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Lian Zuo (arefzuo@gmail.com)
Wei Li (weili_1983@yahoo.com)
Shaojin You (Shaojin.You@va.gov)

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Progesterone reverses the mesenchymal phenotypes of basal phenotype breast cancer cells via a membrane progesterone receptor mediated pathway

Lian Zuo, Wei Li, and Shaojin You*

Atlanta Research & Educational Foundation (151F), Atlanta VA Medical Center,
1670 Clairmont Road, Decatur, GA 30033, USA

***Requests for reprints:** Shaojin You; E-mail: Shaojin.You@va.gov .

Abstract

Introduction: Basal phenotype breast cancers (BPBC) are often associated with apparent epithelial to mesenchymal transition (EMT). The role of progesterone (P4) in regulating EMT of BPBC has not been reported.

Methods: The EMT relevant biology were investigated *in vitro* using human BPBC cell models (MDA-MB468 and MDA-MB231) with P4, PR agonist (RU486), and PR antagonist (R5020) treatments. The essential role of membrane progesterone receptor α (mPR α) in the P4-regulated EMT was demonstrated by knocking down the endogenous gene and/or stably transfecting exogenous mPR α gene in the BPBC cell models.

Results: The expression of snail and down-stream EMT proteins such as occludin, fibronectin, and E-cadherin was significantly regulated by P4 incubation, which was accompanied by cell morphological reversion from mesenchymal to epithelial phenotypes. In searching for the cell mediator of P4' action in the MDA-MB468 (MB468) cells, it was found that mPR α but not the nuclear PR has an essential role in the P4 mediated EMT inhibition. Knocking down the expression of mPR α with specific siRNA blocked the P4's effects on expression of the EMT proteins. In another BPBC cell line – MDA-MB231 (MB231), which is mPR α negative by Western blotting, P4 treatment did not alter cell proliferation and EMT protein expressions. Introduction of the exogenous mPR α cDNA into these cells caused cell proliferation, but not EMT, to become responsive to P4 treatment. In further studies, it was found that activation of the PI3K/Akt pathway is necessary for the P4-induced EMT reversion. To define the potential intermediate steps between mPR α and PI3K, we demonstrated that mPR α , caveolin-1 (Cav-1), and

epidermal growth factor receptor (EGFR) are colocalized in the membrane of caveolar vesicle and the P4-repressed EMT in MB468 cells can be blocked by EGFR inhibitor (AG1478) and PI3K inhibitor (wortmannin).

Conclusions: Our data suggest that the signaling cascade of P4 induced mesenchymal repression is mediated through mPR α and other caveolae bound signaling molecules namely Cav-1, EGFR, and PI3K. This novel finding may have great impact on fully understanding the pathogenesis of BPBC and provide an essential clue for developing a targeted therapeutic strategy for treatment of BPBC.

Introduction

Basal phenotype breast cancer (BPBC) is a subtype of cancer with apparent mesenchymal phenotypes. Boyer and colleagues first described a morphologic change from epithelial-like sheets of cultured cancer cells to scattered, fibroblast-like cells capable of invading the basement membrane, so called epithelial to mesenchymal transition (EMT) [1]. The morphological criteria of EMT *in vitro* involve changes in cell polarity, separation into individual cells and acquisition of cell motility [2]. These changes can be either stable or reversible. The essential changes in gene expression that disrupt cell polarity and cause mesenchymal transition have been identified. Snail, twist, and slug have been shown as key regulators of EMT in both animal and human cancers [3]. Among these genes, snail acts as a transcriptional factor to repress genes that encode the cell-cell junctional apparatus, such as E-cadherin and occludin; and to enhance genes that

encode mesenchymal or tumor interstitial components, such as fibronectin and vimentin, resulting in a dedifferentiated mesenchymal transition characterized by increased cell motility [4, 5].

