個人因為不純熟，也不熟悉實驗方法、實驗環境，所以也頗為辛苦，每天從早忙到晚，也不見得有何成果發現，前半年主要從事分

子生物的研究，主要是卵巢癌中男性素接受器及男性素接器來幫助因子的相互關係，後半年主要從事雌鼠生育能力的檢定，從配種、選種到治療、解剖、組織處理、切片、染色均一手包辦，由過程中也深深了解在國內研究所所學的真的非常非常的重要，事實上台灣的教育真的不比美國差，只可惜我們的軟硬體真的差人一大截，比如在學校中所有當期，即時的期刊，我們都很快立即從網路獲得而且是全文資料，反觀國內環境，這常常比不上，所有最新的訊息在台灣至少有一到兩個月的延遲，所以有時要跟上〝世界的主流〞相當的困難，這是很深的感觸。

後半年主要從事雌鼠生育能力的檢定從配種、篩檢、藥物排卵刺激及卵泡收集、組織處理、切片染色以及分子生物的研究，完全一手包辦，

每日清晨六點不到即到實驗室工作，一直到夜晚九點、十點才拖著疲憊的身軀回家，經過一年慘淡的經營下，我們發現男性荷爾蒙接受器的雌鼠的重要性，遠比我們以前的認知為大，因為失去男性荷爾蒙接受器的雌鼠，其生育能力大幅降低，而且很早就進入卵巢衰竭的時期。詳細研究其機轉，其可能性包括：①卵巢的濾泡發育過程出現瑕疵，導致存在大量不成熟且退化性的濾泡，以及少有成熟濾泡的發現及黃體形成缺陷②所排出卵泡的結構也異常，導玫卵泡存活降低，受精力不佳③子宮的發育及成熟也受到干擾，進一步造成缺乏男性素接受器雌鼠的所生育能力大幅下降

回國前，男性素荷爾蒙接受器剔除雌鼠的生育力，已做一番整理以及投稿出去，期望有一個

好的結果。

心得

美國的居住環境好的無庸置疑，地廣人稀，戶戶有庭院，有翠地、有花草，對幼兒的照顧無微不至，對於殘障人士的保障也極佳，不得不佩服他們對人類的尊重，尤其是弱勢族群，開車的禮讓也遠比台灣好，我想這是環境使然，當然與教育也有關係，另外美國極重視家庭，照顧小孩是父母雙方共同負擔，對小孩多予以正面鼓勵，養成其樂天進取的觀念，雖然在聰明才智感覺其未必比得上東方人，不過唯有讀書高這個觀念並不是很明顯，反倒比較像極為功利主義的社會，只要能賺錢即可，不過，美國人的消費觀念及儲蓄觀念真的大大與國人不同，不知何者為優，何者為劣。

在美國實驗室一年發覺大陸人很多，普遍上

較台灣去的同學認真，這個狀況在最近數年中可能愈來愈嚴重，美國人工作態度非常認真，但無加班觀念(過勞)，這也是與國人大為不同。

建議

國內應整合資源，對抗美國的大規模作戰，比如來說，全文文獻的取得，國內真有待加強，每個醫院、每個學校都付錢訂雜誌，但是每個單位所擁有的期刊卻有限，應統籌所有經費建立國家型網路圖書館，集中所有資源，讓世界立即的訊息、研究能輕易獲得，這樣國內才有萬能趕上瞬息萬變的科學進展。

Subfertility in Female Mice Lacking Androgen Receptor

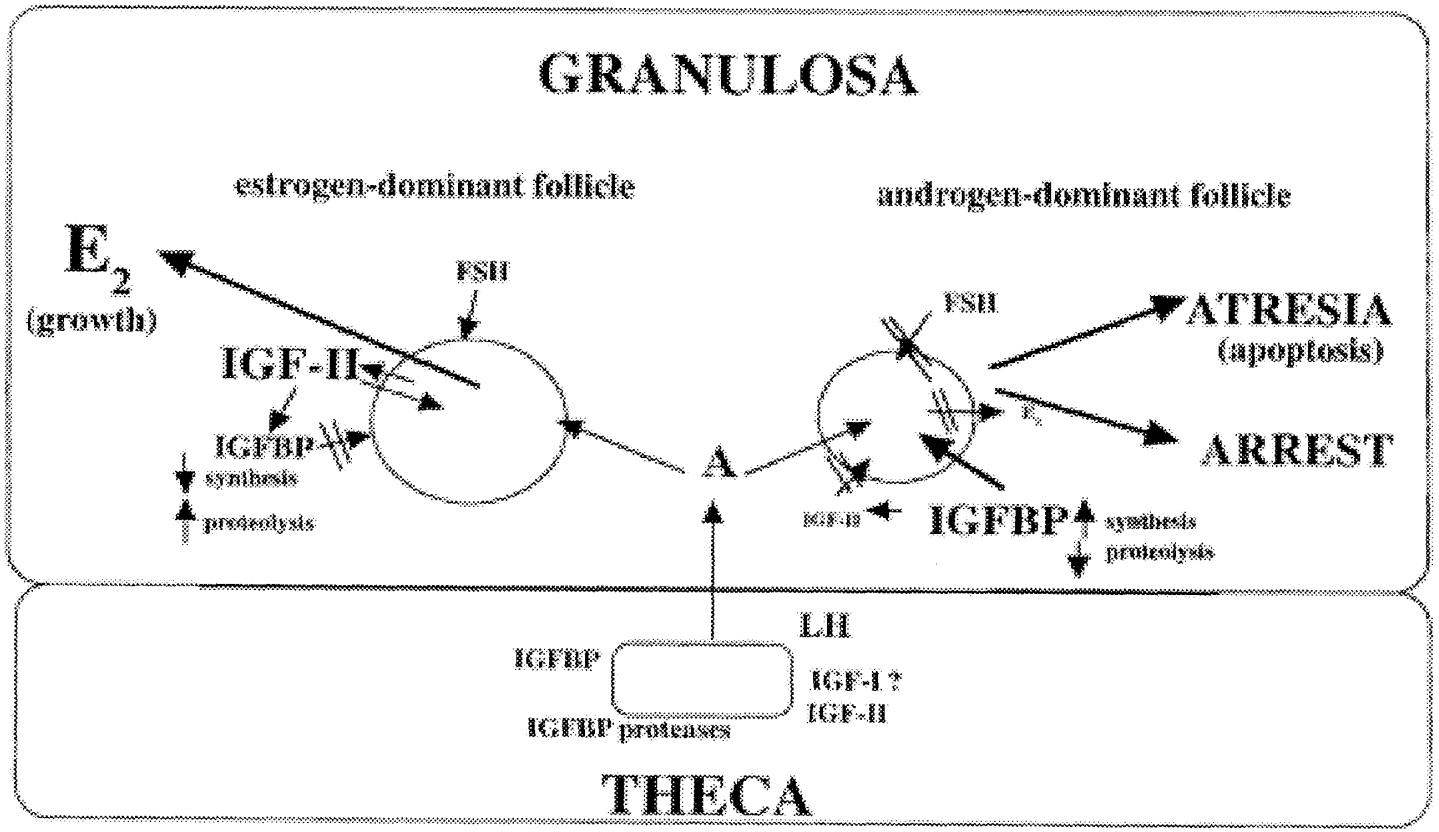
Peng-Hui Wang, M.D., Ph.D.; Chawnshang Chang, Ph.D.

George Whipple Laboratory for Cancer Research, Departments of Pathology and Urology, University of Rochester Medical Center, Rochester, New York 14642, USA

Department of Obstetrics and Gynecology, Taipei Veterans General Hospital and National Yang-Ming University School of Medicine, Taipei 112, TAIWAN

Background ?

Wang PH



Introduction (1)

- **A/AR and E2/ER**
  - **Facilitate FSH actions in early antral follicles, a critical period of FSH influence on follicular development, by positively regulating FSH receptor (FSHR)**
    - Tetsuka and Hillier 1996; Cardenas and Pope 1997; Tetsuka and Hillier 1998; Durlinger et al. 1999; Palter et al. 2001; Rosenfeld et al. 2001.

Introduction (2)

- **How A/AR plays roles in ovarian follicle development remains controversial**
  - **Antiproliferative, atretogenic effect or promoting follicular growth ?**
    - Hsueh et al. 1994; Richards 1994; Billig et al. 1993; Cheng et al. 2002; Daniel and Armstrong 1980; Vendola et al. 1999; Cardenas et al. 2002 .

Introduction (3)

- Controversies: lack of an animal model to study AR roles in females (infertile males without functional AR)
- Using cre-lox conditional knockout strategy → female mice lacking AR.
- Provide *in vivo* roles of AR in female fertility and ovulation.

