The significance of Her2 on AR protein stability in the transition of androgen requirement in prostate cancer cells

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Abstract

Androgen ablation therapy is the most common strategy to suppress prostate cancer progression; however, tumor cells eventually escape androgen requirement and progress into androgen-independent phase. Androgen receptor (AR) plays a pivotal role in this transition. In order to answer this transition mystery in prostate cancer, we established an androgen-independent prostate cancer cell line (LNCaPdcc) by long-term screening LNCaP cells in androgen-deprived condition to investigate changes of molecular mechanisms before and after androgen withdrawal. We found that LNCaPdcc cells displayed the morphology of neuroendocrine differentiation, less aggressive growth, weaker androgen sensitivity, and lower expression levels of cell cycle-related factors, although the cell cycle distribution was similar to parental LNCaP cells. Interestingly, higher protein expressions of AR, phospho-Ser81-AR, and PSA in LNCaPdcc cells were observed. Moreover, nuclear distribution and protein stability of AR increased in LNCaPdcc cells. On the other hand, LNCaPdcc cells expressed higher levels of Her2, phospho-Her2, and ErbB3 proteins than parental LNCaP cells. Notably, these two cell lines exhibited distinct responses toward Her2 activation (by heregulin treatment) and Her2 inhibition (by AG825 or Herceptin treatments) on proliferation. In addition, Her2 inhibitor more effectively caused AR degradation in LNCaPdcc cells. Taken together, our data demonstrate that Her2 plays

59 an important role to support AR protein stability in the transition of androgen

60 requirement in prostate cancer cells. We hope these findings would provide new

61 suggestion on the treatment of hormone-refractory prostate cancer.

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Introduction

Prostate cancer is an age-related carcinoma and the most commonly diagnosed malignancy among men (25). Although the prostate specific antigen (PSA), a biomarker of hypertrophy in the prostate gland, helps to identify prostate cancer in the early stages, the disease still causes high mortality. Traditionally, gonadectomy is the main therapeutic procedure for androgen-dependent prostate cancer. Once the cancer escapes from androgen dependence and becomes androgen-independent, radio- or chemo-therapies are subsequently applied. Unfortunately, the treatment of hormone-refractory prostate cancer in this stage is often ineffective and the mechanisms of prostate cancer progression in this stage remain to be elucidated. Therefore, it is imperative to understand the transition of androgen requirement and to develop strategies for prolonging the survival of patients with recurrent and hormone-refractory prostate cancer.

The androgen receptor (AR), a member of the steroid receptor family, plays a decisive role in the development of the prostate gland and in the pathogenesis and progression of prostate cancer. AR binds to androgen response elements (AREs) and thereby mediates androgen-regulated gene expression (12). A growing number of clinical investigations show amplifications of AR and AR-regulated genes in hormone-refractory prostate cancer, which suggests that the AR signaling pathway is

still activated and important at limiting concentrations of androgen (14). Previous research indicates that the elevated AR expression levels were correlated to resistance to anti-androgen therapy (3). The cross-talk between receptor tyrosine kinases with their cognate ligands and AR signaling in hormone-refractory transition of prostate cancer has also been addressed (6, 11, 26). On the other hand, Her2/ErbB3 signals have been suggested to stabilize AR proteins and to increase the interaction of AR to promote/enhancer regions of AR-regulated gene in androgen-dependent prostate cancer cells (23).

Here, we established an androgen-independent prostate cancer cell line named LNCaPdcc by incubating LNCaP cells in androgen-deprived condition for a long period (eight months). We try to take advantage of this popular strategy of cell model to answer how prostate cancer cells maintain AR protein levels and activation in androgen free environment. Indeed, we observed several characteristics obviously changed after androgen deprivation. Importantly, our data showed that LNCaPdcc cells were more sensitive to Her2 inhibition with increase of AR degradation than parental LNCaP cells. These findings suggest that Her2 activation might be an important support of AR protein stability in prostate cancer cells under adaptation of androgen deprivation.

Materials and Methods

Materials

R1881 (Methyltrienolone; NLP-005) was purchased from PerkinElmer (Boston, MA, USA); Cycloheximide (CHX; C1988) from Sigma (Missouri, USA); MG-132 (474791) from Calbiochem (San Diego, CA, USA); Recombinant human heregulin β1 (396-HB) from R&D Systems, Inc. (Minneapolis, MN, USA); AG825 (121765) from Calbiochem and Herceptin from Roche Applie Science (Mannheim, Germany). Antibodies used for immunoblotting were indicated: Cdk1 (sc-54, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Cyclin A (sc-751, Santa Cruz), Cyclin B1 (sc-752, Santa Cruz), Cyclin D1 (sc-20044, Santa Cruz), β-actin (MAB1501, Millipore, Temecula, CA, USA), phospho-Ser81-AR (07-541, Upstate, Lake Placid, NY, USA), AR (sc-13062 and sc-7305, Santa Cruz), PSA (sc-7316, Santa Cruz), α-tubulin (05-829, Upstate), PARP (06-557, Upstate), phospho-Tyr1221/1222-Her2 (2249, Cell Signaling, Danvers, MA, USA), Her2 (C-18, Santa Cruz; OP-15, Calbiochem) and ErbB3 (sc-285 and 7309, Santa Cruz). Secondary antibodies were peroxidase-conjugated anti-mouse or anti-rabbit (Jackson ImmunoResearch Laboratory, West Grove, PA, USA).

