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Progesterone Receptor (PR) Polyproline Domain (PPD) Mediates Inhibition of Epidermal Growth Factor Receptor (EGFR) Signaling in Non-Small Cell Lung Cancer Cells

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Abstract

Recent evidence has suggested a possible role for progesterone receptor (PR) in the progression of non-small cell lung cancer (NSCLC). However, little is known concerning roles of PR in NSCLC. PR contains a polyproline domain (PPD), which directly binds to the SH3 domain of signaling molecules. Because PPD-SH3 interactions are essential for EGFR signaling, we hypothesized that the presence of PR-PPD interfered with EGFR-mediated signaling and cell proliferation. We examined the role of PR-PPD in cell proliferation and signaling by stably expressing PR-B, or PR-B with disrupting mutations in the PPD (PRBΔSH3), from a tetracycline-regulated promoter in A549 NSCLC cells. PR-B dose-dependently inhibited cell growth in the absence of ligand, and progestin (R5020) treatment further suppressed the growth. Treatment with RU486 abolished PR-B- and R5020-mediated inhibition of cell proliferation. Expression of PR-BΔSH3 and treatment with R5020 or RU486 had no effect on cell proliferation. Furthermore, PR-B expression but not PR-BΔSH3 expression reduced EGF-induced A549 proliferation and activation of ERK1/2, in the absence of ligand. Taken together, our data demonstrated the significance of PR extranuclear signaling through PPD interactions in EGFR-mediated proliferation and signaling in NSCLC.

Keywords

progesterone receptor; SH3 domain; lung cancer; EGFR

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1. Introduction

Lung cancer is a leading cause of cancer mortality worldwide. More deaths are attributable to lung cancer than colorectal, breast and prostate cancers combined [1]. Lung cancer is broadly classified into two types, small cell lung cancers (SCLCs) and non-small cell lung cancers (NSCLCs). NSCLC constitute the majority (80–85%) of all lung cancers at diagnosis [2]. Although survival rates from several types of cancers have improved in recent years, the overall 5-year survival rate for advanced NSCLC remains disappointingly low, at approximately 15% [3, 4]. The epidermal growth factor receptor (EGFR) has been shown to play a key role in the development and progression of NSCLC. EGFR is a transmembrane receptor protein and is activated by binding to ligands, resulting in autophosphorylation and activation of several downstream signal transduction molecules and cascades [5]. These include mitogen-activated protein kinase (MAPK), PI3K/AKT, and JAK/STAT pathways, whose activation results in cell proliferation and inhibition of apoptosis [5, 6]. The EGFR gene is often found to be upregulated, amplified, mutated, or overexpressed in subsets of NSCLC specimens from the clinic [7, 8]. As a result, EGFR is often targeted for treatment of NSCLC. However, the effectiveness of EGFR-targeted therapies for NSCLC, such as tyrosine kinase inhibitors, varies depending on several factors, particularly mutations in the EGFR gene [9]. Therefore, a better understanding of the molecular mechanism of signal transduction in NSCLC is urgently needed to help develop new approaches to treatments and prevention.

It is well-established that steroid hormone receptors such as the estrogen receptor (ER) and progesterone receptor (PR) contribute significantly to the development of cancers in hormone-targeted tissues, such as breast, endometrial, and ovarian cancers [10]. While PR is widely used as a prognostic marker in hormone-sensitive cancers, the potential role of PR in NSCLC is not well understood. PR is expressed from a single gene as two isoforms, PR-A and PR-B. In human cells, PR-A is truncated, lacking the first 164 amino acids at the N-terminal domain, but otherwise, both isoforms have identical sequences in the shared N-terminal domain, DNA binding domain (DBD) and ligand binding domain (LBD). The transcriptional activities of the two PR isoforms vary depending on cell type and promoter context [11, 12]. The ratios of PR-B to PR-A have been shown to correlate with clinical outcomes in breast cancer, with high PR-A levels associating with more aggressive tumors and poor prognosis [13]. However, the potential role of PR isoforms in normal lungs and in lung cancer development and progression remains to be determined [3, 14–16].

PR expression in NSCLC is often reported to correlate with less aggressive disease and better prognosis [14–17], although some studies are equivocal [18]. Of note, progesterone treatment of PR expressing NSCLC cells inhibited cell growth both *in vitro* and in a mouse xenograft model *in vivo* [15, 16]. In contrast, treatment with anti-progestin (Mifepristone or RU-486) reduced the progression of spontaneous lung tumors in mice [19], whereas lung tumors with little to no PR expression were shown to be more aggressive [15]. Lack of PR expression was also reported to correlate with EGFR mutations in NSCLC [20]. Additionally, a recent study suggested that PR expression in cells adjacent to or surrounding tumor cells was associated with improved disease-specific survival [21]. Further reports identified nuclear PR expression as a significant biomarker in stage-I NSCLCs, and the

investigators suggested that PR could serve as a predictor of better prognosis and extened survival time [22]. Interestingly, these protective effects of PR were shown in older men and postmenopausal women, who had little to no circulating progesterone [17], thus suggesting that progesterone-independent mechanisms may have been involved. How PR signals and affects the development and progression of NSCLC remains unknown. Together, these data demonstrate the need for a better understanding of PR signaling in NSCLC, and suggest an alternative PR interactive pathway whereby PR cross-communication with growth factors or growth factor receptors may have a significant impact on NSCLC proliferation and progression. As a first step toward a better understanding of PR functions in NSCLC, it is crucial to develop cell models to help with *in vitro* analyses of PR functions in NSCLC.

Previously, we demonstrated that the N-terminal region of human PR contained a polyproline domain (PPD) at amino acids 421–428. This SH3 recognition motif, a PXXPXR motif, directly binds to the SH3 domain of c-Src and selected SH3 domain-containing molecules, and plays a key role in PR extranuclear signaling [23, 24]. We demonstrated that PR is the only steroid hormone receptor that contains the PPD, a PXXPXR motif, and a consensus sequence for SH3 ligand [23, 25]. The PPD is required for progestin-dependent rapid activation of c-Src and MAPK [23]. More recently, we found that expression of PR-PPD in breast cancer inhibited breast cancer growth in 3-D cultures in the absence of ligand [26]. SH3-PPD domain interactions are often used by many signaling molecules and growth factor receptors, such as EGFR, to transduce signals inside the cell [27]. Because SH3-PPD interactions are essential for EGFR signal transduction, we hypothesized that the presence of PR-PPD could interfere with EGFR-mediated signaling and NSCLC proliferation.

In this study, we aimed to elucidate the role of PR-B and PR-PPD extranuclear signaling on NSCLC cell proliferation and EGFR activation of signaling pathways. We also examined the involvement of ligand in the PR-mediated proliferation of NSCLC. PR-B was chosen for this analysis because PR-B is distributed equally in the cytoplasm and the nucleus in the absence of ligand [28] and is capable of mediating PR extranuclear signaling, whereas PR-A localizes only to the nucleus and is unable to mediate PR extranuclear signaling [28, 29]. Our data demonstrate the significance of PR extranuclear signaling through PPD interactions and suggest that PR inhibition of NSCLC growth is partly mediated through its PPD.

2. Materials and Methods

2.1 Cell lines

HEK293T, T47D and A549 cell lines were obtained from the American Type Culture Collection (ATCC). HEK293T is a human embryonic kidney cell line 293T and was used for Lentiviral production. T47D is a human breast ductal epithelial tumor cell line, which has high constitutive expression of PR. T47D was used as a positive control for PR expression. HEK293T and T47D cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, Logan, USA) supplemented with 10% fetal bovine serum (FBS; Merck Millipore, Darmstadt, Germany) and 1% Penicillin Streptomycin (PenStrep; HyClone Laboratories, Logan, USA). A549 is a human non-small cell lung carcinoma cell line. A549 has wild-type EGFR expression, no PR expression, and was used for the construction of cell models. The identity of A549 was confirmed by genomic DNA comparison to the ATCC

database. A549 was cultured in Roswell Park Memorial Institute medium (RPMI; HyClone Laboratories, Logan, USA) plus 5% FBS and 1% PenStrep. All cells were cultivated in a humidified tissue culture incubator at 37°C with 5% CO_2.

2.2 Plasmid construction and lentiviral production

To construct the tetracycline (Tet)-inducible vectors PR-B and PR-BΔSH3, pHAGE-PR-B and pHAGE-PR-BΔSH3, encoding PR-B or PR-BΔSH3, were PCR amplified from pcDNAI-PR-B and PR-BΔSH3 vectors [23]. PR-B and PR-BΔSH3 PCR products were inserted into pENTR/D-TOPO (Invitrogen, USA), resulting in pENTR/D-PRB and pENTR/D-PR-BΔSH3. To block the expression of PR-A, the PR-A transcriptional start site ATG was mutated to GCG in PR-B and PR-BΔSH3 constructs using the Quick-Change II kit as described [30]. PR-B and PR-BΔSH3 cDNA were then cloned in between attR1 and attR2 of pHAGE-Dest (pINDUCER20, a Tet-inducible bicistronic lentiviral vector for the inducible expression of a gene of interest driven by the tetracycline response element, TRE, and constitutive expression of the neomycin resistance gene driven by the ubiquitin C promoter, Ubc [31]) using the Gateway cloning system with LR Clonase II as suggested by the manufacturer (Invitrogen/Thermo Fisher, USA). The pINDUCER20 plasmid was a gift from Stephen Elledge (Addgene plasmid # 44012). The pHAGE-Dest lentiviral vector is a Tet-on vector that encodes a recombinant tetracycline controlled transcription factor (rtTA3) gene [31]. Therefore, expression of PR-B and PR-B-ΔSH3 was induced by increasing the concentration of a tetracycline analog, doxycycline (Dox). PR-B and PR-BΔSH3 sequences were verified by DNA sequence analysis to confirm the fidelity of the constructs. A similar cloning strategy using a Green Fluorescence Protein (GFP) cDNA was also used to generate a control Tet-inducible pHAGE-GFP lentiviral construct.

To generate pHAGE lentiviral particles, HEK293T cells were seeded at 100,000 cells per well in a 6-well tissue culture plate and incubated for 24 hours to achieve 50–60% confluency. Cells were treated with Opti-MEM I medium (Gibco/Life Technologies, Gaithersburg, USA) mixed with packaging plasmid (psPAX2), envelope plasmid (pMD2G), a DNA construct (pHAGE-PRB, pHAGE-PR-BΔSH3 or pHAGE-GFP), and X-tremeGENE HP DNA transfection reagent (Roche, Mannheim, Germany). psPAX2 and pMD2G plasmids were gifts from Didier Trono (Addgene plasmid # 12260 and # 12259, respectively). The ratio of lentiviral DNA construct:psPAX2:pMD2G was 1:2:1, and the ratio of the DNA construct:X-tremeGENE HP DNA transfection reagent was 1:3. Cells were incubated with the DNA-XtremeGENE mixture for 48 hours, and media containing viral particles were subsequently collected, filtered with 0.45 μM sterile filter PVDF membranes (Merck Millipore, Ireland), and stored at −80 °C [32].