Subfertility

Pup number per litter

Genotype	Pup No./ Litter
$AR^{+/+}$	8.3 ± 2.1
$AR^{+/-}$	7.2 ± 2.2
$AR^{-/-}$	4.6 ± 2.5

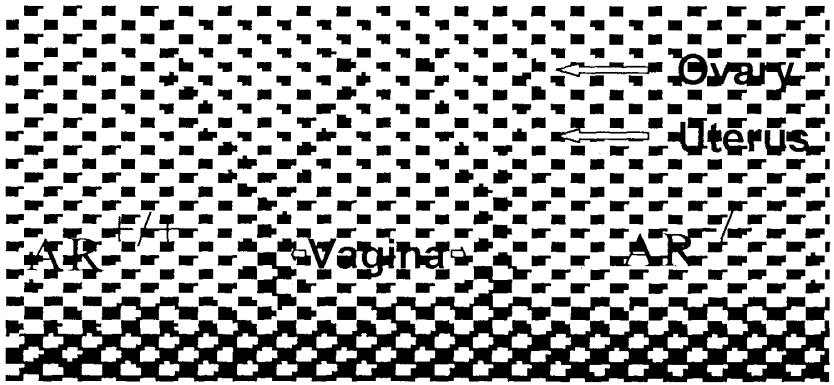
Shown for each is the mean ± SEM.

T test, age-matches $AR^{+/+}$ vs $AR^{+/-}$ vs $AR^{-/-}$

1. **The smaller litter-size**
2. **30% $AR^{-/-}$ female mice become infertile after 5-6 months (continuing mating test)**

Smaller size of reproductive organs

	$AR^{+/+}$ (mg)	$AR^{-/-}$ (mg)
Ovary	4.3 ± 0.3	3.5 ± 0.4
Oviduct	2.1 ± 0.2	2.7 ± 0.2
Uterus	34.3 ± 1.6	44.3 ± 3.5
Vagina	50.9 ± 5.2	63.3 ± 2.5



Fewer oocytes during superovulation test

Oocyte yield after superovulation

Genotype	Age	N	Oocyte Count	
			Average	Range
$AR^{+/+}$	25	4	26.5 ± 7.9	25-34
$AR^{+/-}$	25	4	25.5 ± 4.9	20-32
$AR^{-/-}$	25	4	16.3 ± 8.2	7-27

Shown for each is the mean ± SEM.

T test, age-matches $AR^{+/+}$ vs $AR^{+/-}$ vs $AR^{-/-}$

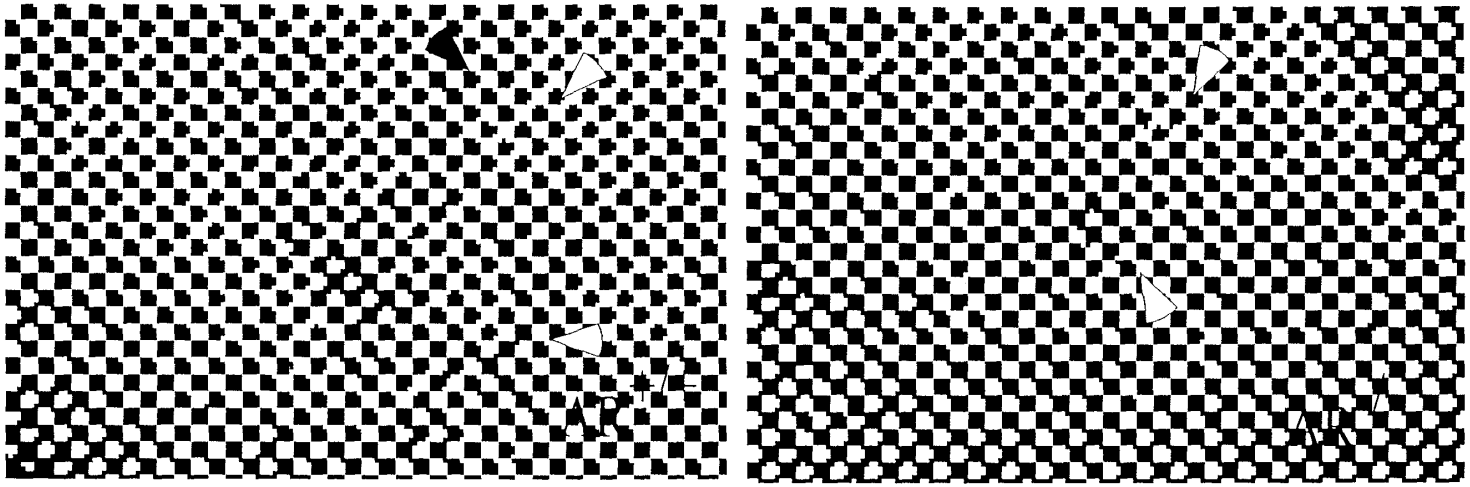
Wang PH

Decreased viability of ovulated oocytes

- Oocytes free of cumulus granulosa cells

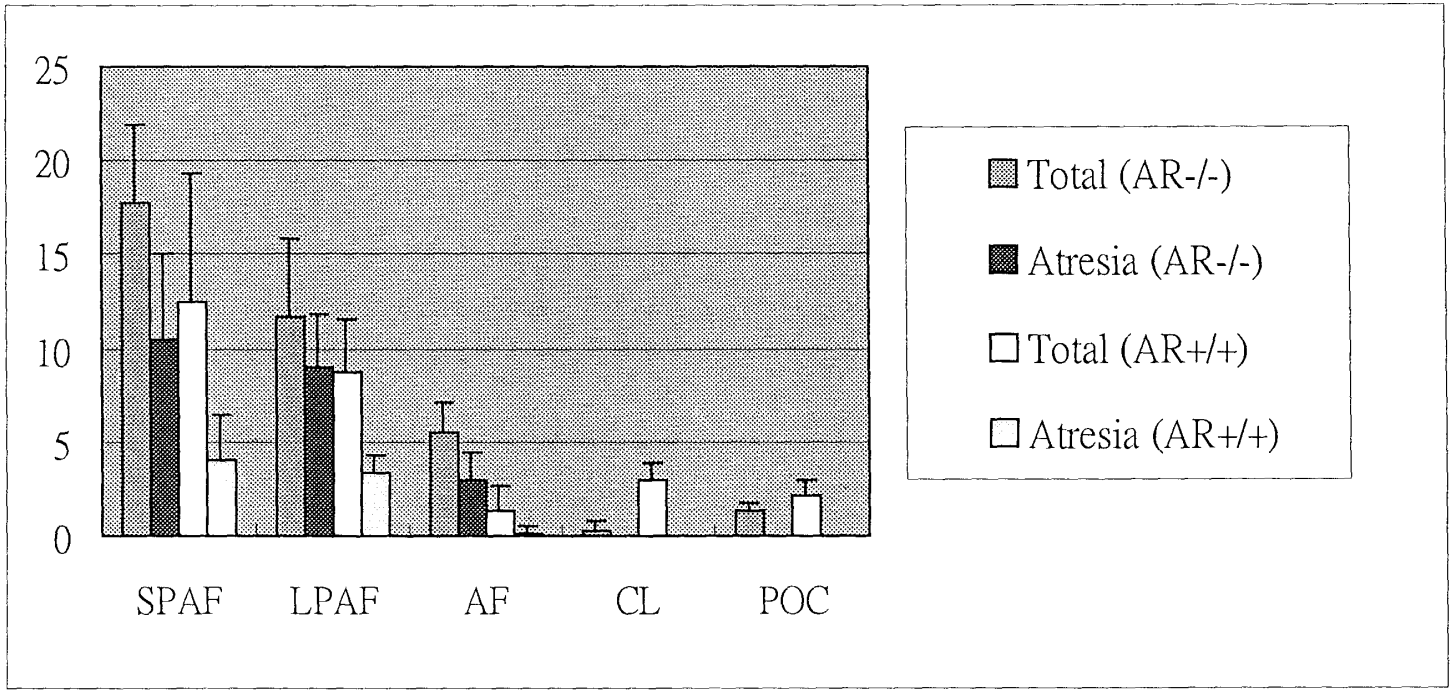
  $AR^{+/+}$: $AR^{-/-}$ = 23% : 36%

- COC (cumulus-oocyte-complex) less condense and less intact



Increased immature follicles
Increased atretic follicles
Less corpus luteal formation

Wang PH



SPAF: small preantral follicle, LPAF: large preantral follicle, AF: antral follicle, CL: corpus luteum, POC: postovulation cyst

Wang PH

Reduced reproductive life span and enhanced termination of breeding capacity

- Same number of primordial follicles in most of mice
- Increased immature follicles
- Increased atretic follciles

⇩

- Exhaustion of the primordial follicles

⇩

- Ovarian failure & reproductive life span

Wang PH

Molecular Background

Model of cAMP Actions in Granulosa Cells

FSH via activation of A kinase induces the expression of specific genes, such as Sgk.