Cell Culture

Human prostate carcinoma cell lines derived from lymph node carcinoma of the prostate (LNCaP clone FGC (fast growing colony), BCRC 60088) (13) were purchased from Food Industry Research and Development Institute, Taiwan. LNCaP cells were maintained in complete medium: phenol red-positive RPMI-1640 culture medium (Gibco, Carlesbad, CA, USA) supplemented with 1.5 g/L sodium bicarbonate ($NaHCO_3$) (Sigma), 10% fetal bovine serum (FBS) (Gibco), and penicillin/streptomycin (P/S) (100 IU/mL and 100 μg/mL, respectively) (Gibco). Cells were cultured at 37 ^{o}C in a humidified atmosphere with 5% CO_2 (18). Cells were routinely passaged by trypsin/EDTA (0.05% and 0.02%, respectively) (Gibco) twice a week in the ratio 1:3. LNCaPdcc cells, a subline from LNCaP cells, was designed to be an *in vitro* model for investigating the progression of androgen-independent prostate cancer (7). LNCaPdcc cells were established by domesticating LNCaP cells in a long-term androgen-ablated condition over 14 passages. To deprive cells of steroid hormones, FBS was incubated with dextran-coated charcoal (dcc) (Sigma) by rotating at a low speed at 4 °C for 12-16 h. The charcoal-FBS mixture was then centrifuged twice at 500 *g* for 10 min. Then the supernatant was stored at –20 °C until use. LNCaPdcc cells were grown in phenol red-free RPMI-1640 medium (Sigma) plus 10% dcc-stripped FBS, 1.5 g/L $NaHCO_3$, and P/S (100 IU/mL, 100 μg/mL) at 37 ^{o}C in a humidified atmosphere at 5% CO_2. Cells were split once a week in the ratio

1:2. All experiments on LNCaPdcc were performed between passage 25 and 45.

Cell Viability Assay

The modified colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was manipulated to quantify the viability of LNCaP and LNCaPdcc cancer cells. Yellow MTT compound (Sigma) is converted by living cells into blue formazen, which is soluble in isopropanol. The intensity of blue staining in culture medium is proportional to the number of living cells and measured by using an optical density reader (Athos-2001, Australia) at 570 nm (background, 620 nm) (1, 18, 19).

Immunoblotting and Fractionation Analyses

Cell lysates were obtained in lysis buffer (50 mM Tris-HCl [pH 8.0], 0.5% Nonidet P-40 [NP-40], 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM sodium orthovanadate [Na_3VO_4], and protease inhibitor cocktail [Roche Applied Science]). Lysates were then analyzed for immunoblotting using methods modified from those previously described (1, 18, 19). To isolate subcellular proteins, cells were collected and washed in PBS/Na_3VO_4. Pelleted cells were resuspended in hypotonic buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM

158 EDTA, 0.1 mM EGTA, 0.5% NP-40, 1 mM PMSF, 2 mM Na_3VO_4, and protease
159 inhibitor cocktail). Nuclei were pelleted and the supernatant was harvested as the
160 cytosolic fraction. The nuclear pellet was washed three times with hypotonic buffer
161 before lysing in nuclear extraction buffer (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1
162 mM EDTA, 1 M EGTA, 20% glycerol, 1 mM PMSF, 2 mM Na_3VO_4, and protease
163 inhibitor cocktail) in a procedure modified from those described previously (1, 18, 19).
164 Protein samples were analyzed by direct immunoblotting (25-35 μg/lane) or blotting
165 after immunoprecipitation (0.5-1 mg /immunoprecipitation). ECL detection reagent
166 (PerkinElmer Life Science) was used to visualize the immunoreactive proteins on
167 membranes (polyvinylidene difluoride, PVDF; Perkin Elmer Life Science) after
168 transfer using a Trans-Blot SD (Bio-Rad, Berkeley, CA, USA).
169

170 **Trypan Blue Assay**

171 LNCaP and LNCaPdcc cells were seeded in a 24-well plate in the complete
172 culture medium. Cells were trypsinized, stained with 0.2% trypan blue (Sigma), and
173 counted by hemocytometer to distinguish the live and dead cells.
174

175 **Analysis of Cell Cycle Distribution**

176 Propidium iodide staining was used for DNA content measurement. Cancer cells,

trypsinized and fixed in 70% ethanol, were washed once with PBS and treated with RNase A for 30 minutes, followed by staining with propidium iodide (0.1% sodium citrate, 0.1% Triton X-100, and 20 μl/mL propidium iodide). DNA content was measured by using flow cytometry (FACS Calibur, Germany). Percentage of cells in each phase of the cell cycle was analyzed by the software, Cell Quest.

Statistics

All values are given as the mean ± standard error of the mean (SEM). Student's *t*-test was used in the cell proliferation. A difference between two means was considered statistically significant when $p < 0.05$.

Results

Comparisons of characteristics between LNCaPdcc and parental LNCaP cells

The LNCaPdcc cells displayed the dendritic-like morphology in neuroendocrine differentiation as compared with parental LNCaP cells (Fig. 1A). In addition, growth curves of two cell lines were determined by cell counting (Fig. 1B). The comparison of doubling time (inset table of fig. 1B) showed that LNCaPdcc cells grew much slower than parental LNCaP cells. By using the flow cytometry, the differences of cell cycle distribution between parental LNCaP and LNCaPdcc cells were identified. The data showed that S phase distribution of LNCaPdcc cells was obviously higher than parental LNCaP cells although G1 and G2/M phase distributions of two cell lines were similar (Fig. 2A). Therefore, it is interesting to understand the levels of cell cycle-related proteins expressed in two cell lines. The results revealed that the protein levels of Cdk1, cyclin A, cyclin B1, and cyclin D1 were all lower in LNCaPdcc cells (Fig. 2B), which might explain why LNCaPdcc cells grew slowly and stuck in S phase.

AR-related features in two cell lines

Compared to parental LNCaP cells, LNCaPdcc cells expressed higher protein levels of phospho-Ser81-AR, AR, and PSA (AR-regulated gene) (Fig. 3A). In addition,

the protein fractionation was utilized to investigate the subcellular distribution of AR proteins. Interestingly, compared to parental LNCaP cells, LNCaPdcc cells contained higher levels of nuclear AR protein (Fig. 3B), indicating that AR in LNCaPdcc cells are still activated even in the absence of androgen. Then, cycloheximide (CHX) was used to block protein synthesis and the degradation of existing protein was then monitored. The result exhibited that AR protein in LNCaPdcc cells was more stable than that in parental LNCaP cells (Fig. 3C). Subsequently, the cell proliferation in response to androgen treatment was investigated by using MTT assay. Parental LNCaP cell proliferation was sensitive to synthetic androgen R1881 under steroid-deprived condition, especially at the limiting concentrations (0.1 and 1 nM) of androgen. However, the proliferation of LNCaPdcc cells was inhibited by these concentrations (Fig. 4). The result illustrates that the proliferation of LNCaPdcc cells is androgen-independent.