2.3 Construction of A549 cells expressing tetracycline (Tet)-inducible proteins

A549 cells were seeded at 800,000 cells per well in a 6-well dish and incubated 24 hours to achieve 50–60% confluency. Cells were treated with 2.5 ml of medium containing pHAGE-PRB or pHAGE-PRBΔSH3 viral particles mixed with 2.5 ml serum-free RPMI medium and 8 μg/ml of polybrene transfection reagent [32] (Merck Millipore, Darmstadt, Germany), and incubated at 37 °C for 6 hours. After 6 hrs, 1 ml of DMEM supplemented with 10% FBS was added to each well, and cells were incubated with the viral particles for an additional 48

hours. To select for A549 cells with inducible PR-B or PR-BΔSH3 expression, viral transduced A549 cells were grown in A549 medium supplemented with 500 μg/ml G418 (Gibco/Life Technologies, Gaithersburg, USA) for 14 days. After 14 days, various concentrations of Dox (Merck Millipore, Darmstadt, Germany) were added to A549-PR-B and A549-PR-BΔSH3 cells to induce the expression of PR-B and PR-BΔSH3 proteins [33]. PR-B and PR-BΔSH3 protein expression patterns in cell models were determined by Western blot and immunohistochemistry using 1294 PR-specific mouse monoclonal antibody [34].

2.4 Gel electrophoresis and western blot analysis

PR-B and PR-BΔSH3 protein expression patterns in A549 cells were examined by western blotting as previously described [23, 35]. Briefly, cells were washed once with ice-cold phosphate buffered saline (PBS; HyClone Laboratories, Logan, USA), and incubated with RIPA lysis buffer (Merck Millipore, Billerica, USA) containing proteinase inhibitor cocktail (Roche, Mannheim, Germany) on ice for 5 minutes. Cells were scraped and lysed. Lysates were rotated end-over-end for 30 minutes, and then centrifuged at 8000 rpm at 4°C for 10 minutes. Total protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, USA). Cell lysates were resolved on a 10% SDS-PAGE gel, and proteins were transferred onto PVDF membranes. Blotted membranes were blocked with blotting-grade Blocker (Bio-Rad, Hercules, USA) for 1 hour at room temperature, and washed with TBST buffer (1 M Tris-base pH 7.4, 5 M NaCl, 0.05% Tween-20). Blots were incubated with (1:2000 v/v) primary antibody recognizing PR-B (1294 PR, a specific mouse monoclonal antibody [34]), or GAPDH antibody (1:2500 v/v, Santa Cruz Biotechnology, CA, USA) overnight at 4°C. Following incubation with primary antibodies, blots were washed three times with TBST buffer, and incubated with secondary antibodies, (1:2500 v/v) anti-mouse IgG HRP-linked antibody (7076s; Cell Signaling Technology, Danvers, USA) or anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, Danvers, USA) for 1 hour at room temperature. Protein bands were visualized on X-Ray film by a chemiluminescence reaction using Pierce® ECL Western Blotting Substrate (Thermo Scientific, Rockford, USA). Protein bands on X-Ray film were quantitated and analyzed by a gel documentation system (Syngene, Frederick, MD, USA) using GeneTools software (Image Analysis Software).

2.5 Immunofluorescence

The intracellular localization of PR-B and PR-BΔSH3 proteins in A549-PR-B and A549-PR-BΔSH3 cells was examined by immunofluorescence staining. A549-PR-B, A549-PR-BSH3 and T47D cells (positive controls for PR expression [36, 37]) were seeded on a coverslip that was placed in a 6-well plate at 37°C overnight, and then Dox (500 ng/ml) was added to induce PR-B and PR-BΔSH3 expression for 24 hours. Thereafter, cells were treated with 10 nM progestin agonist (R5020) (PerkinElmer, Boston, USA), for 1 hour. Cells were then washed with ice-cold PBS at pH 7.4, fixed in 4% paraformaldehyde for 20 minutes, washed with PBS with 0.5% Triton X-100 3 times, and then blocked with 1% FBS-BSA (Merck Millipore, Billerica, USA) for 1 hour. Cells were incubated with primary antibodies for PR (1294 mouse monoclonal antibody [34]) at 1:1000 in 1% BSA-PBS (v/v) at 4°C overnight. Following primary antibody incubation, cells were washed three times with PBS, and incubated with secondary antibody, rabbit anti-mouse IgG-Alexa555 at 1:3000 in 1%

BSA-PBS (v/v), for 1 hour at room temperature. Coverslips were washed with PBS three times, incubated with DAPI (4',6-diamidino-2-phenylindole) at 1:5000 in PBS (v/v) for 10 minutes. Thereafter, samples were washed with PBS three times, and mounted on microscope slides with Prolong® Gold antifade reagent (Invitrogen, Carlsbad, USA). The localization patterns of PR-B and PR-BΔSH3 proteins were analyzed using a LSM700 laser confocal scanning microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Fluorophores were excited with a 405-nm laser for DAPI and a 561-nm laser for Alexa555. PR immunofluorescence staining images were analyzed by ImageJ v 1.50 software (National Institute of Health, USA). Fluorescence intensity for each cell was obtained by drawing area of interest (ROI) around the whole cell or nucleus that showed PR staining to measure average gray value within the selection. Average fluorescence intensity for each image represented mean average gray value from cells or nuclei in each image.

2.6 Real-time PCR

Total RNA was extracted from T47D and A549 cell lines using Genezol reagent (GeneAid, Taiwan). One microgram of total RNA was used to generate cDNA using Accupower RT Premix (Bioneer, Korea). cDNA was analyzed by real-time PCR with primers for the PR gene (forward primer: 5’ TCGCCTTAGAAAGTGCTGTC 3’, reverse primer: 5’ GCTTGGCTTTCATTTGGAACG 3’) with a 138 bp PCR product. cDNA from T47D breast cancer cells was used as a positive control. A negative control was performed on RNA samples without the addition of reverse transcriptase to confirm the absence of genomic DNA contamination. PCR products were analyzed by DNA gel electrophoresis.

2.7 Luciferase assay

A549, A549-PR-B or A549-PR-BΔSH3 cells were seeded at 3×10^4 cells/well in RPMI supplemented with 5% FBS and 1% PenStrep in a 24-well plate and incubated overnight. The following day, medium was removed and cells were rinsed twice with PBS before medium was replaced with RPMI supplemented with 5% DCC-FBS (Dextran-coated charcoal stripped FBS; Gibco/Life Technologies, Gaithersburg, USA). Cells were transfected with 50 ng pRL-CMV (Renilla) and 450 ng PRETK-Luciferase reporter construct [35]. Transient transfections were carried out using the Lipofectamine® 3000 transfection kit (Thermo-Fisher Scientific, Waltham, USA). At twenty-four hours after transfection, cells were treated with 500 ng/ml Dox and 10 nM R5020 or ethanol (vehicle control) for an additional 24 hours. Cells were then harvested, and lysates were analyzed for luciferase activity using the Dual-Glo® Luciferase Assay System (Promega, Madison, USA) according to the manufacturer’s recommendation.

2.8 Cell proliferation assay (MTT assay)

Cells were seeded in RPMI supplemented with 5% DCC-FBS (Gibco/Life Technologies, Gaithersburg, USA) and 1% PenStrep at 3,000 cells per well in a 96-well tissue culture plate, and incubated for 24 hours to achieve 50–60 % confluence. The following day, cells were induced with increasing concentrations of Dox (0, 50, 200 and 500 ng/ml) for 24 or 48 hours to determine the optimal Dox concentration for maximum PR expression. In subsequent experiments, cells were seeded for 24 hours and then treated with Dox for an additional 24 hours for maximal PR expression, prior to treatment with other chemicals. To

determine the effects of EGF, cells pretreated with 500 ng/ml Dox for 24 hours and then treated with different concentrations of EGF (0, 10, 20, 50 and 100 ng/ml) for an additional 24 hours [38]. To determine the effects of progestin or anti-progestin, cells were pretreated with 500 ng/ml Dox for 24 hours, then culture medium was removed and replaced with RPMI supplemented with 5% DCC-FBS and 1% PenStrep containing 500 ng/ml of Dox and either 10 nM R5020 or 100 nM RU486 or the combination of 10 nM R5020 and 100 nM RU486 [30, 39] (Sigma-Aldrich, St. Louis, USA) for an additional 24 or 48 hours.

Following treatments, 20 μl per well of 50 mg/ml 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) solution (Applichem, Darmstadt, Germany) was added to cells; cells were incubated for 4 hours, until a purple precipitate was visible. Precipitates were dissolved with DMSO by gentle shaking to completely dissolve the precipitate, and 200 μl was transferred to a new 96-well plate. Absorbance was measured at 550 nm using a microplate spectrophotometer (Biotek Synergy Mx microplate reader, Biotek, USA). All experiments were carried out in quadruplets, and the data were calculated from three independent experiments as means ± SEM. Results are presented as % cell viability compared with the control value of each experiment.

2.9 Western Blotting of MAPK and the AKT Signaling Pathway

A549, A549-PR-B or A549-PR-BΔSH3 cells were seeded in RPMI supplemented with 2% DCC-FBS (Gibco/Life Technologies, Gaithersburg, USA) and 1% PenStrep at 200,000 cells per well in a 6-well tissue culture plate, and incubated for 24 hours. The next day, cells were induced with 500 ng/ml Dox for 24 hours. Following Dox treatment, cells were treated with increasing concentrations of EGF (0, 10, 20 and 50 ng/ml) [40] for different durations (0, 10, 30 and 60 minutes). After EGF treatment, cells were lysed using RIPA lysis buffer (Merck Millipore, Billerica, USA), proteinase inhibitor cocktail (Roche, Mannheim, Germany), 5 mM sodium fluoride and 2 mM sodium vanadate. Equal amounts of cell lysate proteins were loaded on SDS-PAGE gels, electrophoresed, and subjected to Western blot analysis, as previously described [28]. Blots were probed with primary antibody recognizing phospho-p44/42 MAPK (Erk-1/-2, Thr202/Tyr204, rabbit monoclonal antibody, 4370, Cell Signaling Technology, Danvers, USA), p-Akt (S473, D9E rabbit monoclonal antibody, 4060, Cell Signaling Technology, Danvers, USA), total MAPK (p44/42 MAPK, or Erk-1/-2, rabbit monoclonal antibody, 4695, Cell Signaling Technology, Danvers, USA) and total AKT (pan Akt, c67E7, 4691, Cell Signaling Technology, Danvers, USA) as suggested by the manufacturer.

2.10 Statistics

Statistical analysis was performed by employing a paired t-test or a one-way ANOVA method as appropriate with a Bonferroni post-test correction using SPSS statistical software version 20. Values were represented as means ± SEM, and p values <0.05 were considered to be significant.