FSH via PI3-K and its downstream targets, PDK1 (but not A-kinase), phosphorylates PKB in a manner that mimics and enhances IGF-I-induced phosphorylation and activation of PKB.

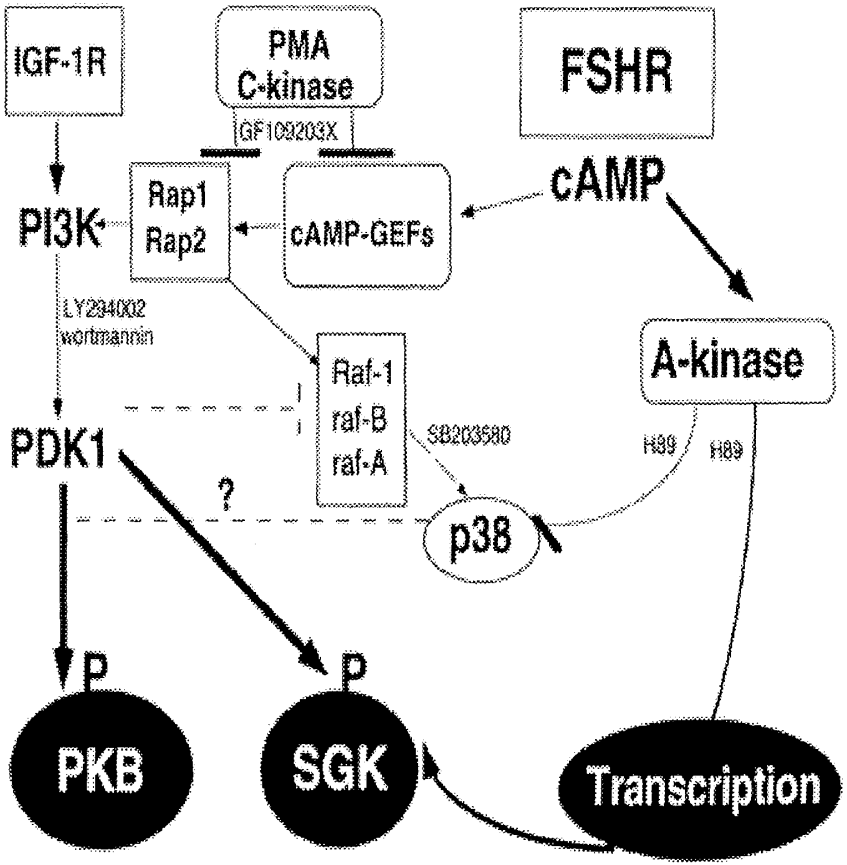
IGF-I via PI3-K also phosphorylates PKB and Sgk.

PMA (via C kinase) inhibits cAMP-mediated (but not IGF-I-mediated) phosphorylation of PKB and Sgk.

FSH likely acts via a cAMP-GEF/ras-Rap/Raf pathway, which is upstream of PI3-K.

cAMP, by mechanisms that are also independent of A-kinase and PI3-K, also leads to the phosphorylation of p38MAPK, likely by a cAMP-GEF/ras-Rap/Raf pathway.

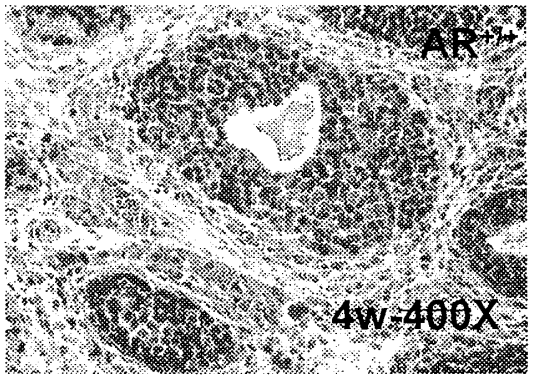
Thus, FSH appears to activate both A kinase-dependent and A kinase-independent pathways in granulosa cells, each of which controls specific kinase cascades.

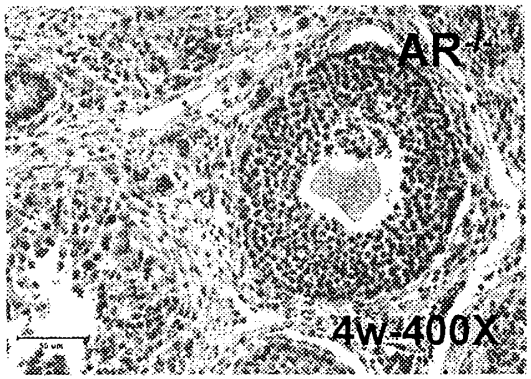


Wang PH

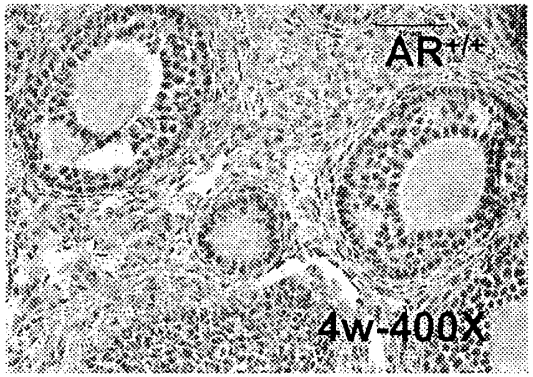
Possible molecular mechanism

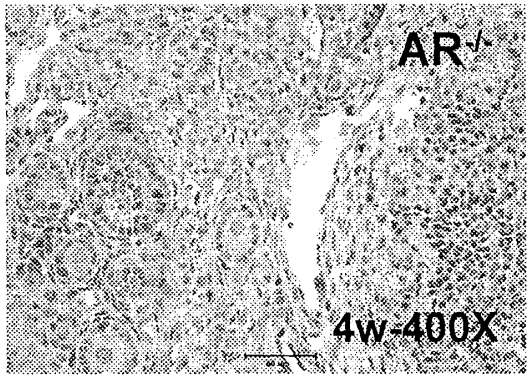
1. IGF-I/IGF-IR→Akt pathway
   (a) IGF-IR mRNA and protein ⇩





   (b) Akt phosphorylation ⇩

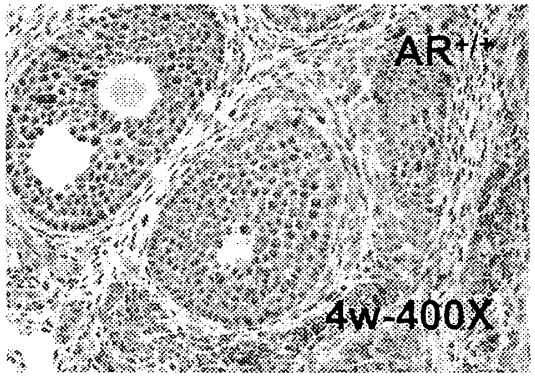


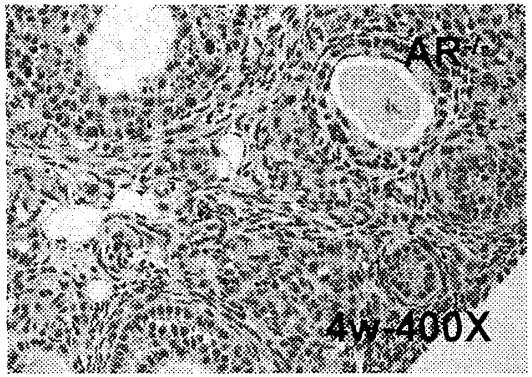


   1) increased apoptosis of granulosa cells
   2) progesterone decrease 50%

Wang PH

2. IGF-I/IGF-IR-FOXO3a transcriptional factor



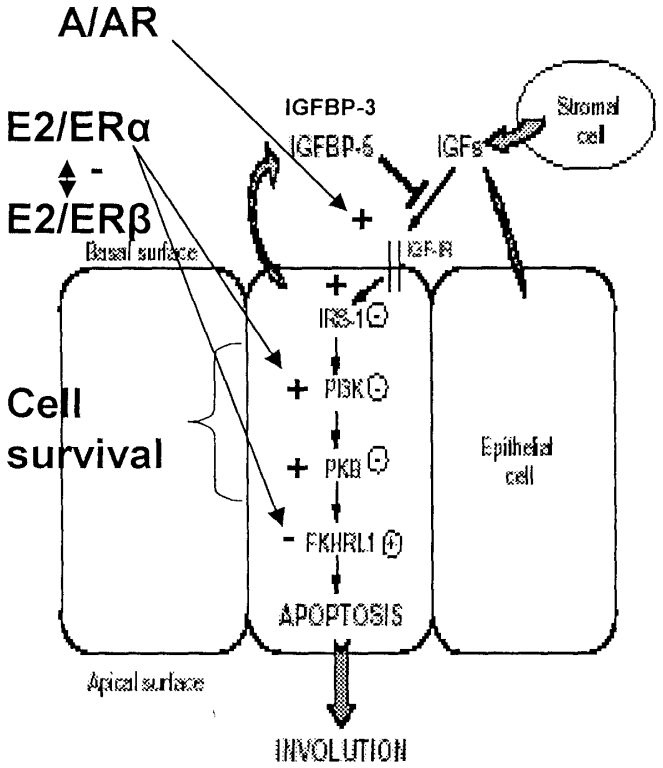


- More nuclear accumulation of FOXO3a will trigger the transcription of target genes needed in apoptosis then result in acceleration consumption of follicular pool in $AR^{-/-}$