Her2-related features in two cell lines

According to previous research, there is a correlation between AR and Her2 signals in androgen-dependent prostate cancer cells (23). Therefore, the protein expressions of Her2 and its activation partner, ErbB3, in two cell lines were investigated. The data showed that LNCaPdcc cells expressed higher levels of

phospho-Y1221/1222-Her2, Her2, and ErbB3 (Fig. 5A). Since Her2 activation was correlated to its phosphorylation status, these data imply that Her2 is more active in LNCaPdcc cells than parental cells. In addition, parental LNCaP and LNCaPdcc cells were both treated with 10 ng/mL of heregulin (HRG, ligand of Her2/ErbB3) in a time-course manner. HRG-induced Her2 activation in LNCaPdcc cells could sustain for 24 hours after treatment; however, that activation in parental cells simply dropped since 1 hour after treatment (Fig. 5B). In order to understand the physiological functions of Her2 in different cell lines, the effects of Her2 inhibitors on proliferation of two cell lines were evaluated by MTT assay. AG825 and Herceptin (monoclonal antibody of Her2 for clinical use) were treated to both cell lines. Parental LNCaP cell proliferation displayed weak response to both Her2 inhibitors whereas LNCaPdcc cell proliferation was significantly declined by Her2 inhibition (Fig. 5C). It might be due to the high levels of Her2 in LNCaPdcc cells. Accordingly, Her2 in LNCaPdcc cells might take more charge on LNCaPdcc proliferation comparing to parental cells.

AR stability in LNCaPdcc cells depends on high Her2 activation

AR is a short half-life protein and tends to be degraded through the ubiquitin-proteasome pathway (27). It has been reported that AR proteins can be stabilized under Her2/ErbB3 activation (23). In addition, our data indicated that AR

protein levels were correlated to Her2 activation in both cell lines (data not shown). In order to determine whether Her2 is involved in the increase of AR stability in LNCaPdcc cells (Fig. 3C), Her2 inhibition (by AG825) was performed and AR stability in two cell lines was monitored. The results showed that Her2 inhibition accelerated AR degradation in LNCaPdcc cells (Fig. 6A), although the initial level of AR protein in LNCaPdcc cells was still higher than that in parental cells (time=0, Fig. 6A). After 9-hour treatment of CHX, the AR degradation percentage of LNCaPdcc (quantitative ratio) was 79% which is much higher than 32% of parental cells. Furthermore, Ser81 phosphorylation of AR has been reported to be responsible for itself stability (21). Corresponding to previous research (23), Her2 inhibitor effectively reduced AR Ser81 phosphorylation in both cell lines (Fig. 6B). Interestingly, LNCaPdcc cells were more sensitive to AG825 treatment on the inhibition of AR Ser81 phosphorylation (40% inhibition in LNCaPdcc cells *versus* 20% inhibition in parental cells). Taken together, higher Her2 activation might make more contribution to AR protein stability through Ser81 site phosphorylation in LNCaPdcc cells.

Discussion

Prostate carcinoma is a leading cause of death in male malignancy. Since the prostate is an androgen-dependent gland, androgen ablation therapy is the most frequent strategy used to suppress prostate tumor pathogenesis. Nevertheless, cancer cells eventually escape the androgen requirement and progress to an androgen-independent phenotype. The cure for the hormone-refractory prostate cancer remains a main clinical challenge. In the progression of prostate cancer, AR emerges as an important determinant. AR protein controls cell cycle, cell proliferation, inhibition of apoptosis, regulation of angiogenic growth factors, and stimulation of cellular migration among other functions (5). In order to investigate the roles of AR activity in prostate cancer progression following androgen withdrawal, the authors established LNCaPdcc subline by long-term screening LNCaP cells in an androgen-stripped condition. The LNCaPdcc cells revealed a dendritic-like morphology (Fig. 1A) and a lower growth rate (Fig. 1B) indicating the adaptation of LNCaPdcc cells to androgen-free condition.

Interestingly, AR proteins of LNCaPdcc cells were even more active in the absence of androgen because higher levels of AR Ser81 phosphorylation, PSA proteins (Fig. 3A) and nuclear AR proteins (Fig. 3B) in LNCaPdcc cells were

observed. It might be due to the excessive recruitment of coactivators (10) or crosstalk with several polypeptide growth factors as well as cognate receptors (22, 28) in the transition of prostate cancer. On the other hand, cyclin D1 was reported to interact predominantly with the N-terminal domain of AR and this interaction depends on the presence of the AR ^{23}FxxLF^{27} motif, which is also important for interaction between the N- and C-termini of AR. Through this motif, cyclin D1 protein prevents the interaction between the two termini of AR, consequently inhibiting AR activity (2). Our data revealed that cyclin D1 proteins dramatically declined in LNCaPdcc cells (Fig. 2B), illustrating that the decrease of cyclin D1 levels might help to increase AR activation. In addition, we found that the proliferation of LNCaPdcc cells was not dependent on androgen (Fig. 4). It has been reported that AR in LNCaP cell line is a T877A mutant that can be activated not only by androgens but also by non-androgenic steroid hormones and anti-androgens (31). Our unpublished data showed that parental LNCaP cell proliferation was significantly stimulated by estradiol bezoate (EB, synthetic estrogen) in dose-dependent manner while LNCaPdcc displayed insensitive to EB.