3. Results

3.1 Characterization of tetracycline-inducible PR-B and PR-BΔSH3 expression in A549 NSCLC cell models

Previous studies demonstrated that PR is expressed in archival human NSCLC specimens from clinics [15, 16], and the loss of PR expression in the early stage of NSCLC is associated with poor clinical outcomes [22]. However, a reliable *in vitro* cell model to analyze PR biological functions in NSCLC is not currently available. As a first step toward understanding PR function in NSCLC, we constructed an *in vitro* model to study PR function in A549 NSCLC cells. Like several NSCLC cell lines, A549 expresses wild-type EGFR and is sensitive to EGF-mediated cell proliferation [41], thereby allowing us to explore crosstalk between PR and EGFR in NSCLC cells. Using RT-PCR with PR specific primers and western blotting with a PR-specific antibody, we were able to detect PR mRNA and protein expression in T47D breast cancer cells, but failed to detect any PR mRNA or PR protein expression in A549 cells (Figure 1 A–C). While mRNA from A549 showed a small increase in fluorescence signal in the 31st cycle as shown in the RT-PCR amplification plot (Figure 1A), the signal was below the threshold limit for the assay. Together, these data indicated that A549 cells did not express any significant amount of endogenous PR under our experimental conditions. This allowed us to analyze the role of ectopically expressed PRs without interference from endogenous PR.

To examine the role of PR and the PR PPD-SH3 interaction in NSCLC, we stably expressed tetracycline (Tet)-inducible wild-type PR-B, or PR-B with point mutations (ΔSH3) in the SH3 domain binding motif [28], in A549 NSCLC cells using lentiviral transduction as described in Materials and Methods. Point mutations in the PPD of PR in PR-BΔSH3 cells (P422A, P423A, P427A) abolished PR interactions with c-Src and other SH3-containing signaling molecules, but had no detectable effect on other functional activities of PR, including hormone binding and progestin-dependent transcriptional activity of a progesterone response element (PRE) regulated reporter construct [23, 25]. Therefore, the ability of PR to interact with Src or other SH3 domain-containing molecules seems to be independent of other functions of the receptor and is dissociable by point mutations in the PPD [23, 25]. We limited our analysis to only the PR-B isoform because PR-B exhibits nuclear and cytoplasmic localization, and has extra-nuclear functions whereby it interacts with cytoplasmic/membrane signaling molecules. PR-A, on the other hand, exhibits only nuclear localization and lacks extra-nuclear functions [24, 28]. Thus, the PR-A transcriptional start site ATG was mutated to CAG in both PR-B and PR-BΔSH3 constructs to block PR-A expression to ensure that only PR-B was expressed our cell models, thus allowing us to analyze the role of PR-B in NSCLC, independent of PR-A.

Tet-inducible expression of PRs permitted us to directly compare how PR-B or PR-BΔSH3 expression affected cellular properties under the same cellular background. Cells were transduced with lentiviral constructs [31] for Tet-inducible expression of PR-B or PR-BΔSH3, and selected with 500 μg/ml G418 for 14 days for cells expressing inducible PRs as described in Materials and Methods. Selected cells were treated with increasing doses of tetracycline analog (doxycycline, Dox) for 24 hours, lysed, and analyzed for PR expression

by western blots using a PR-specific monoclonal antibody [34]. No PR expression was detected in A549-PR-B or A549-PR-BΔSH3 cells in the absence of Dox. Dox treatment (50–1000 ng/ml) dose-dependently increased PR-B and PR-BΔSH3 expression, including the maximum dose of 500 ng/ml (Figure 2A and 2B). Dox-treated A549-PR-B and A549-PR-BΔSH3 cells showed equivalent PR protein levels as determined by Western blot analysis (Figure 2C). Furthermore, these cells did not overexpress PR-B, and expressed approximately 15% as much PR-B as T47D breast cancer cells (Figure 2C). To determine the optimal time for PR-B and PR-BΔSH3 expression, A549-PR-B and A549-PR-BΔSH3 cells were treated with 500 μg/ml Dox, and cells were analyzed for PR expression at 24, 48 and 72 hrs. Maximum PR expression was observed 24 hours after Dox treatment (Figure 2D and 2E).

We next examined the intracellular localization of ectopically-expressed PR-B and PR-BΔSH3 in A549 cells. A549-PR-B and A549-PR-BΔSH3 cells were induced with Dox for 24 hours and then treated with 10 nM R5020 or ethanol for 60 min. Cells were fixed and analyzed by immunofluorescence using a PR-specific monoclonal antibody. As shown in Figure 3A, no PR staining was detected in A549 cells in the absence of Dox. Dox-induced PR-B and PR-BΔSH3 in A459 cells exhibited a distribution between the nucleus and cytoplasm in the absence of hormone. Addition of synthetic progestin (R5020) for 60 min caused an increased accumulation of PR-B and PR-BΔSH3 in the nucleus, with little PR remaining in the cytoplasm (Figure 3A, panels A–C and D–F). A similar intracellular localization of PR was observed in T47D breast cancer cells expressing endogenous PR-B and PR-A (Figure 3A, panels G–I). Dox treatment of A549-PR-B and A549-PR-BΔSH3 resulted in an increase in the average PR-immunofluorescence intensity as shown in Figure 3B. These findings were similar to results obtained in a previous study of ectopically expressed PR-B and PR-BΔSH3 in PR-null breast cancer cells [28]. We next examined the transcriptional activity of Dox-induced PR-B and PR-BΔSH3 expressed in A549 cells by transiently transfecting A549-PR-B and A549-PR-BΔSH3 cells with a luciferase reporter construct containing the progesterone response element (PRE2-TK-Luc) [12]. R5020 failed to induce PRE2-TK-Luc reporter activities in A549 cells. In the absence of Dox, R5020 treatment did not induce reporter activity in A549-PR-B or A549-PR-BΔSH3 cells. Dox treatment induced PR-B and PR-BΔSH3 expression, which mediated an equivalent induction of the PRE2-TK-Luc reporter by R5020 in A549 cells (Figure 3C). Together, these results demonstrated that Dox-induced PR-B and PR-BΔSH3 proteins in A549 cells were functional, transcriptionally active, and exhibited normal and appropriate PR localization, similar to endogenous PR in T47D breast cancer cells.

3.2 The PPD of PR is required for PR-mediated inhibition of NSCLC cell proliferation in the absence and presence of progestin

The growth of NSCLC cells such as A549 cells is often dependent upon the activation of the EGFR signaling cascade, which requires PPD-SH3 interactions to transmit downstream signals in cells. We asked whether the presence of the PPD, a high affinity ligand for the SH3 domain [23], in PR could inhibit NSCLC cell proliferation. A549-PR-B and A549-PR-BΔSH3 cells were treated with increasing concentrations of Dox (0–500 ng/ml) for 24 hrs. Under these conditions, Dox treatment was shown by Western blotting to induce the

expression of PR-B and PR-BΔSH3 proteins in a dose-dependent manner. Expression of PR-B protein, in the absence of progestin, dose-dependently inhibited A549 cell proliferation (Figure 4A) with maximum inhibition at 500 ng/ml Dox, whereas expression of PR-BΔSH3 or control GFP had little to no effect on A549 cell proliferation at all Dox concentrations tested. Our data suggested that a single Dox treatment (500 ng/ml) could induce PR expression for up to 48 hrs (Figure 2D and 2E); therefore, we tested whether addition of fresh Dox every 24 hour would help maintain high levels of PR expression. As shown in Figure 3B, PR expression levels were maintained in A549-PR-B, A549-PR-BΔSH3 for up to 72 hrs when the culture medium containing 500 ng/ml Dox was replaced every 24 hrs. To determine whether Dox-induced PR-B expression could inhibit A549 cells at later times, A549-PR-B, A549-PR-BΔSH3 and A549-GFP (control) cells were treated with 500 mg/ml Dox for 24 hr and the culture medium containing 500 ng/ml Dox was replaced every 24 hour up to 48 hrs. The %cell viability was determined by an MTT assay at 24 and 48 hrs after Dox treatment. Expression of PR-B in A549 cells significantly inhibited cell proliferation at 24 and 48 hrs after Dox treatment, while expression of PR-BΔSH3 and GFP (control) had little to no effect on A549 cell proliferation at both time points (Figure 4C).

To determine whether the presence of PR-PPD, in the absence of ligand, could interfere with and reduce the EGF-mediated proliferation of A549 NSCLC cells, cells were treated with Dox for 24 hrs prior to EGF treatment (0–50 ng/ml). In the absence of EGF, PR-B expression reduced the basal cell proliferation of A549 cells (Figure 4D). Addition of EGF elicited a dose-dependent increase in cell proliferation. PR-B expression significantly reduced EGF-induced cell proliferation at all EGF concentrations tested (Figure 4D). These data suggested that PR mediated the inhibition of A549 basal and EGF-induced cell proliferation through its PPD. PR-B mediated inhibition of cell proliferation is independent of hormone, suggesting that this property of the PR PPD is independent of its nuclear transcriptional activity.

We next examined whether a progestin agonist (R5020) and antagonist (RU486) affected the proliferation of A549 cells expressing PR-B and PR-BΔSH3. A549-PR-B cells were treated with 500 ng/ml of Dox for 24 hr before the culture medium was changed to medium containing 500 ng/ml Dox with ethanol (vehicle), 10 nM R5020, 100 nM RU486, or a combination of 10 nM R5020 and 100 nM RU486 and then incubated for an additional 24 or 48 hrs. On addition of Dox every 24 hrs, A549 cells expressing PR-B showed a significant reduction in cell growth by 15 ± 5.2 and 40 ± 5.96 percent at 48 and 72 hr after Dox treatment, respectively, compared with control A549-PR-B cells with no Dox treatment, in the absence of progestin (Figure 5A). Addition of R5020 further suppressed the proliferation of A549 cells expressing PR-B by 29 ± 5.7 and 54 ± 0.4 percent at 24 and 48 hrs, respectively, compared with control A549-PR-B cells with no Dox treatment (Figure 5A). Previous studies have shown that RU486 inhibited both nuclear and extra-nuclear activities of PR-B [23, 42]. Treatment of A549 cells expressing PR-B with RU486, in the absence of R5020, abolished PR-B mediated inhibitory growth effects. The addition of RU486, in combination with R5020, blocked R5020-mediated inhibition of cell growth in A549 cells expressing PR-B (Figure 5A), suggesting that the inhibition of cell growth was mediated through PR. However, R5020 and/or RU486 treatment had little to no effect on the growth of A549 cells expressing PR-BΔSH3 (Figure 5B). The differences in the PR ability to inhibit

cell proliferation was not due to differences PR levels. As shown in Figure 5 C and D, PR levels in A549-PR-B and A549-PR-BΔSH3 at the last time point after 72 hr of Dox treatment were similar and were similarly reduced after 48 hr of R5020 and/or RU486 treatment, as previously reported [43, 44]. Together, these data suggested that the inhibition of cell proliferation observed in A549-PR-B cells was likely mediated through the PR-PPD by progestin-dependent and -independent mechanisms that could be blocked by RU486.