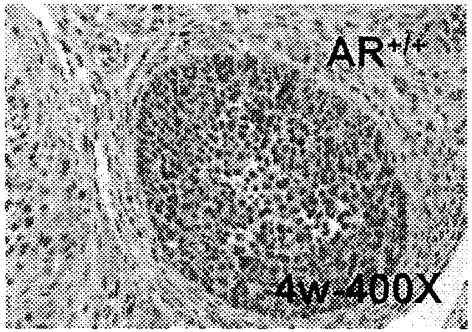
Wang PH

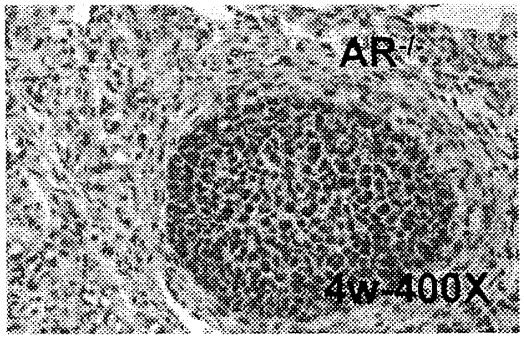
The target genes of Akt involving cellular survival

- Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor (FKHRL1)→ Brunet et al Cell 1999;96:857
- Presence of phosphorylated form in cytoplasm indicated cell survival → in contrast, presence of nuclear form indicated cell undergoing apoptosis

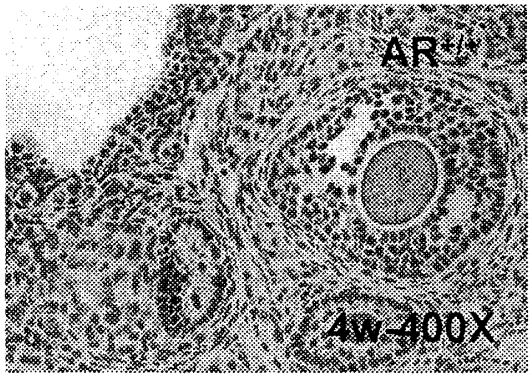


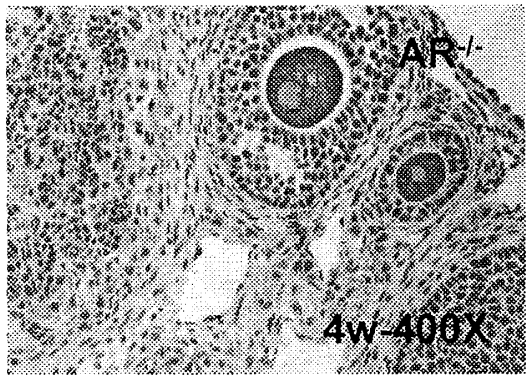
Increased ERβ in granulosa cells





AR$^{-/-}$ → ERβ ⇧→ FSH/FSHR ⇧





Results: AR-/- → ER β ⇧→ FSH/FSHR ⇧

⇩

Granulosa cell is highly proliferative

Wang PH

Wang PH

Summary of AR roles in folliculogenesis

1 $AR^{-/-}$ → IGF-I/IGF-IR ⇩ → Akt ⇩ → Apoptosis ⇧

2 $AR^{-/-}$ → IGF-I/IGF-IR ⇩ → Foxo3a nuclear accumulation ⇧ → Apoptosis ⇧

3 $AR^{-/-}$ → ERβ ⇧→ FSH/FSHR ⇧→ Proliferation ⇧

Conclusion

Wang PH

Subfertility & Defective Folliculogenesis

- Ineffective folliculogenesis with increased immature follicles and increased atretic follicles and disregulation of granulosa cell proliferation and apoptosis
- Disorders of follicular maturation and decreased rates of spontaneous ovulation with less corpus lutea formation
- Defects of COC with increased percentage of oocytes free from cumulus cells and easy dissociation of oocytes from COC
- Abnormal maturation of uterus with smaller size, luteal defects, and poor development of pregnancy-associated decidualization

ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Biochemical and Biophysical Research Communications 305 (2003) 462 469

BBRC

www.elsevier.com/locate/ybbrc

Interleukin-6 differentially regulates androgen receptor transactivation via PI3K-Akt, STAT3, and MAPK, three distinct signal pathways in prostate cancer cells☆

Lin Yang,[1] Liang Wang,[1] Hui-Kuan Lin, Pu-Yeh Kan, Shaozhen Xie, Meng-Yin Tsai, Peng-Hui Wang, Yen-Ta Chen, and Chawnshang Chang*

George Whipple Lab for Cancer Research, Departments of Pathology, Urology, Radiation Oncology, and The Cancer Center, University of Rochester Medical Center, Rochester, NY 14642, USA

Received 10 March 2003

Abstract

The effects of IL-6 on prostate cancer cells are well documented yet remain controversial. Some reports suggested that IL-6 could promote prostate cancer cell growth, while others showed that IL-6 could repress prostate cancer cell growth. Here, we systemically examined various IL-6 signaling pathways in prostate cancer cells and found that IL-6 could go through at least three distinct pathways to modulate the functions of androgen receptor (AR), a key transcriptional factor to control the prostate cancer growth. Our results show that IL-6 can enhance AR transactivation via either the STAT3 or MAPK pathways. In contrast, IL-6 can suppress AR transactivation via the PI3K-Akt pathway. Co-existence of these various signaling pathways may result in either additive or conflicting effects on AR transactivation. Together, our results indicate that the balance of these various pathways may then determine the overall effect of IL-6 on AR transactivation.

Keywords: AR; Transactivation; IL-6; PI3K; MAPK; STAT3

Prostate cancer is the second most prevalent cancer in males in the United States. There is evidence that IL-6 may play an important role in metastatic prostate cancer. It has been demonstrated that all of the commonly used prostate cancer cell lines (PC-3, DU145, and LNCaP) express receptors with a high affinity for IL-6 [1–4]. In addition, prostate cancer cell lines, PC-3 and DU145, have been demonstrated to secrete varying amounts of IL-6, whereas the hormone dependent cell line, LNCaP, does not secrete IL-6 [2–4]. Clinical data show that serum IL-6 levels are elevated in men with hormone-refractory prostate cancer and that these high serum IL-6 levels are accompanied by high levels of serum prostate specific antigen (PSA) [5]. However, proliferation studies of IL-6 in LNCaP cells have resulted in contrasting results. Addition of exogenous IL-6 to the culture media of LNCaP cells showed that cell growth was inhibited in a dose-dependent manner [2,6,7]. In contrast, other reports revealed cell growth stimulation after treatment with IL-6 [3,8,9]. The reasons for these differences have not been clarified to date, but suggest that IL-6 may exert divergent effects in human prostate cancer.

The androgen receptor (AR) is a 110-kDa nuclear protein that consists of several domains, including transactivation, DNA binding, nuclear localization, dimerization, and ligand binding domains [10–13]. The AR is expressed in normal prostate tissue and prostate cancers, and is a key transcription factor to control prostate cell growth. Activation of the AR in prostate cancer is being intensively investigated. After a ligand

☆ *Abbreviations:* AR, androgen receptor; PI3K, phosphatidylinositol 3(OH)-kinase; IL6, interleukin 6; wtAR, wild-type AR; DHT, 5α-dihydrotestosterone; MMTV, mouse mammary tumor virus; PSA, prostate specific antigen; luc, luciferase.

* Corresponding author. Fax: 1-585-756-4133.
E-mail address: chang@URMC.rochester.edu (C. Chang).

[1] Authors contributed equally to this work.

0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)00792-7

binds to AR, the ligand–receptor complex translocates into the nucleus and binds to specific androgen response elements (AREs) [14,15]. Some evidence shows that the AR could also be stimulated in the absence of its cognate ligand by a number of nonsteroidal compounds, such as various growth factors and protein kinase pathways [16,17].