The roles of female sex hormones such as progesterone (P4) in the pathogenesis of BPBC remain unclear. Classically, the actions of P4 on cancer cells are attributed to the binding of nuclear progesterone receptor (PR), translocation of P4/PR complex into the nucleus and subsequent activation of target genes over the course of several hours. These mechanisms, however, are not applicable to BPBC due to lack or very low level of PR expression in these cancers. The mechanisms for P4's actions in modulating the cancer biology of BPBC remain largely unknown. Recently, the cell membrane hormonal receptors, such as mPR family and PGMRC1, were identified and demonstrated functional in BPBC [6, 7]. It is believed that the rapid responses of P4 are initiated at the cell surface by binding to the membrane receptors [8-10]. For examples, progestin, a synthetic P4, has been shown to activate a variety of signaling pathways through mPR α [6]. The binding of progestin to mPR α alters the secondary messenger pathways through activation of the pertussis toxin-sensitive inhibitory G-proteins and then activates the MAPK/Erk 1/2 pathway [6, 7, 11, 12]. However, this theory has been debated since others failed to demonstrate mPRs on the cell surface or mediate progesterone-dependent signaling events, such as coupling to G proteins [13]. Moreover, mPRs were shown primarily situating in the endoplasmic reticulum [13, 14]. In this study, we co-localized mPR α , Cav-1, and EGFR at a specified membrane structure, so called caveolar vesicle, and demonstrated that P4 reverses the mesenchymal phenotypes of human BPBC cells (MB468 and MB231) via a caveolae bound signaling complex namely mPR α , Cav-1, EGFR, and PI3K/Akt. Further study

on this unique molecular pathway may afford great potential to discover novel molecular targets for treatment of basal phenotype breast cancer.

Materials and methods

Chemicals and antibodies. RU486 (Mifepristone), AG1498, Wortmannin, PP1 and PD98052 were purchased from EMD Chemicals (Gibbstown, NJ, USA); R5020 (Promegestone) and bpV(phen) were from PerkinElmer (Waltham, MA, USA) and Thermo Fisher Scientific (Pittsburgh, PA, USA) respectively. Anti-snail antibody was from Abcam (1:1000, Cambridge, MA, USA); anti-E-cadherin and anti-fibronectin antibodies were obtained from EPITMICS (1:1000, Burlingame, CA, USA); anti-mPR α , anti-GAPDH (1:500) and secondary antibodies (1:2000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-occludin antibody was from BD transduction (1:500, San Jose, CA, USA); and anti- α -tubulin antibody was from Sigma (1:2000, St. Louis, MO, USA).

Cell culture. The human breast cancer cell lines MDA-MB468 (MB468), MDA-MB231 (MB231) and Human Embryonic Kidney 293 (HEK 293) cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Both human breast cancer cell lines were negative for ER and Her-2 and classified as “basal phenotype A” cells [15]. The cultured MB468 cells at early passages typically appear like epithelial cells with oval and/or polygonal shapes [16]; and after multiple passages (50+), these cells exhibit apparent mesenchymal phenotypes with spindle and elongated shapes (Supplementary figure S1 in Additional file 1), which suite for the proposed studies. Long term cell culture *in vitro* may generate genetic instabilities and the derived cell lines with altered cell biological features have been utilized as cell models for *in vitro* studies [17, 18]. The late passage MB468 cells and early passage MB231

cells with apparent mesenchymal phenotypes [16] were cultured and maintained at 37 °C with 21% O₂ and 5% CO₂ (without 5% CO₂ for MB231 and HEK293 culture) in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, Carlsbad, CA, USA) and maintained in a humidified incubator. The XTT cell proliferation assay kit was from Cayman Chemicals (Ann Arbor, MI, USA).

Immunoblotting. Western blot assays were performed as described previously [19]. After treatment with or without P4 and/or diverse pathway inhibitors, the growth-arrested cells were lysed with 500 µl ice-cold lysis buffer, pH 7.4 (50 mM HEPES, 5 mM EDTA, 50 mM NaCl), 1% Triton X-100, protease inhibitors (10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin) and phosphatase inhibitors (50mM sodium fluoride, 1mM sodium orthovanadate, 10mM sodium pyrophosphate). Cell lysates (25 µg) were separated using SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, blocked overnight in PBS containing 6% nonfat dry milk and 0.1% Tween 20, and incubated for 1 hr with primary antibodies at proper dilutions. After incubation with secondary antibodies, proteins were detected by ECL chemiluminescence. Image J was used for quantitative analysis.

Cell morphological changes of MB468 treated with P4. MB468 cells (10⁵ cells/dish) were seeded and grown in 35 mm cell culture dishes (Bioprotechs Inc., Butler, PA) for 24 hours. The medium was changed to complete culture medium with or without 30ng/ml of P4 for 48 hrs and then continue to culture as indicated. Nomarski differential interference contrast (DIC) images were taken using a confocal microscopy (Olympus FV1000, Japan) with a transmitted light at 400× magnification.

Cell proliferation assay. The XTT cell proliferation assay was performed according to the manufacturer's protocol. Briefly, cells were seeded in a 96-well plate in 100 μ l of culture medium with or without the compounds to be tested and incubated for 24-48 hrs at 37 °C. The reconstituted XTT mixture (10 μ l/well) was added and the cells were incubated for 2hrs. The absorbance of each sample was subsequently measured using a microplate reader at a wavelength of 450 nm.