3.3 PR-PPD mediated inhibition of EGF-induced ERK1/2 activation

Activation of the MAPK (ERK1/2) signaling pathway is associated with cell proliferation, survival and motility [45]. Because the PPD of PR can mediate the inhibition of EGF-induced A549 cell proliferation, we were prompted to investigate whether the presence of PR or the PR-PPD affected EGF-induced ERK1/2 activation, in the absence of hormone. To study PR PPD functions independent of PR transcriptional activities, these studies were carried out in the absence of hormone. A549-PR-B and A549-PR-BΔSH3 cells were induced with Dox for 24 hr before treatment with increasing concentrations of EGF (10–50 ng/ml) for 30 min, and then analyzed for activated ERK1/2. Consistent with the effect of PR-PPD on EGF-induced cell proliferation, A549 cells expressing wildtype PR-B showed significantly reduced ERK1/2 activation, compared with cells expressing PR-BΔSH3 (Figure 6A) at all EGF concentrations tested, with maximum inhibition when induced with 50 ng/ml of EGF. We have determined whether the presence of PR-PPD could change the kinetics of EGF-induced ERK1/2 activation. A549 cells expressing PR-B or PR-BΔSH3 were treated with 50 ng/ml EGF for 10–60 mins, and analyzed for activated ERK1/2. Expression of PR-B not only reduced ERK1/2 activation but also shortened the duration that the MAPK remained active (Figure 6B). EGF-induced ERK1/2 activation in A549 cells expressing PR-B was significantly reduced in as few as 30 mins, while EGF-induced ERK1/2 activation in A549 cells expressing PR-BΔSH3 showed prolonged ERK1/2 activation and did not show a significant reduction in MAPK activation until 60 min after EGF treatment (Figure 6B). Interestingly, expression of PR-B or PR-BΔSH3 had no effect on EGF-induced Akt activation at all concentrations of EGF tested, and at all times of EGF treatment tested (Figure 7A and 7B). Together, these data suggested that expression of PR through its PPD could reduce both the magnitude and the duration of EGF-induced activation of ERK1/2 in the absence of progestin.

4. Discussion

PR is routinely used as a diagnostic marker for several endocrine-related cancers, and most of our understanding about PR functions comes from studies of endocrine-related tissues, such as breast, endometrium and ovary. Recent data suggest that PR may also have important functions in non-endocrine tissue [20, 22]. Increasing evidence suggests that PR could play a vital role in the pathogenesis and progression of NSCLC. However, most studies on PR and lung cancer have been based on receptor expression in archival specimens from clinic [15, 16], and precise mechanistic role of PR in NSCLC remained to be determined. Several studies show that expression of PR in NSCLC is associated with better prognosis and improved patient survival [15, 17, 20]. On the other hand, other reports suggest that PR expression in NSCLC is associated with poor prognosis and shorter survival

[46]. These differences may be attributable to a number of experimental issues such as antibodies that are not validated and application of immunohistochemical protocols that are not standardized [14–17] Due to the lack of an appropriate, reproducible *in vitro* model to examine PR function in NSCLC, the role of PR function in NSCLC has remained unclear.

In this study, we successfully constructed an *in vitro* model to investigate PR-B and its PPD functions in human A459 NSCLC cells. We generated a system for Tet-inducible expression of PR-B and PR-BΔSH3 in A549 cells. Tet-inducible cell models allowed us to examine PR-B and PPD functions in the presence and absence of hormone under the same cellular background and were not affected by clonal variation often found in conventional stable transfectants. We demonstrated that Dox treatment dose-dependently induced equal expression of both PR-B and PR-BΔSH3 in A549-PR-B and PR-BΔSH3 cell lines (Figure 2A–E). Furthermore, Dox-induced PRs were transcriptionally active and exhibited normal intracellular localization, similar to endogenous PRs in T47D breast cancer cells (Figure 3A and 3B).

Previous studies showed that A549 cells expressed both PR-A and PR-B, as evidenced by western blotting with PR antibodies, albeit at very low levels as compared to control breast tumor cells [15, 16] and one report showed progesterone-dependent transcriptional activation of a PRE-luciferase construct [15]. A549 cells used in this study were obtained from ATCC and the identity was confirmed by genomic DNA comparison to the ATCC database. However, we failed to detect any PR expression by western blotting or by progestin-dependent activation of a PRE reporter in A549 cells in this study. This could be due to differences in the specificity of antibodies used for PR protein detection. The 1294 monoclonal antibody used in this study was shown to be highly specific for PR by western blotting and immunohistochemistry staining in several previous PR-related studies [23, 35, 47]. Additionally, although the PRE reporter was activated with 1 μM progesterone in a previous study [15] we noted that activation was achieved with 10 nM of non-metabolizable progestin (R5020). Thus, it is possible that activation of the PRE-reporter at such a high progesterone concentration could be due to other nonspecific factors, such as progesterone metabolites, or non-specific progesterone binding and activation of other receptors or signaling pathways that affected PRE-reporter activation. To exclude problems with PR antibody specificity and PR transcription activity, we examined PR mRNA expression and transcriptional activity in A549 cells using RT-PCR and a PRE-luciferase reporter assay, and failed to detect any significant amount of PR mRNA expression or PR transcriptional activity (Figure 1A–C and Figure 3B). While the amplification plot of the quantitative RT-PCR indicated that there was a small increase in fluorescence intensity from the A549 mRNA sample after more than thirty-one rounds of amplification. However, an increase in fluorescence intensity generated from A549 mRNA was below the threshold limit for the assay (Figure 1A), thus suggesting that A549 cells might express very low to undectable amounts of PR. Based on these data, we conclude that the A549 cells used in this study do not express any significant amount of endogenous PR, thereby allowing us to examine the functional role of ectopically expressed PR without interference from endogenous PR.

Expression of PR-B significantly inhibited A549 cell proliferation by 24 and 48 hr, and mutations in the PPD of PR-BΔSH3 cells reversed this inhibition in the absence of hormone.

These results suggested that PR-B inhibition of A549 cell proliferation is likely mediated through non-transcriptional and extranuclear non-genomic activities of the PR PPD. Interestingly, addition of progestin antagonist (RU486) alone reversed PR-B-mediated inhibition of A549 cell proliferation (Figure 5A). RU486 has been shown to antagonize PR nuclear and extranuclear activities of PR [23, 34, 42, 48], suggesting that PR-B inhibition of NSCLC growth is likely mediated, in part, through the extranuclear non-genomic function of the PR-PPD. Addition of R5020 suppressed A549 cell proliferation in cells expressing PR-B, and treatment with RU486 reversed this inhibition (Figure 5A). The effects of R5020 and RU486 were absent in PR-BΔSH3 cells, suggesting that PR-PPD is likely required for both hormone-dependent and hormone-independent inhibition of A549 cell proliferation.

Previously, we demonstrated that the PR PPD is required for extranuclear nongenomic signaling of PR-B [28]. However, a recent study suggested that the PR PPD could mediate the nuclear transcriptional function of PR as well. The expression of tissue factor required the nuclear transcriptional function of the PR PPD [49]. These data suggested that PR-B-mediated inhibition of A549 cell proliferation requires both the nuclear transcriptional and the extranuclear non-genomic activities of PR-B and the PPD. In breast cancer cells, R5020 induced a transient increase in cell proliferation at 24 hours. in cells expressing PR-B [28]. However, R5020 suppressed NSCLC cell proliferation, as shown in the current study (Figure 5A). This difference in R5020 response could well be due to differences in cell type as well as in the ability of PR-B to interfere with other signaling pathways in NSCLC cells.

To explore PR-B extranuclear activities of PR-B and crosstalk with EGFR signaling, we treated cells expressing PR-B or PR-BΔSH3 with EGF, in the absence of R5020 to eliminate PR transcriptional activity. A459 cells expressing PR-B showed significantly reduced EGF-mediated growth, compared with cells expressing PR-BΔSH3 or no PR-B, at all concentrations of EGF tested (Figure 4C). Analysis of EGF-mediated MAPK (ERK-1/-2) activation in A549 cells expressing PR-B and PR-BΔSH3 revealed that cells expressing PR-B, but not PR-BΔSH3, showed a reduced magnitude and duration of EGF-induced MAPK activation (Figure 6A and 6B). However, the ability of PR-B to inhibit signaling pathways is not a general nonspecific effect, as no inhibition was observed in EGF-induced Akt activation in both PR-B and PR-BΔSH3 expressing cells (Figure 7A and 7B). These data suggested that PR-B, through its PPD, inhibited EGF-induced MAPK activation, and could in part lead to a reduction in A549 cell proliferation. Previously, we demonstrated that PR, through its PPD, could interact with other SH3 domain containing signaling molecules [25]. Overexpression of a PPD, which served as a Grb2-SH3 ligand, inhibited growth of HER2+ cancer cells [50]. The SH3-PPD interactions are important in EGFR endocytosis and trafficking [51, 52]. Thus, it is likely that the PPD of PR may compete with other PPD-SH3 interactions that are important for intracellular signal transduction and the trafficking of EGFR. More studies will be needed to identify these important SH3 containing signaling molecules that bind to the PR PPD and mediate PR inhibition of EGF signaling.

Previously, we demonstrated that the PR PPD mediated progestin-dependent activation of c-Src and its downstream MAPK signaling in breast cancer cells [23, 28]. Faivre et al. demonstrated that rapid, transient, progestin-dependent activation of c-Src could lead to sustained MAPK activation by increasing EGFR expression and the availability of EGF

through PR transcriptional activities in breast cancer cells [53]. These differences in PR PPD activities could be because this study focused mainly on determining PR PPD activities in the absence of hormone, in order to examine PR PPD activities independent of its transcriptional functions. Additionally, NSCLC cells may well have different PR and PR PPD activities, compared with breast cancer cells.

Several questions about PR activity in NSCLC remain to be determined. PR exists in most tissues as two isoforms, PR-B and PR-A. Higher expression of PR-A is associated with poor prognosis and high-grade breast cancer [13]. This study only addressed the role of the PR-B isoform in NSCLC. Whether PR-A functions similar to PR-B in NSCLC is not known. Additionally, PR-B is reported in several cell types to mediate rapid progestin-dependent extranuclear signaling, leading to activation of several signaling pathways [28, 53, 54]. Although the current data indicate that extranuclear signaling mediated via the PPD domain of PR allows interactions with EGFR-mediated proliferation in NSCLC, further studies will be required to determine the precise role of progestin-dependent extranuclear signaling, and its biological significance, in NSCLC cells.

Several clinical studies suggested that PR expression in NSCLC correlates with less aggressive tumors and better prognosis [14–16]. Additionally, the protective effects of PR could involve hormone-independent mechanisms because the protective effects of PR were demonstrated in older men and postmenopausal women, in whom there is little to no circulating progesterone [17]. To date, most studies on PR in NSCLC were retrospective reports and only investigated correlates between PR expression and clinical prognosis or outcome. This study, for the first time, provides direct evidence that expression of PR-B, independent of ligand, inhibits basal and EGF-induced NSCLC cell proliferation. We also identified the PR PPD, a PXXPXR motif, as a crucial PR domain required for PR-mediated inhibition of NSCLC growth and EGF signaling, thus suggesting that PR-B or the PR PPD could have future therapeutic potential for NSCLC patients. Furthermore, the *in vitro* NSCLC cell model described in this study should prove useful for future studies examining other biological functions of PR or the PR PPD interaction with other growth factor receptors or signaling molecules in NSCLC cells.