According to previous study, AR is a short half-life protein in the absence of androgen (10) and tends to be degraded through the ubiquitin-proteasome pathway

(27). Ubiquitin-proteasome degradation is important to transcriptional regulation (20) and ubiquitin-ligase E6-associated protein may be a cofactor of steroid receptors (24). Therefore, it is of interest to investigate what delays AR degradation in LNCaPdcc cells (Fig. 3C). In addition to ligand-dependent regulation, post-translational modification of AR has also been extensively discussed (8). The existence of AR Ser81 phosphorylation is correlated to protein stability (21). On the other hand, the Her2/ErbB3 axis has been reported to provide signals to AR which protects AR protein stability (23). It also demonstrates that the androgen-induced Ser81 phosphorylation of AR is declined by a small molecule Her2 inhibitor PKI-166 (23). Additionally, our findings indicated that AR protein levels seem to be positively regulated by Her2 activity but not by epidermal growth factor receptor (EGFR) activation (data not shown). These results suggest the existence of a specific and enhanced regulation between Her2 activation and AR stability in LNCaPdcc cells. In addition, our findings also indicated that AR Ser81 phosphorylation was inhibited by Her2 inhibitors (Fig. 6B), which suggests that AR Ser81 site is a downstream substrate of Her2 pathway. As regards to Her2-downstream serine-threonine kinases, Akt/protein kinase B (PKB) has been reported not to be the kinase that responds to AR Ser81 phosphorylation due to the analysis of phosphorylation consensus sequence sites (23). Although the Ser81 site occurs in the consensus sequence of protein kinase

C (PKC), PKC inhibitors fail to reduce AR Ser81 phosphorylation (9). Several kinases are implied or predicted to be the candidates responding to AR Ser81 phosphorylation such as Cdk1, Cdk5 (4), and Erk (29). However, Cdk1 activation is inhibited by Her2 *via* phosphorylation on tyrosine 15 site (30). Moreover, Cdk1 proteins diminished in our LNCaPdcc cells (Fig. 2B), illustrating that the increasing levels of Her2-dependent AR Ser81 phosphorylation might be irrelevant to Cdk1 activity. On the contrary, we have reported that Cdk5 activity is elevated by Her2 activation through Tyr15 phosphorylation in thyroid cancer cells (16). In addition, Cdk5 is also reported to modulate androgen production (17) and cell fate of prostate cancer (15, 18) by us. With regards to Erk, we found that both phospho-Erk and Erk levels increased in LNCaPdcc cells as compared to those in parental LNCaP cells (data not shown). The specific kinases regulated by Her2 and responsible for Ser81 phosphorylation of AR need to be further investigated.

According to the results in Fig. 3, LNCaPdcc cells displayed higher level of AR Ser81 phosphorylation and longer half-time of AR proteins in androgen-stripped environment. Coincidentally, LNCaPdcc cells expressed higher levels of phospho-Her2 and Her2 proteins (Fig. 5A). By using Her2 inhibitor, Her2 in LNCaPdcc cells was more sensitive to its inhibitor and resulted in the drops of either

AR Ser81 phosphorylation or AR protein stability (Fig. 6). These results suggest that Her2 not only plays a role of growth factor receptor, but also protects AR protein stability through Ser81 phosphorylation in LNCaPdcc cells after cells escape the androgen requirement.

In conclusion, we used a new-established prostate cancer cell subline, LNCaPdcc, to elucidate different characteristics and protein expressions comparing to parental LNCaP cells. LNCaPdcc cells display features of androgen-independent prostate cancer. We found that, in LNCaPdcc cells, Her2 activation becomes more important to protect AR protein from degradation through Ser81 phosphorylation and subsequently modulates cell proliferation. We hope our findings would be helpful in understanding the transition of androgen deprivation. Besides, we also suggest that Her2-AR axis would become a diagnostic and therapeutic target in hormone-refractory prostate cancer in the near future.

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Disclosures

The authors have no conflicts of interest to declare.

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Figure Legends

Fig. 1. Comparisons of morphology and cell growth between parental LNCaP and LNCaPdcc cells. *A*: The morphology of two cell lines was photographed in 16X and 160X magnification. *B*: LNCaP cells were seeded into 24-well plates at a density of 4×10^4 cells/well in phenol red-positive RPMI-1640 culture medium (10% serum). After 24 hours, the cell counting were carried out every day and lasted for six days by trypan blue staining assay (n = 4). The LNCaPdcc cells were seeded into 24-well plates at a density of 5×10^4 cells/well in phenol red-negative RPMI-1640 culture medium (10% charcoal-stripped serum). After 48 hours, the cell counting were carried out every two days and lasted for 12 days (n = 4). The values of error bars indicated the mean ± standard error of the mean (SEM).

Fig. 2. Analyses of cell cycle distribution and cell cycle-relate protein expressions in both cell lines. *A*: Cells were stained by propidium iodide for 30 min and followed by the analysis of flow cytometry as described in "Materials and Methods" (n=3). The figure indicated the average distribution of cell cycle. The values of error bars are given as the mean ± SEM. *B*: Immunoblotting was performed and specific antibodies were utilized to investigate the expression levels of proteins indicated. β-actin served as an internal control.

Fig. 3. Comparisons of AR-related proteins, AR subcellular distribution, and AR stability between two cell lines. *A*: Immunoblotting was performed and specific antibodies were utilized to investigate the levels of protein expression and phosphorylation. *B*: Protein fractionation was performed on LNCaP and LNCaPdcc cell lysates. AR proteins were immunoblotted in both nuclear (N) and cytosolic (C) fractions. PARP and α-tubulin served as markers for the cytosolic and nuclear fractions, respectively. *C*: Cycloheximide (CHX) (10 ng/mL) was treated on LNCaP and LNCaPdcc cells for 0, 2, 4, and 8 hours in respective culture conditions. The endogenous AR protein degradation was monitored by immunoblotting.

Fig. 4. Difference of androgen sensitivity on proliferation of two cell lines. The cells were seeded separately into 96-well plates at densities of 1.5×10^4 cells/well (LNCaP) and 2×10^4 cells/ well (LNCaPdcc) in steroid-deprived medium. After 48 hours, the R1881 (synthetic androgen) was added to the medium at the concentration of 0, 0.1, 1, and 10 nM for four days. Cell proliferation was analyzed by using MTT assay (n = 8). Control value of cell proliferation was set at 100%. The values of error bars are given as the mean ± SEM. **, $P < 0.01$ *versus* control group of LNCaP cells; ##, $P < 0.01$ and #, $P < 0.05$ *versus* control group of LNCaPdcc cells.