Recent studies revealed that IL-6 could activate AR transactivation in an androgen-independent manner in LNCaP cells [18–20]. The PI3K pathway has been reported to be a major contributor in the signaling of IL-6, however, the role of PI3K in the activation of AR by IL-6 is controversial. Some studies suggested that PI3K may play a role in the activation of AR by IL-6, but another report showed that IL-6-mediated AR activation was not dependent on the PI3K pathway [18,21–24]. Moreover, although IL-6 is able to activate AR transactivation in an androgen-independent manner in LNCaP cells, IL-6 alone did not induce PSA expression and mouse mammary tumor virus (MMTV) promoter activity in PC-3 and DU145, in which AR was transiently expressed [18]. These results suggest that IL-6 may have distinct pathways in various prostate cancer cells. Accordingly, in the current report, we explored the availability of various IL-6 signaling pathways in various prostate cancer cells and compared what pathway is dominant in these various cell lines.

Materials and methods

Materials. pCDNA3-cAkt (a constitutively active Akt with a deletion at amino acids 4 129 replaced with a consensus myristylation domain) and pCDNA3-dAkt (a kinase deficient mutant, K179A) were from Dr. Robert Freeman [25]. pSG513-STAT3 and pSG513-STAT3β (a dominant-negative STAT3 with a point mutation) were from Dr. Rolf P. de Groot [26]. LY294002, U0126, and PD98059 were from Calbiochem and DHT was from Sigma. pCMV-AR, pSG5-AR, MMTV-luciferase (MMTV-luc) promoter, and a reporter containing 4 copies of ARE promoter-luciferase ((ARE)4-luc), pRL-SV40, and pRL-TK have been previously described [11]. Phospho-p44/42 MAP kinase (Thr202/Thr204) antibody and p44/42 MAP kinase antibody were purchased from Cell Signaling Technology.

Cell culture and transfections. The human prostate cancer PC-3 and DU145 cells were maintained in Dulbecco's minimum essential medium containing penicillin (25 U/ml), streptomycin (25 μg/ml), and 10% fetal calf serum (FCS). The human prostate cancer LNCaP cells were maintained in RPMI-1640 with 10% FCS.

Luciferase reporter assays. The cells were plated at 8×10^4 on 24-well plates and incubated with RPMI 1640 or Dulbecco's modified Eagle's medium containing 10% FBS for 24 h. Transfection was performed by SuperFect (Qiagen) according to the standard protocol. In brief, the total amount of plasmid DNA was adjusted to 1 μg/well by addition of control plasmid. After 3 h transfection, the medium was replaced with serum-free medium or 10% charcoal dextran treated FBS and treated with DHT, IL-6, or inhibitors. The cells were washed with PBS and harvested after 24 h. Cell lysates were prepared and used for luciferase assay according to the manufacturer's instructions (Promega). The results were obtained from at least three sets of transfection and presented as means ± SD.

PI3K activity assay. PI3K activity was determined as previously described [27]. Briefly, cells were washed twice with ice-cold PBS and lysed in RIPA buffer. The lysates were centrifuged and the protein content was determined. Five-hundred microgram aliquots from each sample were immunoprecipitated with 40 μl aliquots of pre-conjugated, monoclonal anti-phosphotyrosine (PY20) agarose beads (Santa Cruz, CA) by incubating overnight at 4 °C in 500 μl of immunoprecipitation buffer (190 mM NaCl, 50 mM Tris HCl, pH 7.4, 6 mM EDTA, and 2.5% Triton X-100). All subsequent steps were exactly as described [27]. Briefly, 30 μl aliquots from the kinase reaction assays were separated on thin layer Silica Gel 60 chromatography plates (EM Separations Technology), dried at room temperature, and autoradiographed.

Western blot analysis. Protein samples were prepared by lysing cells over ice in ice-cold RIPA buffer (50 mM Tris HCl, pH 7.4, 1% NP-40, 0.1% SDS, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, and 1 mM NaF). Cell lysates were centrifuged at 14,000*g* at 4 °C for 15 min. Protein content was determined using the *DC*-protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (50 μg) from cell lysates were denatured in sample buffer, subjected to SDS PAGE on 4 20% gels (Novex/Invitrogen, San Diego, CA), and transferred to nitrocellulose membranes. The blots were probed with specific primary antibodies as recommended by the suppliers. Appropriate HRP-conjugated secondary antibodies were used (1:5000) and visualized by enhanced chemiluminescence (Bio-Rad Laboratories, Hercules, CA).

Results

IL-6 differentially induces AR transactivation in various prostate cancer cells

We first investigated the effect of IL-6 on AR transcriptional activity in LNCaP cells by transient transfection with the MMTV-luc reporter plasmid. The region of the MMTV promoter that contains the AREs is required for androgen induction. As shown in Fig. 1A, IL-6 had minimal effect on MMTV-luc activity in the absence of DHT in the LNCaP cells. We then treated the LNCaP cells with a low concentration of DHT (0.1 nM) and a maximum induction (45-fold) of MMTV-luc reporter activity was obtained and addition of IL-6 (50 ng/ml) resulted in a 70-fold increase in MMTV-reporter activity relative to the control. However, in contrast to the LNCaP cells, co-transfection of MMTV reporter and wild-type AR with 1 nM concentration of DHT and 50 ng/ml of IL-6 in DU145 and PC-3 cells did not show the similar induction pattern as in LNCaP cells. In contrast, a slight suppressive effect on AR activity was observed (Figs. 1B and C), suggesting that the IL-6 signal may differentially modulate AR activity in various prostate cancer cells.

Synergistic increases in the induction of MMTV promoter activity by IL-6 with blocking of the PI3K pathway

The fact that AR target gene reporters were poorly induced in PC-3 and DU145 cells upon stimulation by

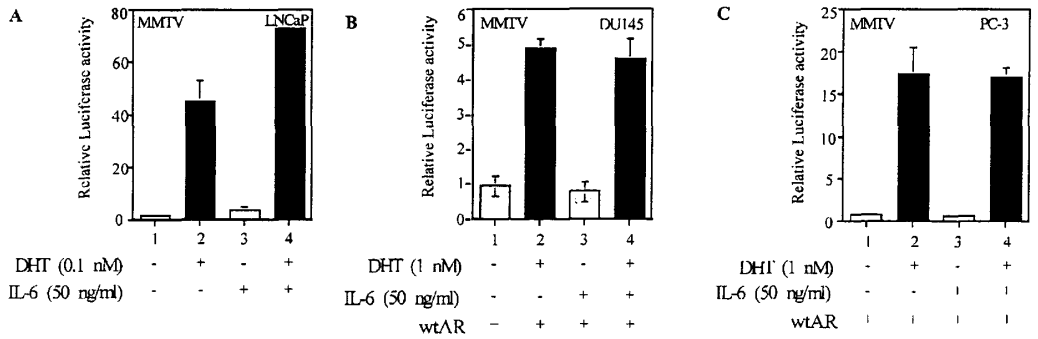


Fig. 1. IL-6 differentially modulated AR target gene in prostate cancer cell lines. (A) LNCaP cells were transiently transfected with MMTV-luc (300 ng/well) for 24 h and then incubated with 0.1 nM DHT or vehicle for additional 24 h under serum-free condition. (B,C) DU145 and PC-3 cells were co-transfected with wild-type AR and 300 ng/well MMTV-CAT or MMTV-luc, respectively, and then incubated with 1 nM DHT, 50 ng/ml IL-6, or vehicle for additional 24 h under serum-free condition, and then harvested. The internal control was 10 ng/well pRL-SV40 and the total amount of plasmid DNA transfected was adjusted to 1 μg/well by addition of the empty vector. The error bars represent the means ± SE of three independent experiments.

IL-6 led us to investigate the PI3K signaling in these two prostate cancer cell lines. We first investigated the effects of Δp85, the dominant-negative form of PI3K and P110, the active form of PI3K. As shown in Figs. 2A and B, addition of Δp85 enhanced AR transactivation in a dose-dependent manner. In contrast, addition of P110 repressed AR transactivation in a dose-dependent manner in DU145 cells. These data suggest that activation of PI3K pathway may result in the suppression of AR transactivation. We then investigated the effect of IL-6, the upstream regulator of PI3K, on the AR transactivation. As shown in Fig. 2C, addition of 1 nM DHT enhanced MMTV-chloramphenicol transferase (CAT) reporter activity 5-fold. Addition of IL-6, from 10 ng/ml to 100 ng/ml, slightly reversed this DHT-induced reporter activity (lanes 3, 4, and 5 vs lane 2). Addition of LY294002, a selective PI3K inhibitor, further enhanced DHT-induced reporter activity from 5-fold to 14-fold (lane 2 vs 6). Simultaneous addition of LY294002 and IL-6 synergistically enhanced reporter activity from 5-fold to 23–28-fold, suggesting that under blockade of PI3K condition, IL-6 may go through other pathways to stimulate AR activity, probably via MAPK signaling pathway, because MAPK inhibitor U0126 suppresses AR transactivation, which can further repress AR activity when the cells were treated with IL-6 (Fig. 2C, lanes 10–13). Together, results from Figs. 2A–C suggest that the lack of induction effect of IL-6 on AR transactivation may be due to enhancement of PI3K activity. To further confirm this hypothesis, we also assayed the PI3K activity upon addition of IL-6. As shown in Fig. 2D, addition of IL-6 enhanced PI3K activity in DU145 cells as well as PC-3 and LNCaP cells. In conclusion, data from Fig. 2 suggest that the IL-6 → PI3K signal pathway may play negative roles for the AR transactivation.