Knocking down mPR α expression with small interference RNA. Cells were transfected with mPR α siRNA or an equal amount of nonspecific control siRNA (Dharmacon, Lafayette, CO, USA) using the Oligofectamine reagents according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Two days after transfection with siRNA, the cells were incubated with diverse experimental reagents.

Transfection of mPR α DNA plasmid. The MB231 cells were cultured and split when the cell confluence reached about 90%. The human mPR α cDNA constructed in pBK-CMV vector [20] was purified and then transfected into the cells using Lipofectamine 2000 reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Two days after transfection, the mPR α expressing cells were selected with 1000 μ g/ml G418 (Gibco, Carlsbad, CA, USA). The resistant colonies were then isolated and propagated with 500 μ g/ml G418 in order to produce the stably transfected cell lines.

Isolation of caveolar fractions. Caveolae membranes were isolated as described previously [21]. Briefly, MB468 cells were homogenized in 1ml of MES-buffered saline (24 mM MES, pH 6.5, and 0.15 NaCl) plus 1% Triton X-100 and spun down at 3,000 g for 5 min at 4°C. The supernatant (4 ml) was used to dissolve sucrose and compose 40% of sucrose solution. This solution was placed in a 12.5-ml Beckman centrifuge tube (Beckman Coulter, Fullerton, CA)

with a 5–30% sucrose gradient layered on top and then centrifuged at 39,000 rpm for 24 h at 4°C in a Beckman SW-41 rotor. After the centrifuge, 600 µl fractions of the solution were collected and subjected to further analysis.

Immunohistochemical analysis (IHC). In brief, two tissue microarray slides consisting of human breast cancer (140 and 70) cores and adjacent benign breast tissue (10 and 24) cores were purchased from the Biomax US (Rockville, MD). These tissue microarray slides were constructed with complete different sets of tissue blocks. There were total 105 breast cancer and 17 benign breast tissues in these two tissue arrays. Two 1.5 mm-cores from each breast tissue block were constructed in the tissue microarray slides. After deparaffinization, rehydration, antigen retrieval, and endogenous peroxidase blocking, the slides were blocked with 5% normal horse serum for 1 hr and sequentially incubated with anti- mPR α antibody (1:200 dilution) at 4°C overnight and then incubated with a secondary antibody at room temperature (see manual of the ImmPRESS REAGENT kit, VECTOR Lab, CA). The color was developed with the ImmPACT DAB kit (VECTOR Lab, CA). Between the incubations, the slides were washed twice with 1xPBS buffer (5 min each) [19] [22]. Two negative controls were included – (1) control slides were stained without the primary antibody; (2) control slides were incubated with a specific blocking peptide (cat# sc-50111p, Santa Cruz, CA) prior to the primary antibody incubations. The immunostained slides were counterstained with hematoxylin and evaluated using a Nikon microscope with an Olympus digital camera. The IHC result was evaluated using a semi-quantitative scoring system by a trained research pathologist, who was blinded to patients' clinical data provided by the company. The intensity of the immunostaining was defined into four categories (strong positive, moderate, weak, and negative, as shown in Supplementary figure S2 A-D in Additional file 2).

Statistical Analysis. The quantitative data was expressed as mean \pm S.E and statistical significance was assessed by Student's paired two-tailed t-test. The positive rates of mPR α immunostains in different groups of human breast cancers and benign diseases was compared and analyzed by Fisher's exact test. $P < 0.05$ was considered significant.