505

506 **Fig. 5. Comparisons of Her2-related issues between two cell lines.** *A*:
507 Immunoblotting was performed and specific antibodies were utilized to investigate
508 the levels of protein expression and phosphorylation in LNCaP and LNCaPdcc cells.
509 *B*: HRG was treated on both cell lines at the concentration of 10 ng/mL in a
510 time-course manner (0, 1, 12, and 24 hours) under serum-free condition.
511 Immunoblotting was performed and specific antibodies were utilized to investigate
512 the levels of phosphorylation and protein expression. *C*: The cells were seeded
513 separately into 96-well plates as described in Fig 4. After cells attached, AG825 (25
514 μM) and Herceptin (20 ng/mL) added in respective complete medium were treated to
515 cells. Cell proliferation was analyzed by using MTT assay (n=8). Control value of cell
516 proliferation was set at 100%. The values of error bars are given as the mean ± SEM.
517 **, $P < 0.01$ *versus* control group of LNCaPdcc cells.

518

519 **Fig. 6. Comparisons of Her2 activity-dependent AR protein stability between two**
520 **cell lines.** *A*: AG825 (25 μM) was treated to LNCaP and LNCaPdcc cells for 24 hours.
521 AR protein degradation was monitored by immunoblotting after different time
522 intervals of CHX treatment (10 ng/mL, 0, 3, 6, and 9 hours). *B*: AG825 (25 μM, 24
523 hours) was treated on both cell lines. The levels of phospho-Ser81 AR and AR protein

were detected by immunoblotting while β-actin served as an internal control. The numbers below the gel images represent the relative levels of protein expressions after quantification.