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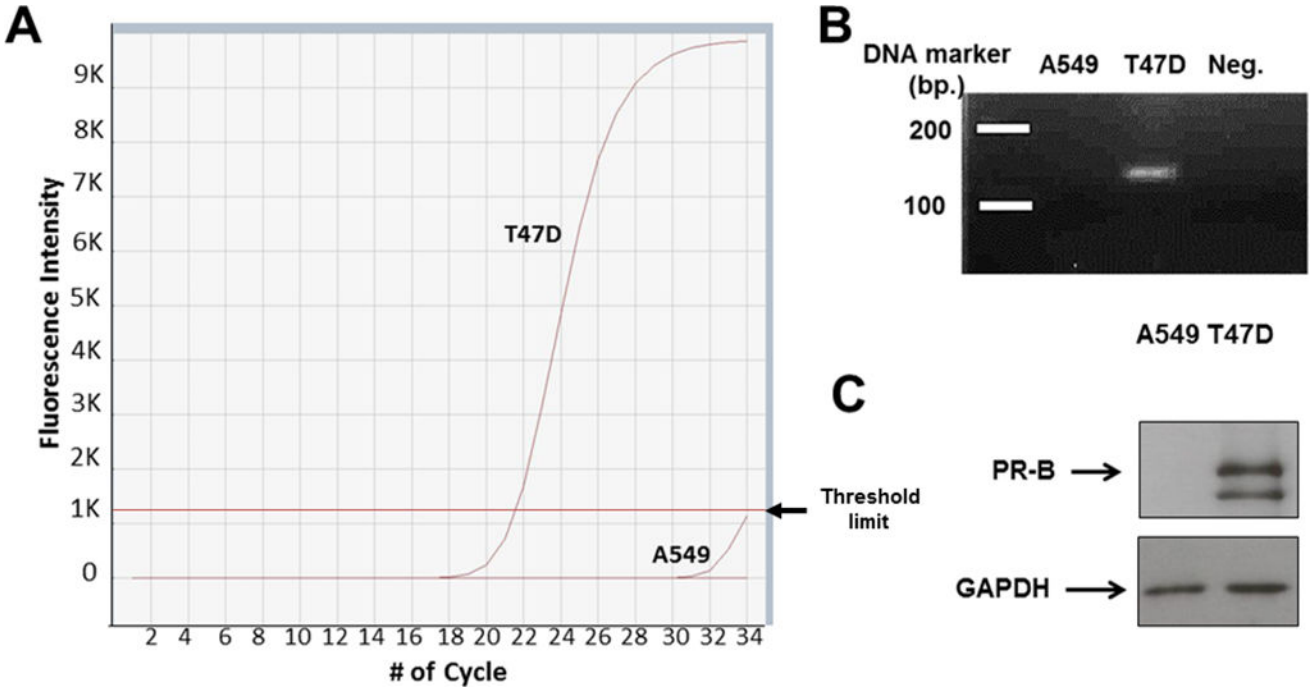


Figure 1.

(A–C) PR mRNA and protein expression in A549 and T47D cells

Real-time PCR analysis of PR expression in A549 and T47D cells. **A)** Amplification plot of quantitative real-time PCR (qRT-PCR) of PR expression in T47D and A549 cells. A549 and T47D total RNA were extracted and cDNA was amplified by qRT PCR as described in Materials and Methods. **B)** PCR products from qRT-PCR of A549 and T47D were electrophoresed on 1% agarose gels, Lane 1: 100 bp DNA ladder, lane 2: A549, lane 3: T47D (positive control), and lane 4: negative control (no template).

C) Western Blot analysis of A549 and T47D cells. Cells were lysed and prepared for western blotting with a PR-specific antibody, 1294 mouse monoclonal antibody. Twenty micrograms of protein was loaded in each lane. Three micrograms of T47D cell lysate was loaded as a positive control for PR expression. GAPDH were used as a loading control.

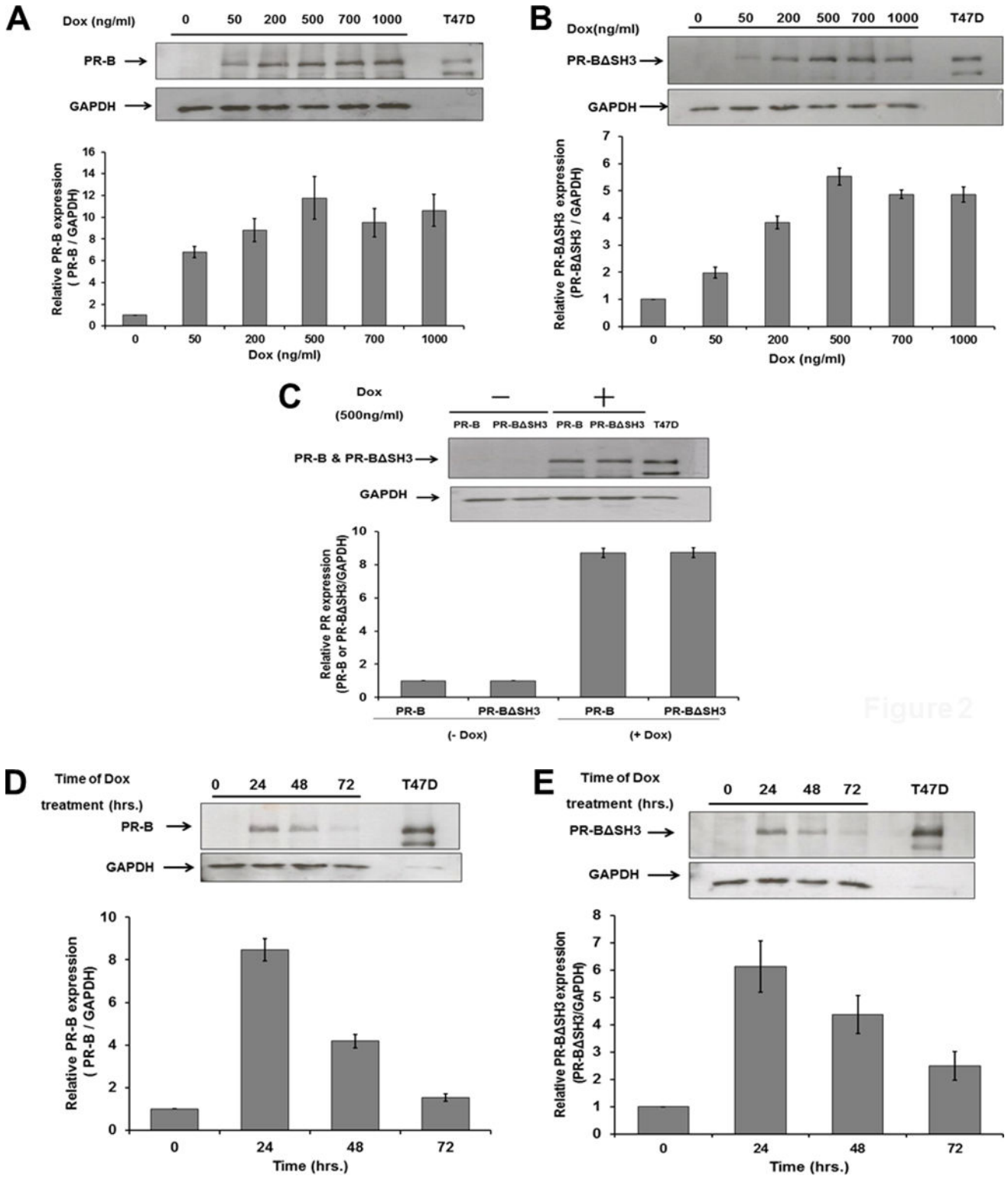


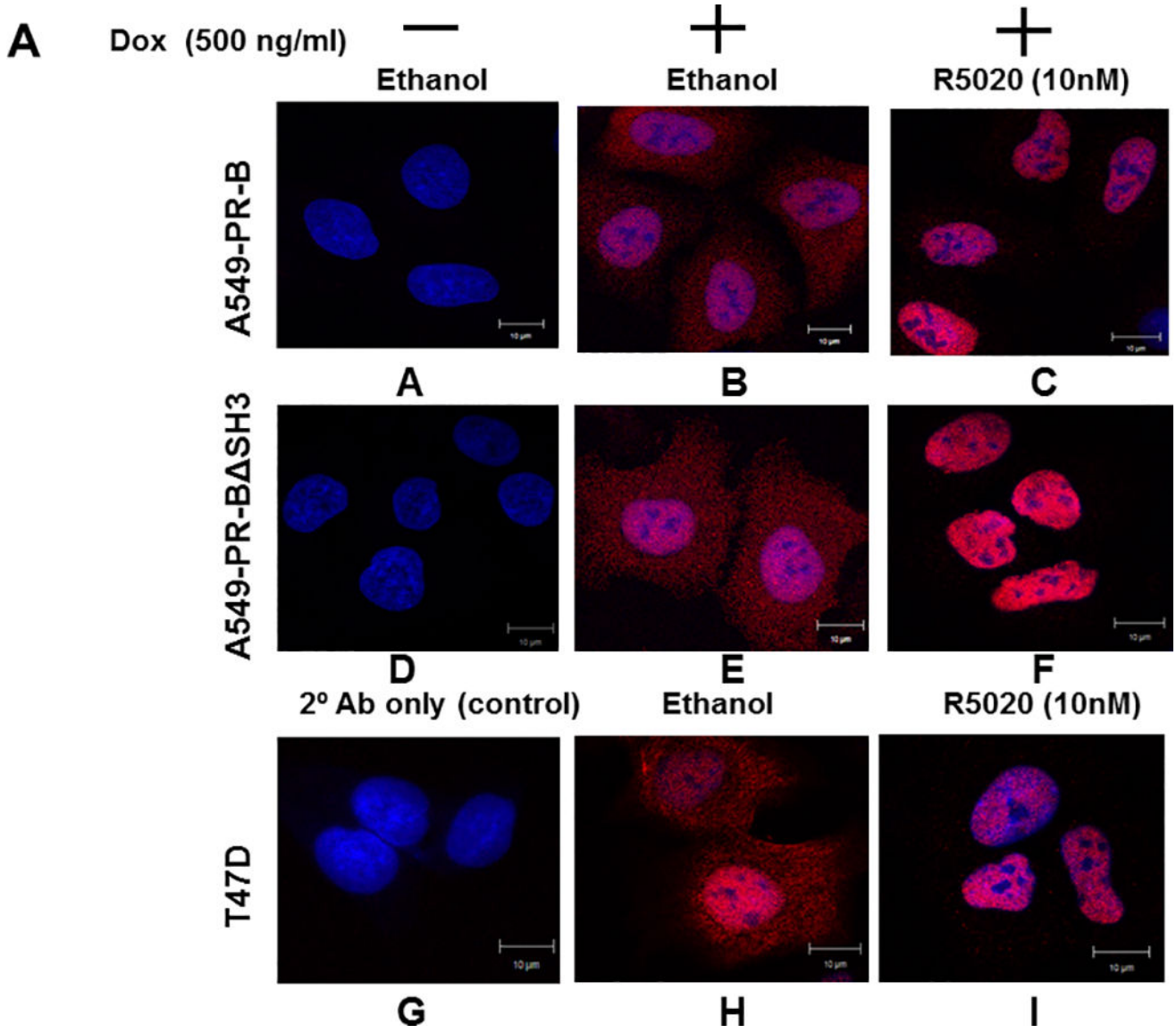
Figure 2.