Results

P4 regulates expression of snail and other EMT relevant proteins in MB468 but not in MB231 cells. In this study, we focused on the effects of P4 on expression of snail and other EMT marker proteins. As shown in the Figure 1A and 1B, the snail expression in the late passage MB468 cells was down regulated by P4 treatment in a dose- dependent (P4 at 15 ng/ml - $47.0 \pm 7.4\%$); 30 ng/ml - $68.3 \pm 6.7\%$; 60 ng/ml - $86.3 \pm 1.6\%$; Figure 1A) and time-dependent manners (6 hrs - $20.0 \pm 2.4\%$; 12 hrs - $24.0 \pm 3\%$; 24 hrs - $87.0 \pm 7.4\%$; 48 hrs - $94 \pm 8.6\%$; Figure 1B). Since snail has been known as a key modulator for EMT [4, 5], the P4 induced EMT relevant changes were further investigated. As shown in Figure 1C, fibronectin expression was significantly inhibited around $74.0 \pm 3.7\%$ by P4 treatment at 30 ng/ml while expression of E-cadherin and occludin was up-regulated about 3.8 fold and 3.5 fold respectively. Furthermore, the changes in expression of snail/other EMT relevant proteins were followed by cell morphological changes. The late passage MB468 cells without P4 exposure showed apparent mesenchymal phenotypes, characterized by diverse sizes and spindle or elongated shapes are usually shown; while with P4 treatment, most of the cells appeared epithelial-like transition, featured by large and polygonal shapes or small oval shapes (Figure 1D at 40x and Supplementary figure S3 in Additional file 3 at 20x). In addition, the cell proliferation of MB468 was also inhibited by P4. As shown in Supplementary figure S4 in Additional file 4, the growth

of MB468 cells was inhibited by P4 treatments in a dose-dependent manner (15 ng/ml - 19%, 30 ng/ml - 29.5%, and 60 ng/ml - 52.3% respectively), which is consistent with the previous report [23]. Interestingly, in the early passage MB231 cells, another BPBC cell line with obvious mesenchymal features, the P4 treatment had no effect on snail expression (Figure 1E) and cell proliferation (data in Figure 2B).

The nuclear PR has no roles on the P4-repressed EMT in MB468 cells. The classical nuclear PR was first considered as a molecular mediator of the P4-repressed EMT in MB468 cells even though they are basically negative for nuclear PR expression in normal culture condition. The cancer progenitor cells, however, may proliferate and express PR in responding to sex hormone treatments [24]. As shown in Figure 3A, MB468 cells were treated by P4 and E2 (estrogen) and the expression of PR was indeed induced by P4 treatment slightly, but not by E2. To explore whether the increase of PR expression is involved in the P4-repressed EMT events, MB468 cells were then co-incubated with P4 plus RU486 (Mifepristone) or R5020 (Promegestone), which are known as PR specific blocker or agonist. Surprisingly, both PR modulators had no effects on the P4 repressed snail expression (Figure 3B) and cell proliferation (Figure 3C), suggesting that other molecular mediators, but not nuclear PR, might be involved in the P4 repressed EMT events.

MPR α plays an essential role in the P4-repressed EMT in MB468 cells. Within the last few years, evidence has been obtained for the involvement of mPR α in the P4's actions in a variety of cell types [6, 7, 11, 12]. In the present study, the expression of mPR α in MB468 cells was up-regulated by P4 treatments in dose dependent manners (15 ng/ml - 1.97, 30 ng/ml - 2.75, and 60 ng/ml - 2.92-folds respectively; Figure 4A). As a control, there were no detectable mPR α protein found in MB231 cells (Figure 4B), which is consistent with a previous report [20]. These

data suggest a potential role of mPR α in the P4 signaling of BPBC cells. To further demonstrate our hypothesis, the expression of mPR α in MB468 cells was knocked down by mPR α specific siRNA. As shown in Supplementary figure S5 in Additional file 5, mPR α siRNA specifically inhibited mPR α expression without affecting α -tubulin expression. After transfection with 50 nM of mPR α siRNA, the P4's effects on the EMT marker proteins were significantly inhibited (Figure 4C).

Activation of PI3K/Akt is necessary for P4's action on EMT but not on cell proliferation. To further prove the involvement of mPR α in P4's action on human BPBC cells, the mPR α -expressing plasmid DNA was introduced into the parent MB231 cells and then treated by P4 as indicated. There was no reduction found in the expression of snail as compared with that of parent MB231 cells (Figure 2A). By comparing the molecular profiles of MB468 and MB231 cells, major differences were noticed between the two cell lines in PTEN expression and PI3K/Akt activation [15]. PTEN is an essential inhibitor for the PI3K/Akt pathway [25]. In MB468 cells, there is no PTEN expression and PI3K/Akt pathway is constantly activated; On the contrary, in MB231 cells PTEN is abundantly expressed and PI3K/Akt pathway is always inactivated. To explore the role of PTEN and PI3K/Akt pathway in the P4 regulated EMT, the mPR α stably-expressing MB231 cells were incubated with the PTEN specific inhibitor - bpV(phen) to transactivate the PI3K/Akt pathway. As shown in Figure 4D, snail expression was clearly down regulated by P4 treatment about $89.8 \pm 1.9\%$. These data strongly suggest that mPR α plays an important role in repression of EMT through activated PI3K/Akt pathway in BPBCs.