Figure 1

Hsu *et al*., 2010

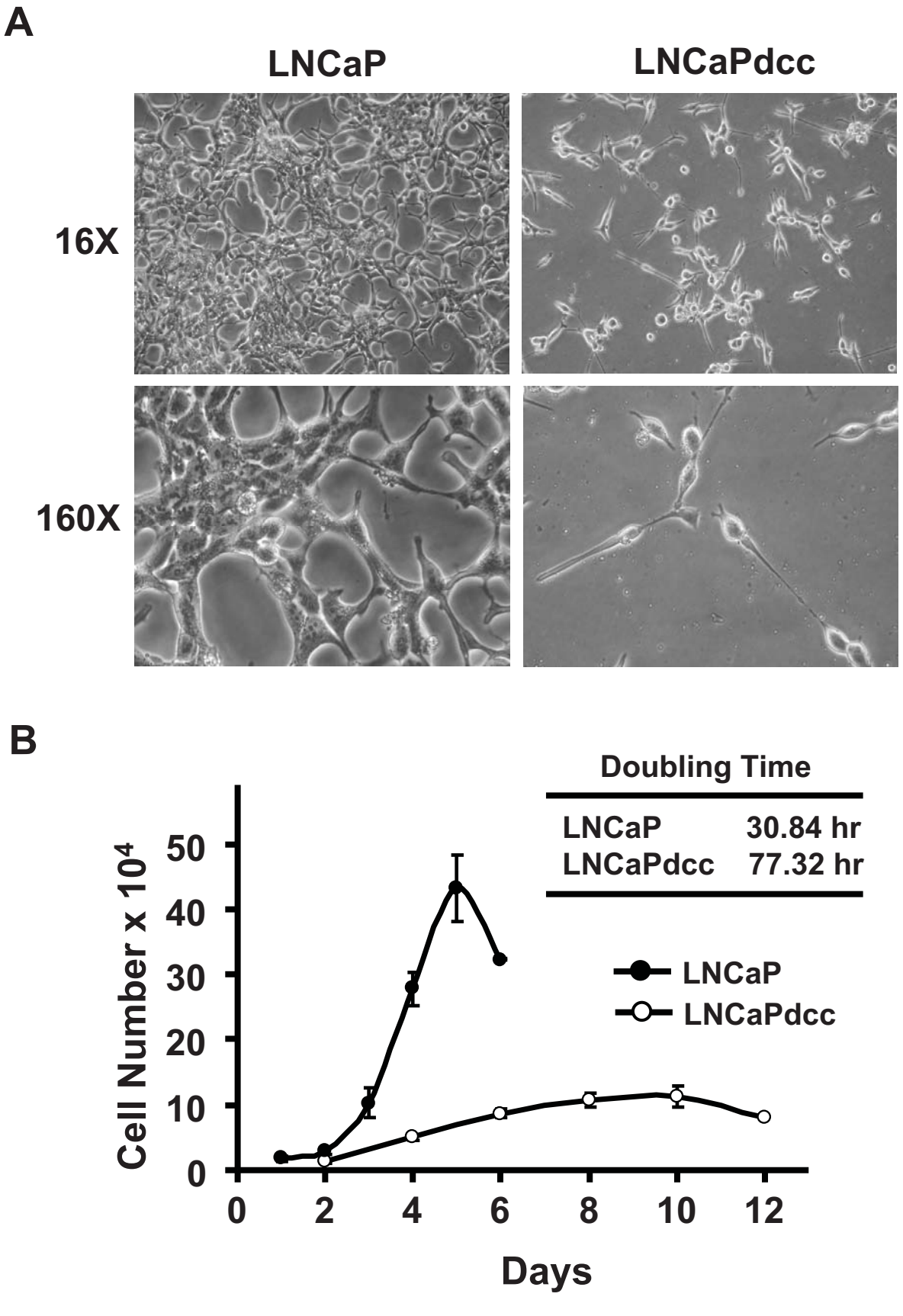


Figure 2 Hsu *et al*., 2010

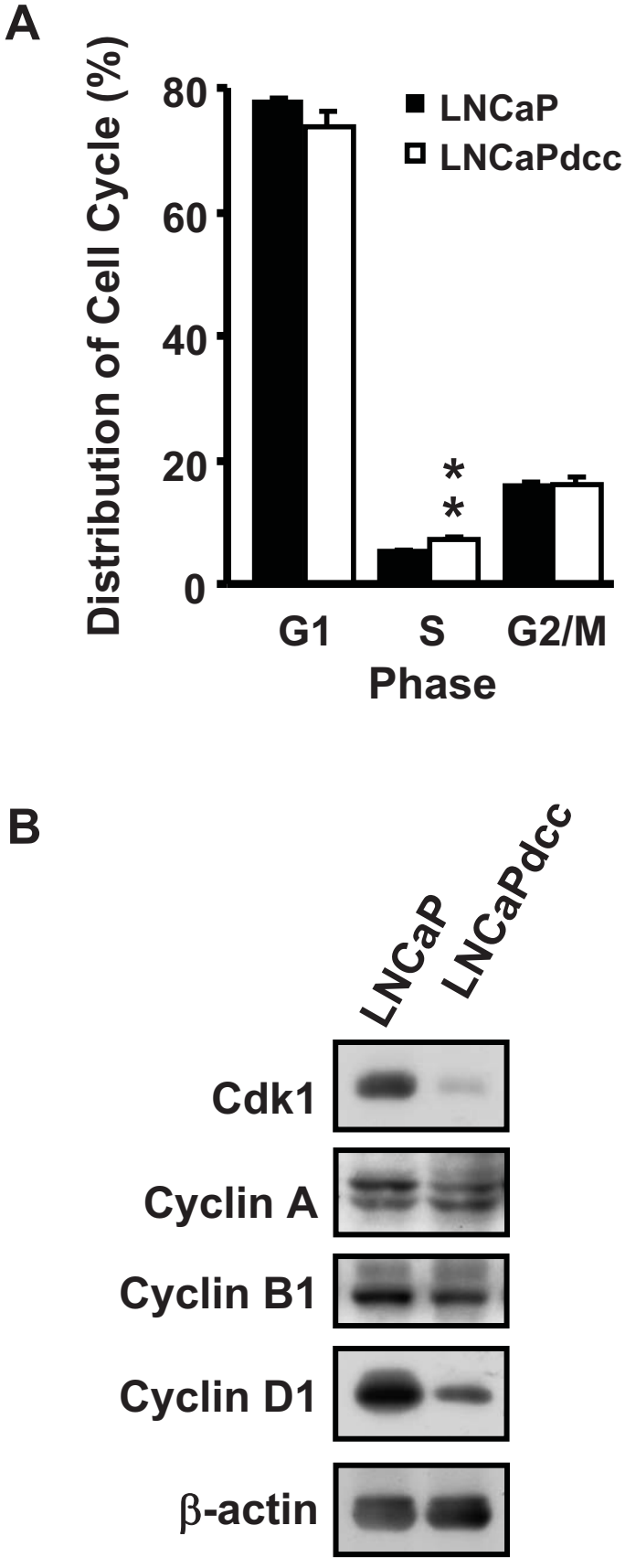
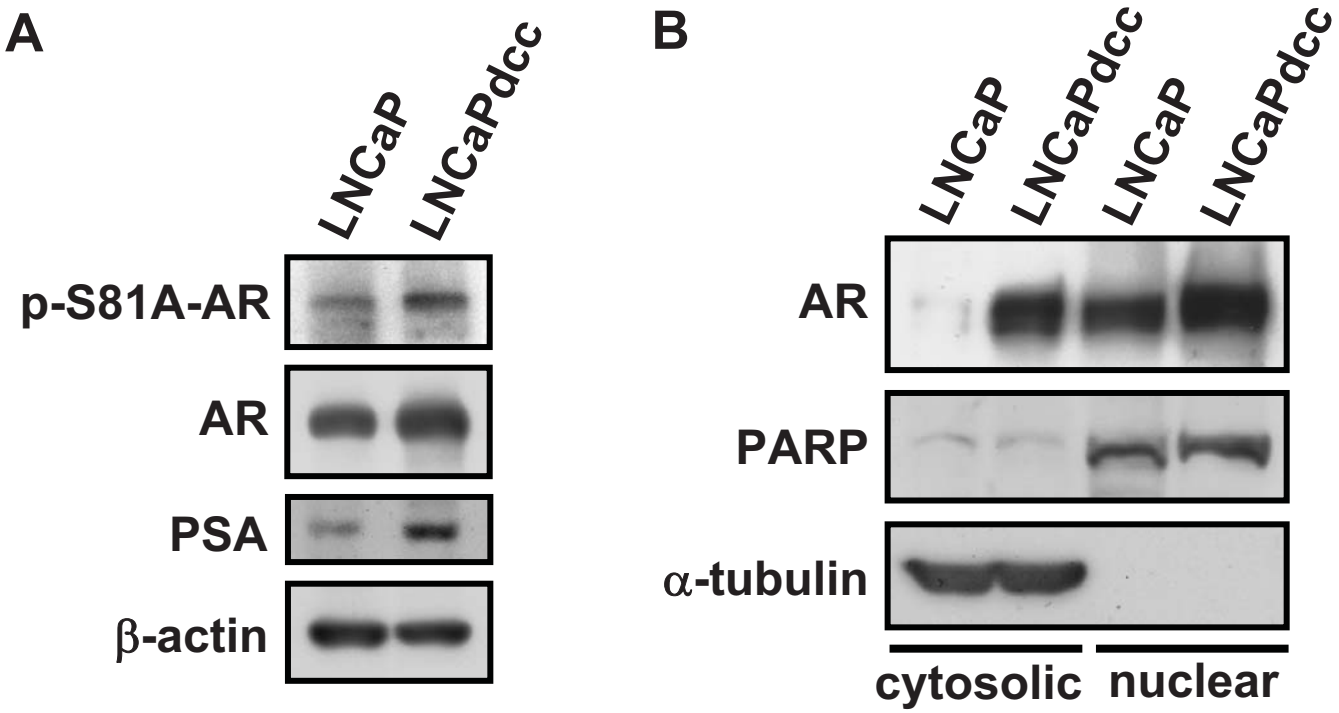