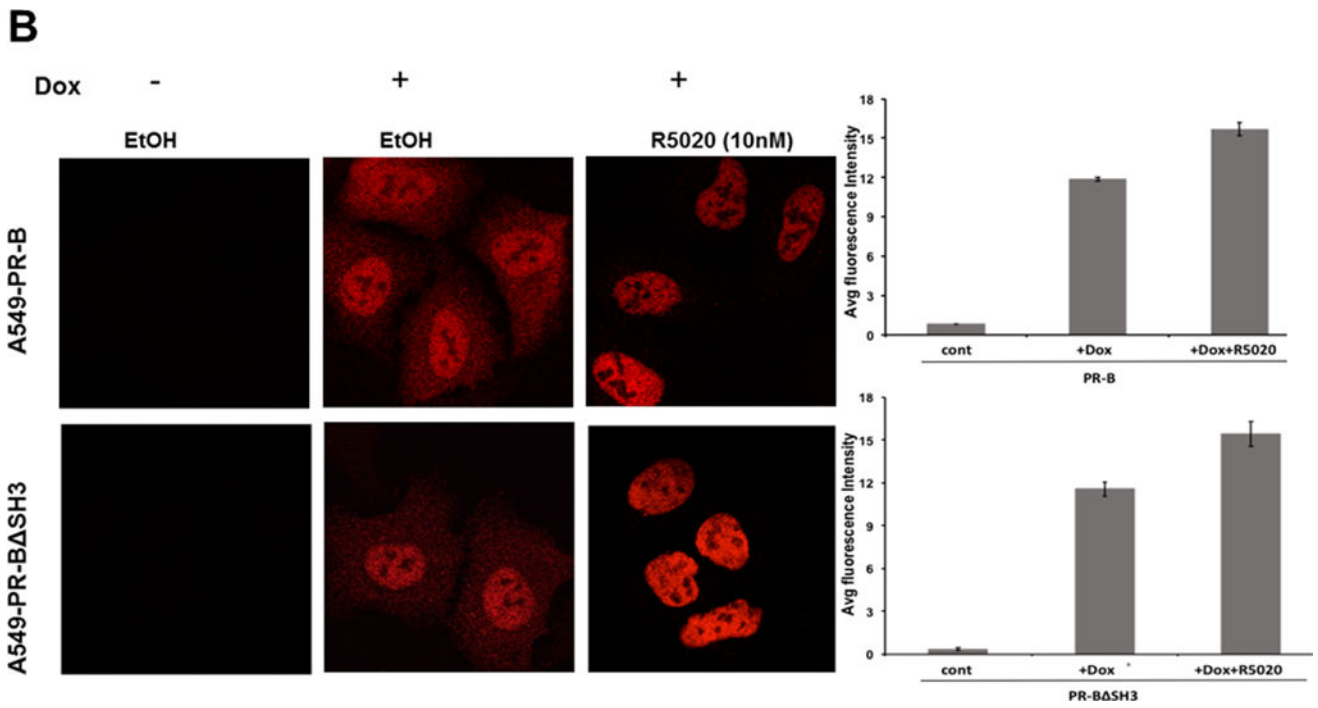
(A–C) Doxycycline (Dox) induction of PR-B and PR-BΔSH3 protein expression in A549 cells

Upper panel **A**) A549-PR-B cells and **B**) A549-PR-BΔSH3 cells were treated with increasing doxycycline concentrations (0, 50, 200, 500, 700 and 1000 ng/ml) to induce the expression of PR-B or PR-BΔSH3 proteins. Cell lysates were prepared and blotted with a PR-specific antibody, 1294 mouse monoclonal antibody. Twenty micrograms of protein was loaded in each lane. Three micrograms of T47D cell lysate was loaded as a positive control. GAPDH was used as a loading control. **C**) Same as A & B except A549-PR-B and A549-PR-BΔSH3 cells were treated with 500 ng/ml doxycycline and incubated for 24 hours to induce expression of PR-B and PR-BΔSH3 proteins. Cell lysates were prepared and blotted as described. *Lower panel* **(A–C)** Plots of relative PR expression. Values represent relative PR expression normalized with GAPDH. Data represent means ± SEM from three independent experiments.

(D&E) Time course analyses of the effects of doxycycline treatment on the expression of PR-B and PR-BΔSH3 proteins in A549 cells

Upper panel **D)** A549-PR-B cells and **E)** PR-BΔSH3 cells were treated with 500 ng/ml doxycycline, and incubated for 24, 48 and 72 hours to induce the expression of PR-B and PR-BΔSH3 proteins. Cell lysates were prepared and immunoblotted with a PR-specific antibody, 1294 mouse monoclonal antibody. Twenty micrograms of proteins was loaded in each lane. Three micrograms of T47D was loaded as a positive control. GAPDH was used as a loading control. *Lower panel* **(D&E)** Plots of relative PR expression. Values represent relative PR expression normalized with GAPDH. Data represent means ± SEM from three independent experiments.





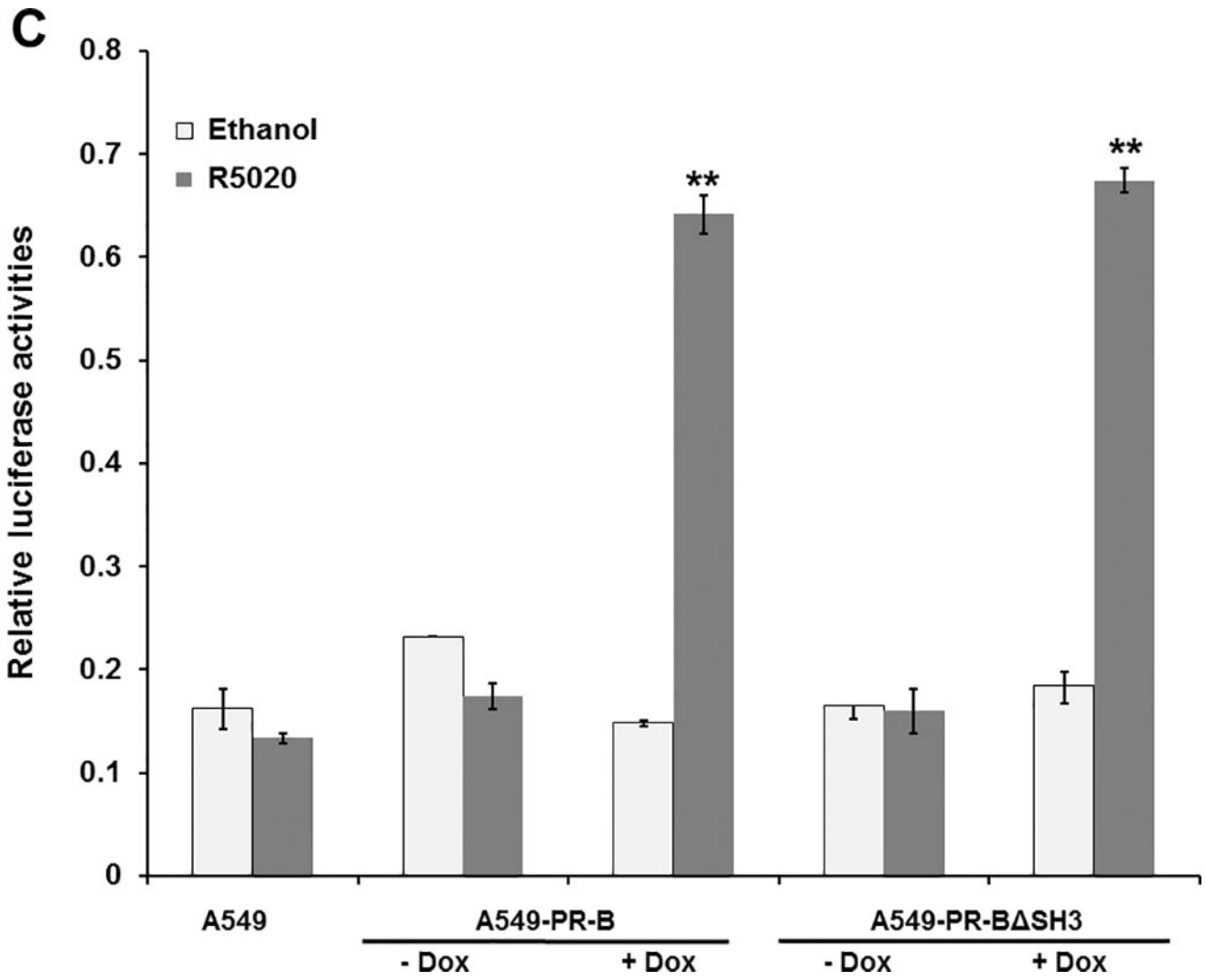


Figure 3.