The effects of IL-6 → PI3K → Akt signal pathway on AR transactivation

As Akt is the downstream signal of IL-6 → PI3K,we were interested to see its effect on AR transactivation. As shown in Fig. 3A, addition of the constitutive-active form of Akt (cAkt) suppresses DHT-induced MMTV-luc reporter activity in PC-3 cells (lane 2 vs 3). In contrast, addition of dominant-negative form of Akt (dAkt) further enhanced DHT-induced MMTV-luc reporter activity (lane 2 vs 4). Similar results were also observed when we replaced PC-3 cells with DU145 cells (Fig. 3B). These data are consistent with Fig. 2 showing that IL-6 → PI3K → Akt signal pathway can suppress AR transactivation.

The effect of IL-6 → MAPK pathway on AR transactivation

MAPK represents another major downstream pathway to mediate IL-6 signal [19]. However, in contrast to the PI3K-Akt pathway that suppresses AR transactivation, our data show that addition of constitutive active MEK1 (cMEK1) further enhanced DHT-induced MMTV-luc reporter activity in PC-3 and DU145 cells (Fig. 3C, lane 2 vs 3; Fig. 3D, lane 2 vs 3). MAPK inhibitor U0126 exerted repression effect on DHT-induced AR transactivation (Fig. 3D, lane 4) in DU145 cells. Together, results from Figs. 3C and D suggest that MAPK may mediate IL-6 signal on AR transactivation in a positive manner.

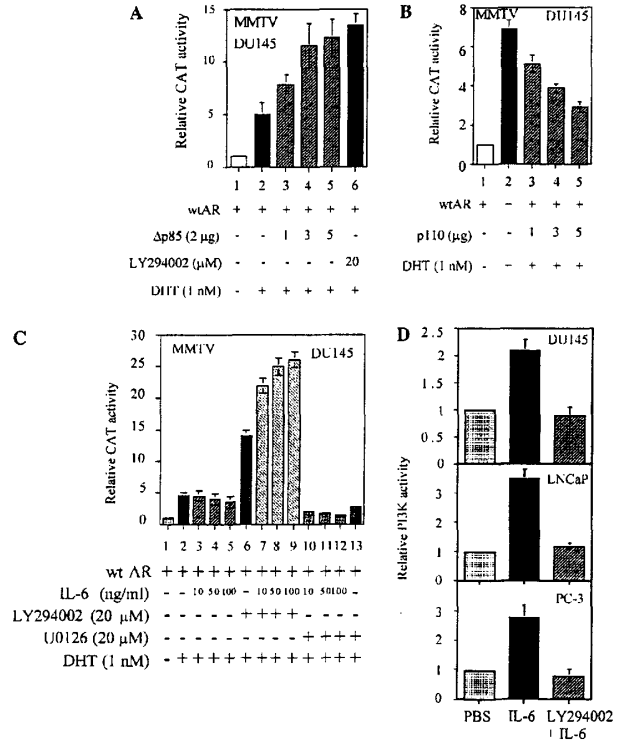


Fig. 2. Additive increases in the induction of MMTV promoter gene by IL-6 with blocking of the PI3K pathway. (A) Enhancement of AR transactivation through the inhibition of PI3K activity by Δp85 and LY294002. After 24 h transfection, the DU145 cells were treated with vehicle or LY294002 for 30 min prior to DHT treatment. The transactivation was measured by CAT activity. (B) Inhibition of AR transactivation by p110 in a dose-dependent manner. (C) Effect of IL-6 on AR transactivation. DU145 cells were transfected for 24 h with AR and MMTV-CAT reporter gene. After transfection, the cells were serum starved for 24 h, then 20 μM LY294002 or PD98059 was added to serum-free medium 30 min prior to IL-6 treatment. After 30 min of treatment with indicated concentrations of IL-6, DHT was added for another 24 h. The cells were then harvested and AR transactivation was measured by CAT activity. (D) Activation of PI3K activity by IL-6 in LNCaP, PC-3, and DU145 cells. After serum starvation for 24 h, LNCaP, PC-3, or DU145 cells were treated with 50 μg/ml IL-6 for 30 min, and then harvested. PI3K activity was measured as described in Experimental procedures.

The effects of combining PI3K-Akt and MAPK on AR transactivation

Fig. 3 suggests that PI3K-Akt and MAPK, which are distinct downstream signals of IL-6, can play opposite roles (suppression vs induction) on the AR transactivation. We were interested in determining their mutual influence on the AR transactivation. As shown in Fig. 4A, addition of cMEK1 alone further enhanced DHT-induced MMTV-luc reporter activity (lane 2 vs 4) and addition of cAkt then suppressed the cMEK-enhanced MMTV-CAT reporter activity (lane 4 vs lanes 6 and 8). Similar conclusions also occurred showing that cMEK1 reversed the cAkt-repressed AR transactivation in PC-3 cells (Fig. 4B). The interactions between two IL-6 downstream signals, MEK1 and cAkt, were further demonstrated using Western blot to assay the MAPK phosphorylation status. As shown in Fig. 4C, MAPK was phosphorylated upon stimulation of IL-6 (lane 2). This IL-6-induced MAPK phosphorylation was suppressed upon addition of U0126, the MEK1 inhibitor (lane 3 vs 4). Interestingly, if we replaced U0126 with LY294002, the inhibitor of cAkt, we found that the phosphorylation of MAPK increased significantly (lane 5 vs 6), suggesting that blocking of the IL-6 → PI3K → Akt pathway may be able to potentiate the IL-6 → MAPK pathway.

IL-6 potentiates STAT3 effect on enhancement of AR transactivation

The third major downstream signal of IL-6 is STAT3. As shown in Fig. 5A, addition of STAT3 enhanced

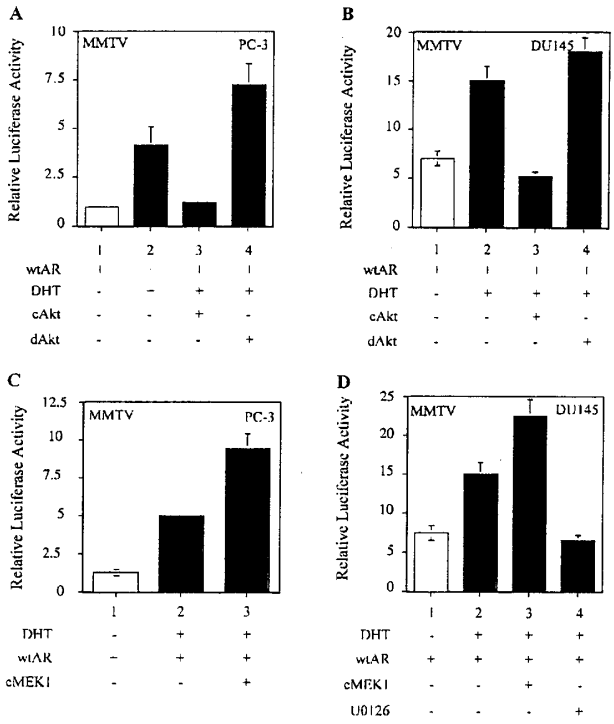


Fig. 3. cAkt suppresses, but cMEK1 enhances AR activity. PC-3 (A) and DU145 cells (B) were transfected with 50 ng pCMV-AR, 150 ng MMTV-luc, 2.5 ng pRL-SV40, 50 ng cAkt, or dAkt. Transfected cells were treated for 24 h with 10^{-9} M DHT or ethanol as vehicle controls. PC-3 (C) and DU145 cells (D) were transfected with 50 ng pCMV-AR, 150 ng MMTV-luc, 2.5 ng pRL-SV40, and 100 ng cMEK1. Transfected cells were treated for 24 h with 10^{-9} M DHT or ethanol as vehicle controls. In DU145 cells (D, lane 4), 20 μM U0126 was added with the DHT. Duplicate samples were analyzed for each data point.