Figure 3

Hsu *et al*., 2010



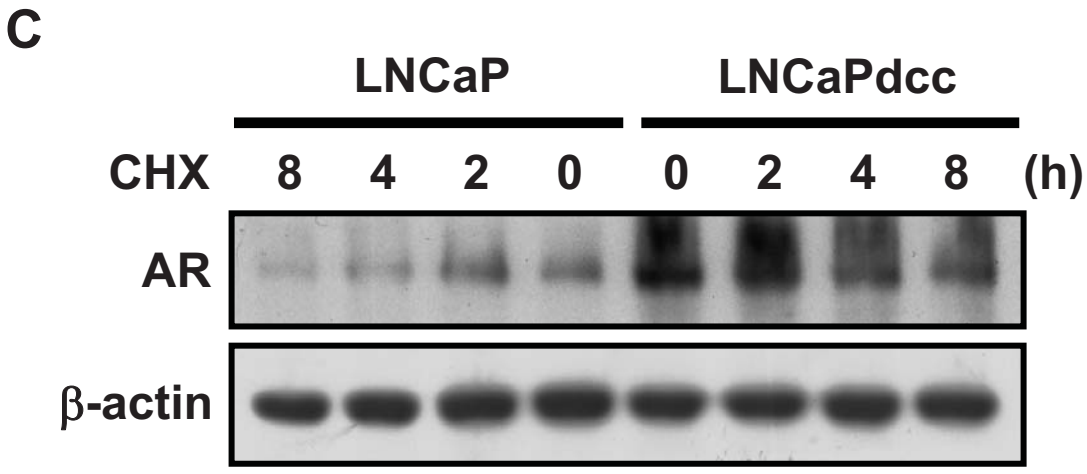


Figure 4 **Hsu *et al*., 2010**

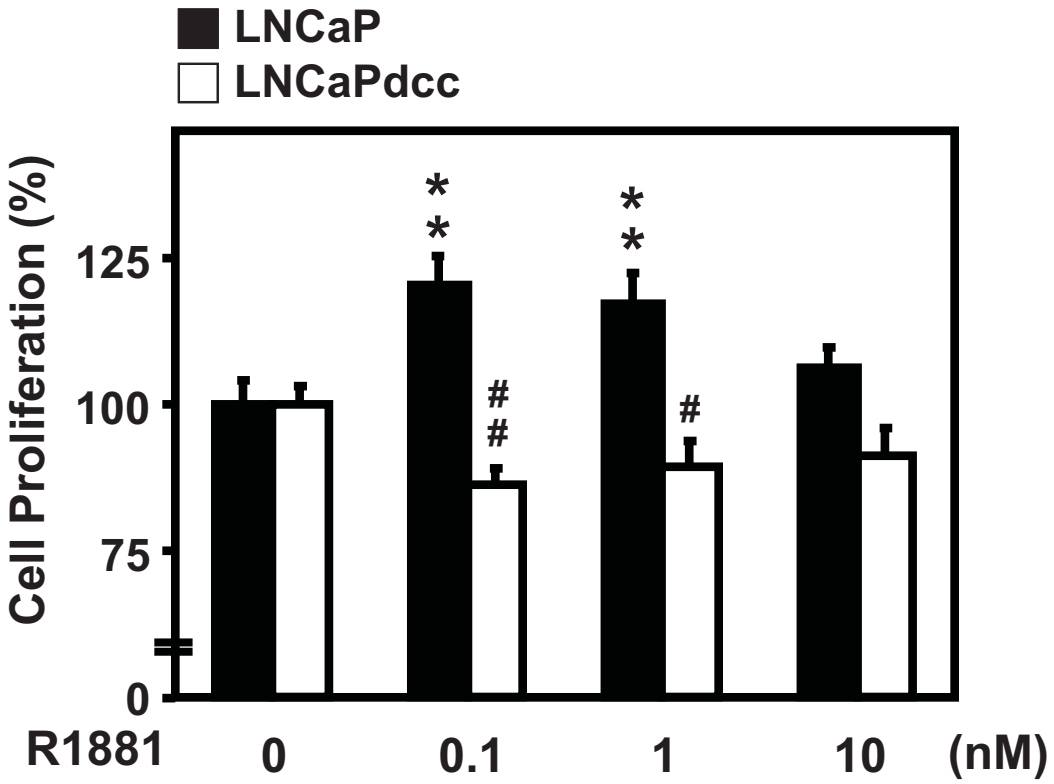
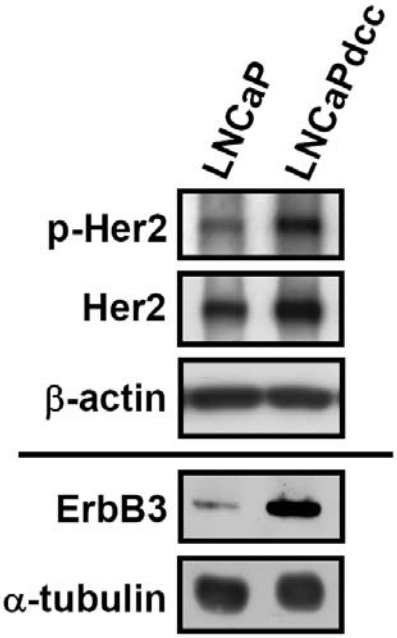


Figure 5

Hsu *et al*., 2010

A



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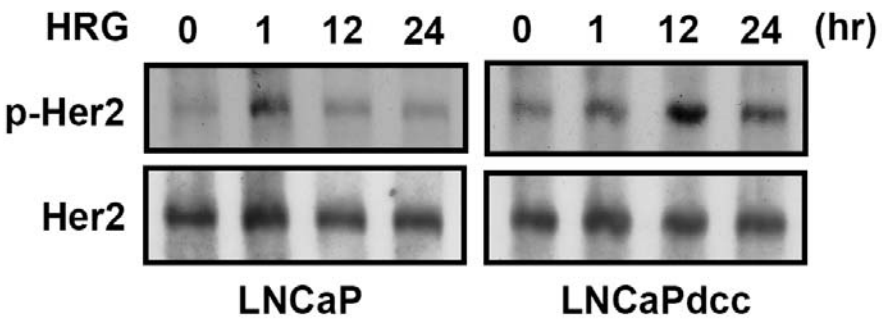