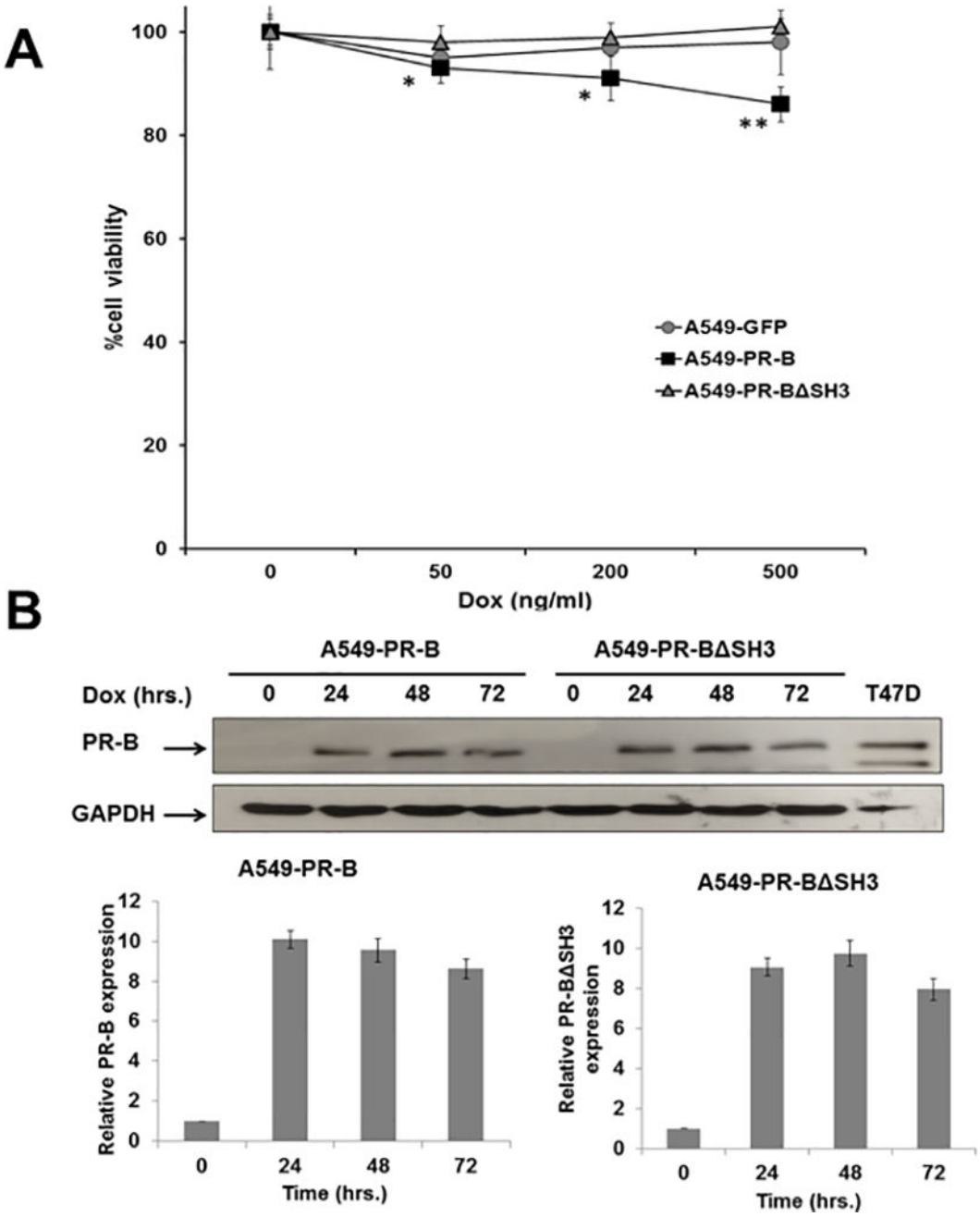
A–C Immunofluorescence staining of PR-B and PR-BΔSH3 expression and luciferase analyses for transcriptional activities in A549-PR-B and A549-PR-BΔSH3 cells

A) Immunofluorescence staining of A549-PR-B and A549-PR-BΔSH3 cells. Cells were fixed and stained as described in Materials and Methods. Panels A & D show untreated (control) cells. Panels B & E show A549-PR-B and A549-PR-BΔSH3 cells, respectively, after the addition of 500 ng/ml of doxycycline for 24 hours. Panels C & F show A549-PR-B and A549-PR-BΔSH3 cells, respectively, after the addition of 500 ng/ml of doxycycline for 24 hours and treatment with R5020 treatment for 60 min. Panel G shows T47D breast cancer cells incubated with secondary antibody alone as a negative control, and panels H & I show T47D breast cancer cells in the presence or absence of R5020, as a positive control for PR staining.

B) PR immunofluorescence staining of A549-PR-B and A549-PR-BΔSH3 cells. Cells were fixed and stained as in A). Images show PR immunofluorescence staining of cells pretreated with Dox (500 ng/ml) for 24 hr before treating with vehicle (EtOH) or 10 nM R5020 for an

additional 24 hr. Values are represented as mean average immunofluorescence intensities ± SEM in each image as described Materials and Methods.

C) The normalized firefly luciferase activities of A549-PR-B and A549-PR-BΔSH3 cells after doxycycline treatment were significantly increased in R5020- treated cells relative to ethanol-treated cells (*$P \leq 0.01$). The relative luciferase activities between A549-PR-B and A549-PR-BΔSH3 cells induced with doxycycline and treated with R5020 were similar. Graphs represent the average firefly luciferase activities relative to renilla luciferase activities. Values are represented as means ± SEM from three triplicates (n=3).



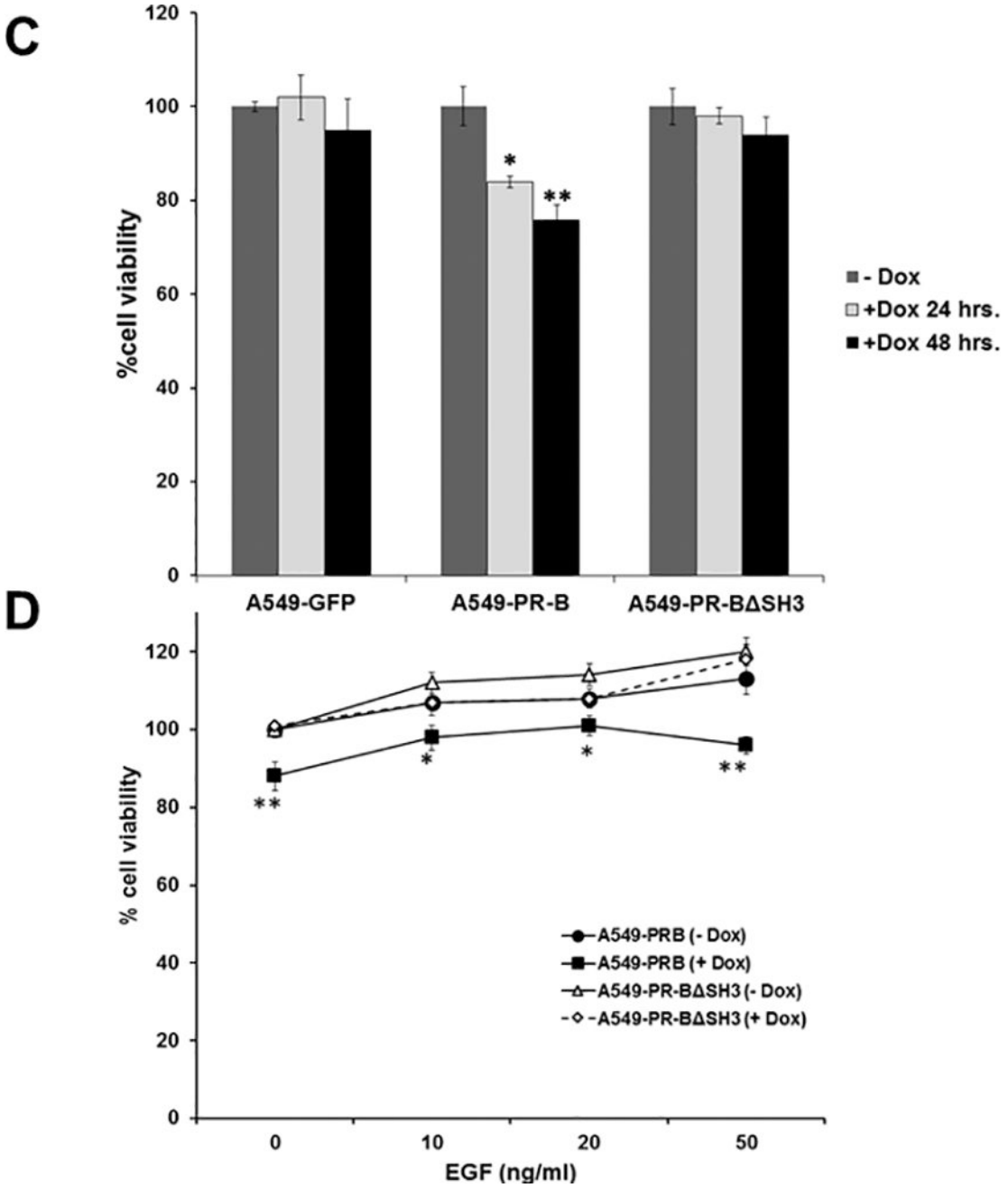


Figure 4.

Effects of PR-B and PR-BΔSH3 expression on A549 cell proliferation

A) Effects of doxycycline concentration on cell viability. A549-PR-B, A549-PR-BΔSH3 and A549-GFP (control) cells were treated with increasing concentrations of doxycycline as indicated for 24 hr, and analyzed for cell viability by an MTT assay as described in Materials and Methods. Percent cell viability levels of A549-PR-B cells were significantly lower than A549-PR-BΔSH3 cells (*$P \leq 0.05$) and A549-GFP cells (*$P \leq 0.05$). Values shown are means ± SEM (n=3). (Percent cell viability is shown as the percent cell viability

of doxycycline-treated cells normalized to the percent cell viability of control untreated cells.)

B) Maintaining PR expression by addition of Dox every 24 hrs. Upper panel: Immunoblots with a PR-specific antibody (1294). A549-PR-B cells and PR-BΔSH3 cells were treated with 500 ng/ml doxycycline, and incubated for 24, 48 and 72 hours with medium changed and replaced with medium containing 500 ng/ml every 24 hrs. Cell lysates were prepared and immunoblotted with a PR-specific antibody, 1294 mouse monoclonal antibody as described in Material and Methods. Lower panel: Plots of relative PR expression. Values represent relative PR expression normalized with GAPDH. Data represent means ± SEM from three independent experiments.

C) Effects of PR-B expression on EGF-induced cell proliferation. A549-PR-B, A549-PR-BΔSH3 and A549-GFP (control) cells were treated with 500 ng/ml doxycycline for 24 and 48 hr with medium change and new 500 ng/ml doxycycline every 24 hr. Cells analyzed for cell viability by an MTT assay. PR-B but not PR-B ΔSH3 or GFP expression significantly inhibited NSCLC proliferation after 24 hours (*$P \leq 0.05$), and 48 hours (**$P \leq 0.01$) after doxycycline treatment. Values are shown as means ± SEM (n=3). (Percent cell viability is shown as the percent cell viability of doxycycline-treated cells normalized to the percent cell viability of control untreated cells.)

D) Effects of PR-B expression on EGF-induced cell proliferation. A549-PR-B and A549-PR-BΔSH3 cells were treated with 500 ng/ml doxycycline for 24 hours, followed by the addition of increasing doses of EGF as indicated (0–50 ng/ml), incubated for additional 24 hr, and analyzed for cell viability. Percent cell viability in PR-B expressing cells was significantly different from control A549-PR-B cells without doxycycline (*$P \leq 0.05$), A549-PR-BΔSH3 cells without doxycycline (*$P \leq 0.05$), and A549-PR-BΔSH3 cells with doxycycline (*$P \leq 0.05$). Values are shown as means ± SEM (n=3).