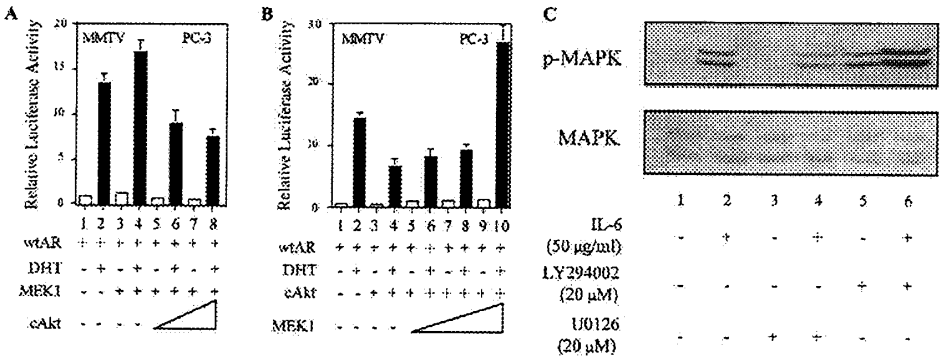


Fig. 4. cAkt and MAPK counteract with each other to modulate AR activity, and IL6 activates MAPK in DU145 cells. (A) PC-3 cells were transfected with 50 ng pSG5-AR, 150 ng MMTV-luc, 100 ng cMEK1, 5 ng pRL-TK, and doses of cAkt (50 and 150 ng). (B) PC-3 cells were transfected with 50 ng pSG5-AR, 150 ng MMTV-luc, 100 ng cAkt, 5 ng pRL-TK, and doses of cMEK1 (50, 150, and 300 ng). Cells were treated for 24 h with 10^{-9} M DHT or ethanol as vehicle controls. Duplicate samples were analyzed for each data point. (C) DU145 cells were treated with 50 ng/ml IL-6, 20 μM LY294002 or 20 μM U0126, or a combination as indicated for 30 min. The phosphorylation status of MAPK was determined by immunoblotting with phospho-p44/p42 MAPK monoclonal antibody (top). The loading control was carried out with anti-MAPK antibody (bottom).

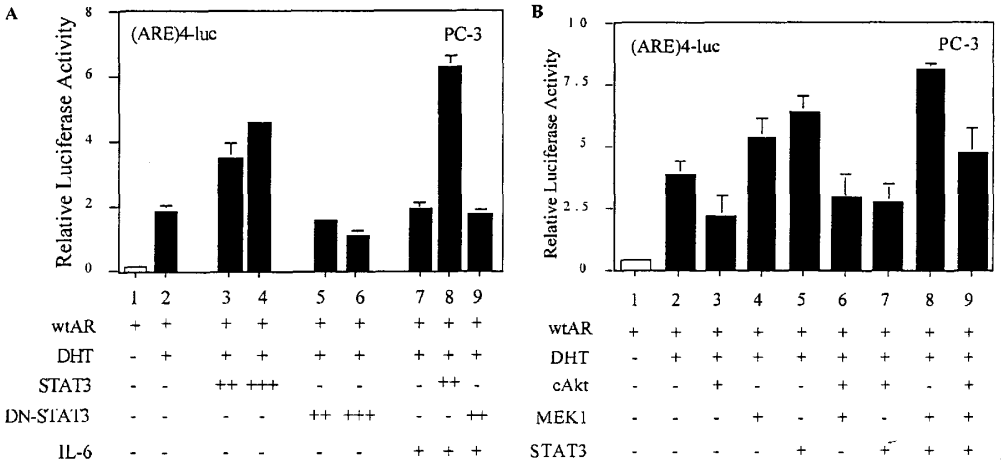


Fig. 5. IL-6 potentiated STAT3 effect on enhancement of AR transactivation and the effects of the co-existence of 3 IL-6 downstream signal pathways. (A) PC-3 cells were transfected with 50 ng pCMV-AR, 150 ng (ARE)4-luc, doses of STAT3 or DN-STAT3 (++, 50 ng and +++, 150 ng), and 5 ng pRL-TK. Transfected cells were treated for 24 h with 10^{-9} M DHT, ethanol, or 50 ng/ml IL6. Duplicate samples were analyzed for each data point. (B) PC-3 cells were transfected with 50 ng pCMV-AR, 150 ng (ARE)4-luc, 5 ng pRL-TK, and 50 ng of cAkt, cMEK1, and STAT3, or combinations of them. Transfected cells were treated for 24 h with 10^{-9} M DHT, ethanol, or 50 ng/ml IL6. Duplicate samples were analyzed for each data point.

DHT-induced AR transactivation in a dose-dependent manner and addition of dominant-negative STAT3 suppressed AR transactivation in PC-3 cells. Addition of IL-6 alone had little effect on the AR transactivation. Addition of IL-6 and STAT3, however, further potentiates STAT-induced AR transactivation (lane 4 vs 8). Together, results from Fig. 5A suggest that IL-6 can enhance AR transactivation through the STAT3 signaling pathway.

The effects of the co-existence of 3 IL-6 downstream signal pathways

To study the potential mutual influences of the 3 IL-6 downstream signal pathways (PI3K-Akt, MAPK, and STAT3) on AR transactivation, we cotransfected these 3 downstream mediators in different combinations in PC-3 cells. As shown in Fig. 5B, addition of cAkt alone suppressed DHT-induced AR transactivation (lane 3). Addition of MEK1 alone enhanced DHT-induced AR transactivation (lane 4). Addition of STAT3 alone enhanced DHT-induced AR transactivation (lane 5). Simultaneous addition of cAkt and MEK1 (lane 6) or cAkt and STAT3 (lane 7) results in the slight suppression of AR transactivation. In contrast, simultaneous addition of MEK1 and STAT3 results in further enhancement of AR transactivation (lane 8). Simultaneous addition of cAkt, MEK1, and STAT3 resulted in the slight enhancement of AR transactivation (lane 9). Together, results from Fig. 5B suggest that IL-6 effects on the AR transactivation may depend on the availability of its three downstream mediators.

Discussion

The role of cytokines in normal prostate biology and prostate cancer is still an emerging area of investigation. IL-6 is significantly elevated in many men with advanced hormone-independent prostate cancer and elevated IL-6 levels may constitute an independent prognostic marker for decreased survival [5]. Thus, it has been predicted that IL-6 signaling plays an important role in androgen-independent progression. IL-6 receptor is expressed in both prostate cancer tissues and prostate cancer cell lines, including the androgen-dependent prostate cell line LNCaP and androgen-independent PC-3 and DU145 cells [28,29]. Binding of IL-6 to its receptor results in activation of JAKs as well as their two major downstream signaling pathways, MAPK and STAT3, in LNCaP cells [8,18,19,29]. IL-6 can also activate the PI3K pathway in LNCaP and PC-3 cells [21–24]. Some reports observed that IL-6 is able to induce AR transactivation in an androgen-independent manner in LNCaP cells, but not in PC-3 and DU145 cells [18,19,30]. However, the mechanism of IL-6 induction of AR transactivation in LNCaP cells still remains largely unknown. To date, results revealed that the induction of AR target gene reporter activity by IL-6 was promoter-specific and cell type-specific. Some studies showed that

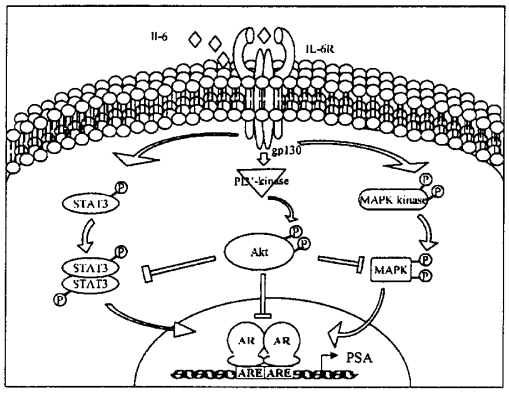


Fig. 6. A hypothetical working model of signal transduction pathways to AR activity upon stimulation by IL-6 in hormone-refractory prostate cancer cell lines. The IL-6 signaling cascade induces up-regulation of AR-regulated genes such as MMTV through the STAT3 and MAPK pathways, but down-regulation of MMTV through the PI3K-Akt pathway.

IL-6 could activate the AR gene promoter resulting in increased AR mRNA and protein level in LNCaP cells, suggesting that IL-6 enhances androgen activity by up-regulating the AR level [18]. However, other reports observed that the whole cell levels of AR were not increased by IL-6 [31].