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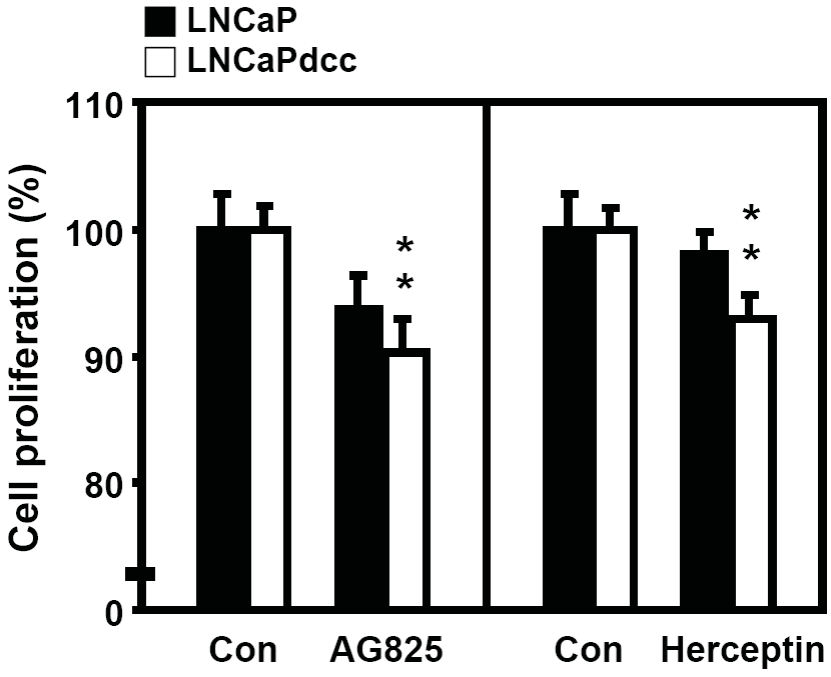

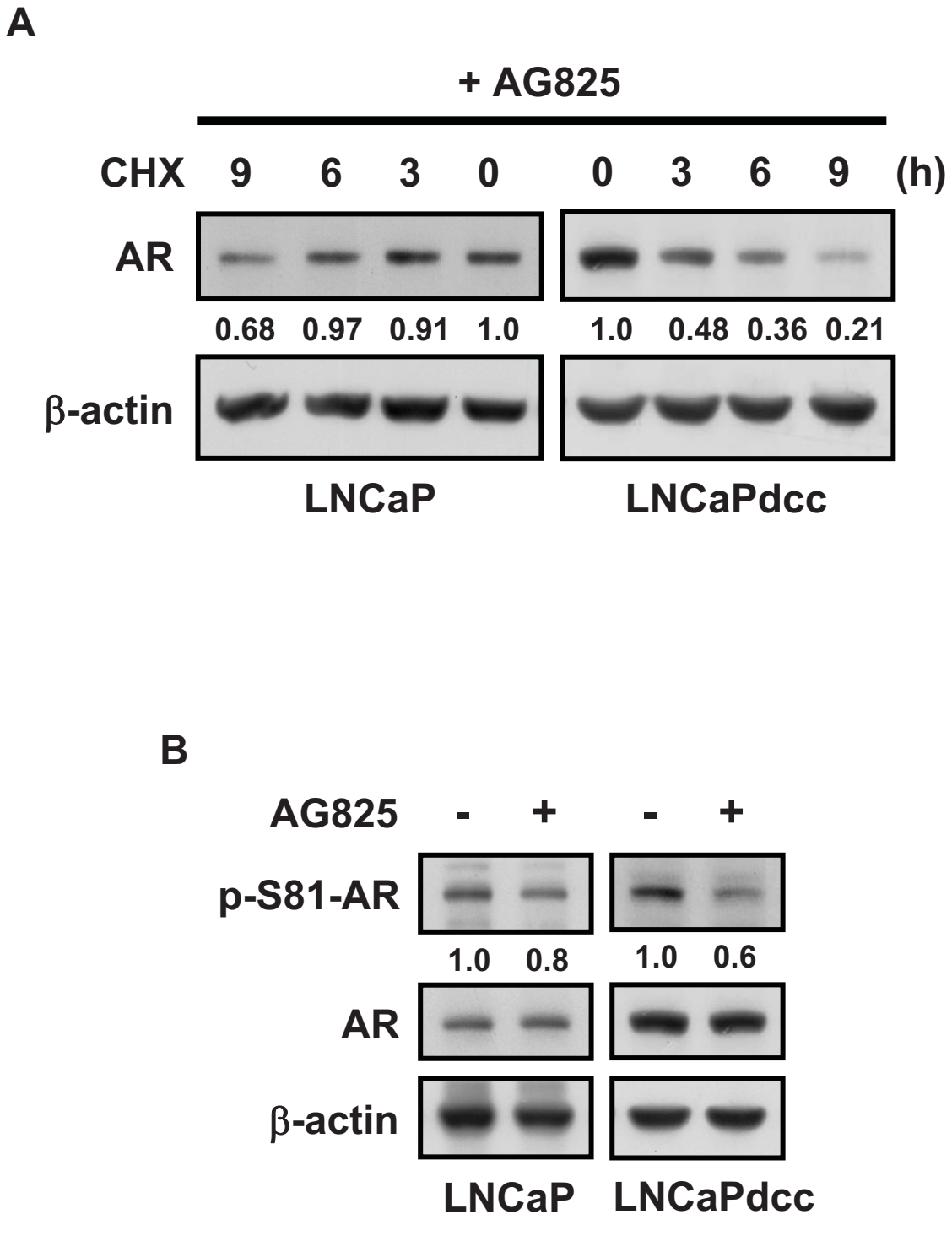
A
+ AG825
CHX 9 6 3 0 0 3 6 9 (h)
AR
0.68 0.97 0.91 1.0 1.0 0.48 0.36 0.21
β-actin
LNCaP
LNCaPdcc
B
AG825 - + - +
p-S81-AR
1.0 0.8 1.0 0.6
AR
β-actin
LNCaP LNCaPdcc