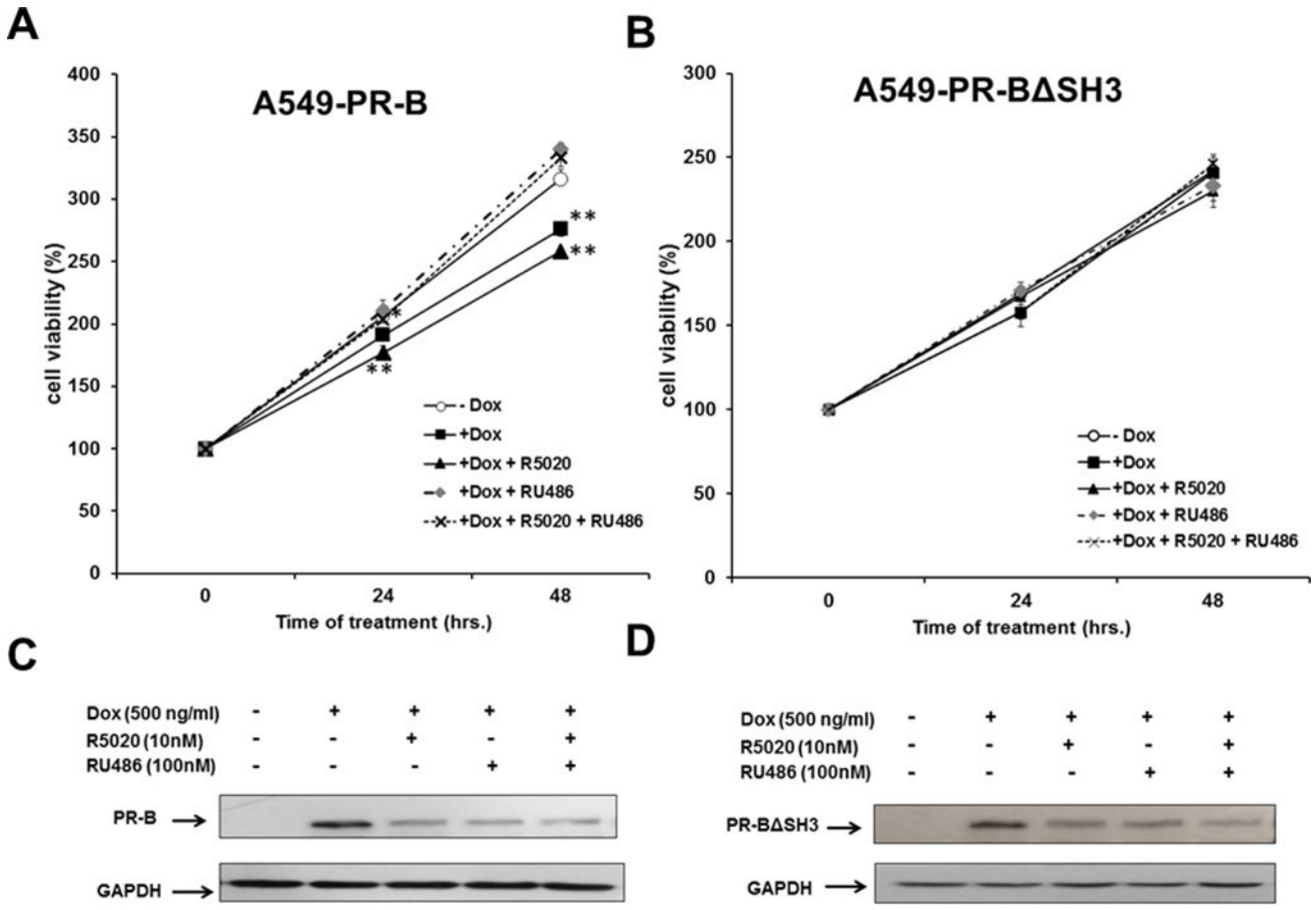


Figure 5.

Effects of progestin agonist (R5020) and progestin antagonist (RU486) on the proliferation of A549 cells expressing PR-B or PR-BΔSH3

A549-PR-B cells **A**) and A549- cells **B**) were treated 500 ng/ml of doxycycline for 24 hr before changing to medium with 500 ng/ml of doxycycline containing vehicle (ethanol), 10 nM R5020, 100 nM RU486, or R5020 and RU486, and incubated for an additional 24 or 48 hr as described in Materials and Methods. The x-axis represents the duration of R5020 or RU486 treatment. Values are represented as means ± SEM (n=3). (Percent cell viability is shown as the percent cell viability of doxycycline-treated cells normalized to the percent cell viability of control untreated cells as described in Materials and Methods.) C and D show Immunoblots of PR-B C) and PR-BΔSH3 D) expression in A549 cells treated with doxycycline for 72 hrs or treated with doxycycline for 24 hrs followed by treatment with R5020 and/or RU486 for an additional 48 hr as described in Materials and Methods.

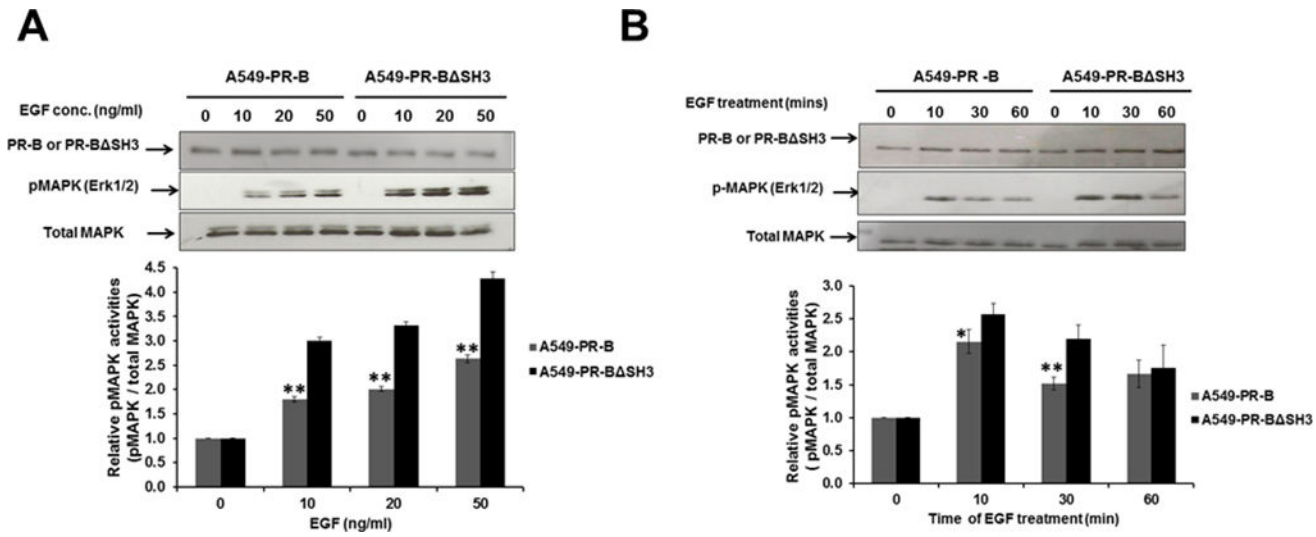


Figure 6.

(A&B) Effects of PR-B and PR-BΔSH3 on EGF activation of ERK1/2 in A549 cells

A) A549-PR-B cells and A549-PR-BΔSH3 cells were treated with increasing concentrations of EGF (0, 10, 20 and 50 ng/ml) as indicated. Cell lysates were prepared and subjected to western blotting analysis with phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204). Total MAPK (p44/42 MAPK, Erk1/2) was used as a loading control. Twenty micrograms of protein was loaded in each lane. Bar graphs show relative pMAPK activities (pMAPK/total MAPK), and data are shown as means ± SEM (n=3). **B)** Same as A) except A549-PR-B cells and A549-PR-BΔSH3 cells were treated with 50 ng/ml EGF and incubated for 0, 10, 30 and 60 mins. Bar graphs show relative pMAPK activities (pMAPK/total MAPK) and data are shown as means ± SEM (n=3).

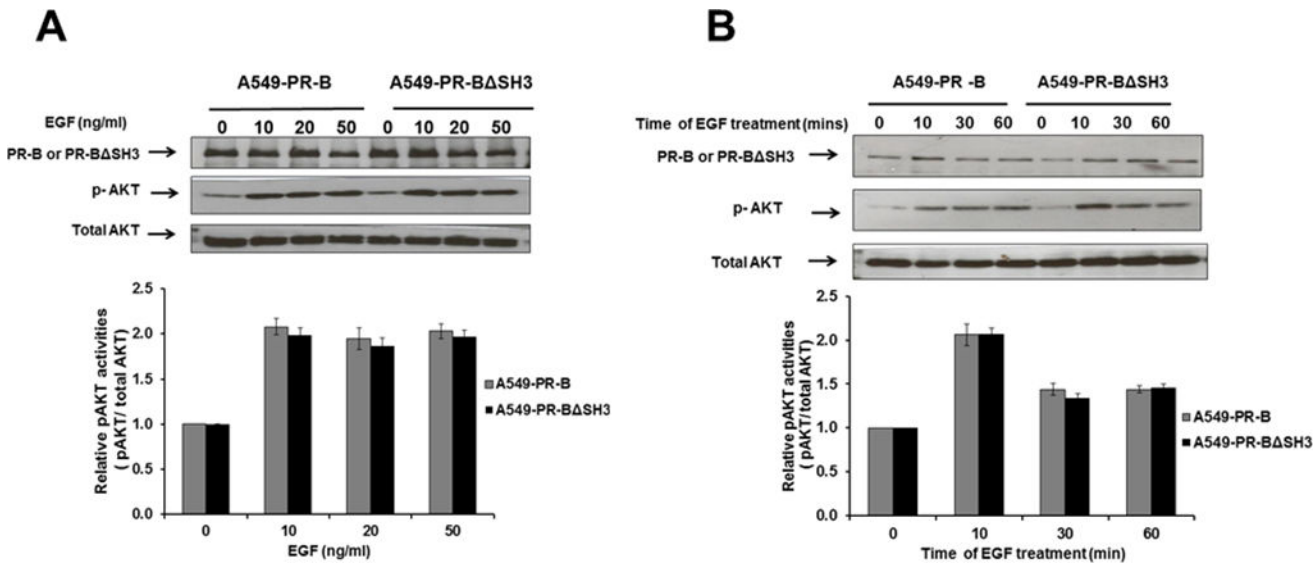


Figure 7.

(A&B) Effects of PR-B and PR-BΔSH3 on EGF activation of Akt

A) A549-PR-B cells and A549-PR-BΔSH3 cells were treated with increasing concentrations of EGF (0, 10, 20 and 50 ng/ml) as indicated. Cell lysates were prepared and subjected to western blotting analysis with pAkt (S473, D9E) for phosphorylated-AKT. Total AKT (pan-Akt, c67E7) was used as a loading control. Twenty micrograms of protein was loaded in each lane. Bar graphs show relative pAkt activities (pAkt/total Akt) and data are shown as means ± SEM (n=3). **B)** Same as A) except A549-PR-B cells and A549-PR-BΔSH3 cells were treated with 50 ng/ml EGF and incubated for 0, 10, 30 and 60 mins. Bar graphs show relative pAkt activities (pAkt/total Akt) and data are shown as means ± SEM (n=3).