The fact that AR target gene reporters were poorly induced in PC-3 and DU145 cells upon stimulation by IL-6 led us to investigate the discrepancies between LNCaP, PC-3, and DU145 cells. The observation that IL-6 was able to activate PI3K, STAT3, and MAPK pathways in PC-3 or DU145 cells, suggested that these three pathways may coordinate with each other to determine the effect of IL-6 on AR transactivation and prostate cancer growth. We found that IL-6 could enhance AR transactivation via the MAPK or STAT3 pathway. Alternatively, IL-6 could repress AR transactivation via the PI3K pathway. We also observed that the PI3K pathway could negatively influence the MAPK and STAT3 pathways, and the PI3K pathway may be more dominant compared to the MAPK and STAT3 pathways upon the stimulation by IL-6 in PC-3 or DU145 cells (Fig. 6). In LNCaP cells, although PI3K pathway is also an IL-6 signal mediator, it has been shown to not be a major signal transduction pathway for IL-6 effect on AR. However, we cannot rule out the possibility that in addition to the availability of these three signal transduction pathways, some mediators of IL-6 to modulate its effect on AR activity are deficient or different in PC-3 and DU145 cells. In conclusion, our data suggest that IL-6 may use multiple pathways to differentially regulate AR transactivation and/or AR-mediated cell growth in prostate cancer cells.

Acknowledgments

We are grateful to Drs. R. Freeman and Rolf P. de Groot for their reagents. We thank Karen L. Wolf for helpful reading of the manuscript. We also thank the members in Dr. Chang's laboratory for technical support and insightful discussion. The work was supported by NIH Grants (DK60905 and DK60948).

References

[1] M.J. Siegsmund, H. Yamazaki, I. Pastan, Interleukin 6 receptor mRNA in prostate carcinomas and benign prostate hyperplasia, J. Urol. 151 (1994) 1396 1399.
[2] T.D. Chung, J.J. Yu, M.T. Spiotto, M. Bartkowski, J.W. Simons, Characterization of the role of IL-6 in the progression of prostate cancer, Prostate 38 (1999) 199 207.
[3] M. Okamoto, M.M. Webber, S. Quader, R. Oyasu, Interleukin-6 and epidermal growth factor promote anchorage-independent growth of immortalized human prostatic epithelial cells treated with *N*-methyl-*N*-nitrosourea, Prostate 35 (1998) 255 262.
[4] D.A. Twillie, M.A. Eisenberger, M.A. Carducci, W.S. Hseih, W.Y. Kim, J.W. Simons, Interleukin-6: a candidate mediator of human prostate cancer morbidity, Urology 45 (1995) 542 549.
[5] D.E. Drachenberg, A.A. Elgamal, R. Rowbotham, M. Peterson, G.P. Murphy, Circulating levels of interleukin-6 in patients with hormone refractory prostate cancer, Prostate 41 (1999) 127 133.
[6] S. Mori, K. Murakami-Mori, B. Bonavida, Interleukin-6 induces G1 arrest through induction of p27(Kip1), a cyclin-dependent kinase inhibitor, and neuron-like morphology in LNCaP prostate tumor cells, Biochem. Biophys. Res. Commun. 257 (1999) 609 614.
[7] A. Hobisch, I.E. Eder, T. Putz, W. Horninger, G. Bartsch, H. Klocker, Z. Culig, Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor, Cancer Res. 58 (1998) 4640 4645.
[8] W. Lou, Z. Ni, K. Dyer, D.J. Tweardy, A.C. Gao, Interleukin-6 induces prostate cancer cell growth accompanied by activation of stat3 signaling pathway, Prostate 42 (2000) 239 242.
[9] Y. Qiu, L. Ravi, H.J. Kung, Requirement of ErbB2 for signalling by interleukin-6 in prostate carcinoma cells, Nature 393 (1998) 83 85.
[10] C.S. Chang, J. Kokontis, S.T. Liao, Molecular cloning of human and rat complementary DNA encoding androgen receptors, Science 240 (1988) 324 326.
[11] S. Yeh, H.C. Chang, H. Miyamoto, H. Takatera, M. Rahman, H.Y. Kang, T.H. Thin, H.K. Lin, C. Chang, Differential induction of the androgen receptor transcriptional activity by selective androgen receptor coactivators, Keio. J. Med. 48 (1999) 87 92.
[12] H.Y. Kang, S. Yeh, N. Fujimoto, C. Chang, Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor, J. Biol. Chem. 274 (1999) 8570 8576.
[13] N. Fujimoto, S. Yeh, H.Y. Kang, S. Inui, H.C. Chang, A. Mizokami, C. Chang, Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate, J. Biol. Chem. 274 (1999) 8316 8321.
[14] C. Chang, A. Saltzman, S. Yeh, W. Young, E. Keller, H.J. Lee, C. Wang, A. Mizokami, Androgen receptor: an overview, Crit. Rev. Eukaryot. Gene Expr. 5 (1995) 97 125.
[15] P.W. Hsiao, T.H. Thin, D.L. Lin, C. Chang, Differential regulation of testosterone vs. 5α-dihydrotestosterone by selective androgen response elements, Mol. Cell. Biochem. 206 (2000) 169 175.

[16] Z. Culig, A. Hobisch, M.V. Cronauer, C. Radmayr, J. Trapman, A. Hittmair, G. Bartsch, H. Klocker, Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor, Cancer Res. 54 (1994) 5474 5478.

[17] N. Craft, Y. Shostak, M. Carey, C.L. Sawyers, A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase, Nat. Med. 5 (1999) 280 285.

[18] D.L. Lin, M.C. Whitney, Z. Yao, E.T. Keller, Interleukin-6 induces androgen responsiveness in prostate cancer cells through up-regulation of androgen receptor expression, Clin. Cancer Res. 7 (2001) 1773 1781.

[19] T. Ueda, N. Bruchovsky, M.D. Sadar, Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways, J. Biol. Chem. 277 (2002) 7076 7085.

[20] T. Ueda, N.R. Mawji, N. Bruchovsky, M.D. Sadar, Ligand-independent activation of the androgen receptor by IL-6 and the role of the coactivator SRC-1 in prostate cancer cells, J. Biol. Chem. 5 (2002) 5.

[21] J. Lin, R.M. Adam, E. Santiestevan, M.R. Freeman, The phosphatidylinositol 3'-kinase pathway is a dominant growth factor-activated cell survival pathway in LNCaP human prostate carcinoma cells, Cancer Res. 59 (1999) 2891 2897.

[22] M. Tezuka-Takahashi, M. Hibi, Y. Fujitani, T. Fukada, T. Yamaguchi, T. Hirano, Tec tyrosine kinase links the cytokine receptors to PI-3 kinase probably through JAK, Oncogene 14 (1997) 2273 2282.

[23] Y. Qiu, D. Robinson, T.G. Pretlow, H.J. Kung, Etk/Bmx, a tyrosine kinase with a pleckstrin-homology domain, is an effector of phosphatidylinositol 3'-kinase and is involved in interleukin 6-induced neuroendocrine differentiation of prostate cancer cells, Proc. Natl. Acad. Sci. USA 95 (1998) 3644 3649.

[24] T.D. Chung, J.J. Yu, T.A. Kong, M.T. Spiotto, J.M. Lin, Interleukin-6 activates phosphatidylinositol-3 kinase, which inhibits apoptosis in human prostate cancer cell lines, Prostate 42 (2000) 1 7.

[25] R.J. Crowder, R.S. Freeman, Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons, J. Neurosci. 18 (1998) 2933 2943.

[26] E. Caldenhoven, T.B. van Dijk, R. Solari, J. Armstrong, J.A. Raaijmakers, J.W. Lammers, L. Koenderman, R.P. de Groot, STAT3β, a splice variant of transcription factor STAT3, is a dominant negative regulator of transcription, J. Biol. Chem. 271 (1996) 13221 13227.

[27] C. Huang, P.C. Schmid, W.Y. Ma, H.H. Schmid, Z. Dong, Phosphatidylinositol-3 kinase is necessary for 12-*O*-tetradecanoylphorbol-13-acetate-induced cell transformation and activated protein 1 activation, J. Biol. Chem. 272 (1997) 4187 4194.

[28] C.B. Siegall, G. Schwab, R.P. Nordan, D.J. FitzGerald, I. Pastan, Expression of the interleukin 6 receptor and interleukin 6 in prostate carcinoma cells, Cancer Res. 50 (1990) 7786 7788.

[29] D. Giri, M. Ozen, M. Ittmann, Interleukin-6 is an autocrine growth factor in human prostate cancer, Am. J. Pathol. 159 (2001) 2159 2165.

[30] T. Chen, L.H. Wang, W.L. Farrar, Interleukin 6 activates androgen receptor-mediated gene expression through a signal transducer and activator of transcription 3-dependent pathway in LNCaP prostate cancer cells, Cancer Res. 60 (2000) 2132 2135.

[31] J.D. Debes, L.J. Schmidt, H. Huang, D.J. Tindall, P300 mediates androgen-independent transactivation of the androgen receptor by interleukin 6, Cancer Res. 62 (2002) 5632 5636.