To test whether the female sex hormone controls cell proliferation of MB468 cells, we incubated the cells with P4 (30ng/ml) for 24 hrs. As shown in Figure 2A, a 34% reduction in cell proliferation was observed in the MB468 cells with treatment of P4, as compared to the cells

with treatment of vehicle alone. As expected, P4 had no effects on cell proliferation of the parental MB231. However, the treatment of the mPR α stably-expressing MB231 cells with P4 induced a significant reduction of cell proliferation (28.1%) (Figure 2B). These data suggest that mPR α is also involved in regulating cell proliferation of BPBC cells.

EGFR and PI3K are involved in the P4-repressed EMT in MB468 cells. To explore the intermediate pathways that regulate expression of snail/EMT proteins in the downstream of P4/mPR α signaling, several pathway specific inhibitors were tested in the current study. As shown in Figure 5A and 5B, the EGFR inhibitor (AG1478) and PI3K inhibitor (wortmannin) significantly blocked the P4-regulated snail/EMT protein expression in MB468 cells; while the ERK1/2 inhibitor (PD98059) did not block the P4's effect on snail/EMT. Supplementary figure S6 in Additional file 6 showed that P4 induced phosphorylation of EGFR, Akt, Src, and ERK1/2; and coordinated pathway inhibitors repressed the P4 induced phosphorylation, indicating the functionality of these inhibitors. These results suggest that the signaling cascades of the P4-repressed snail/EMT in MB468 cells are mainly intermediated through the EGFR and PI3K/Akt pathways. However, the roles of Src family kinase inhibitor (PP1) in modulating EMT remain unclear as compared with that of other pathway inhibitors. PP1 did not block the P4's action on expression of snail and fibronectin, but it did block the P4's action on expression of occludin and E-cadherin.

As it has been reported that human BPBC cells often over express caveolin -1 (Cav-1), which is a major component of caveolae membrane and often negatively regulates the function of other caveolar bound signaling molecules including EGFR [26-29]. To confirm the membrane location of mPR α and potential cross relationship with other caveolae bound signaling molecules, the caveolar fraction proteins were isolated from MB468 cells. As shown in Figure 6A and 6B, Cav-

1 appeared in the fractions 2-4, suggesting these fractions mainly consist of caveolar membrane. Importantly, mPR α appeared in the fraction 3 where EGFR was also located, indicating the potential crosstalk between mPR α and EGFR.

MPR α expression in human benign and malignant breasts. To evaluate expression of mPR α in human breasts, tissue microarray slides were studied by IHC. As shown in **Table 1**, 94 of 105 breast cancer tissues were stained positive for anti-mPR α . The positive signals are mainly observed in cytoplasm (Figure 7A) and/or cell membrane of cancer cells (Figure 7B). There were 14 triple negative cancers (TNBC) among these breast cancer tissues. Most of these TNBC (13/14) were moderate to strong positive for mPR α stain. In addition, mPR α was also detected in all normal and/or benign breast tissues. The ductal and alveolar epithelial cells of breast were showed negative or weak positive while the myoepithelial cells were shown strong positive for mPR α (Figure 7C).

Discussion

Classically, the actions of P4 on breast cancer cells are attributed to the binding of nuclear PR and subsequently activation of the downstream target genes. Lange et al proposed that P4 acts as a priming agent in breast cancer and, in his scenario, breast cells can be directed toward one path or another by cross-talking between the P4/PR complex and other signaling pathways [30]. In the PR negative MB231 cells, P4 showed no effect on cell proliferation and invasion. However, after introducing exogenous PR cDNA into MB231 cells, the PR-expressing MB231 cells exhibited less proliferative activities after P4 treatment than the parental MB231 cells [31]. With this PR-expressing cell model, Lin et al demonstrated that P4 induced remarkable EMT like changes in cell morphology and surface adhesion structures [31]. In the current study, we

showed that P4 treatment *in vitro* inhibited EMT relevant proteins in the late passage MB468 cells. A negative association between P4 and snail expression was observed (**Figure 1B**). Consistent with down regulation of snail expression, other EMT relevant proteins, such as E-cadherin, occludin, and fibronectin, were subsequently modulated by P4 (**Figure 1C**); and these molecular changes were accompanied with cell morphological reversion from mesenchymal- to epithelial-like phenotypes (**Figure 1D**). Our results indicate that P4 functions as an anti-EMT hormone in MB468 cells *in vitro*. It is still unclear how P4 regulates these EMT events and what the cell mediators of P4 are.

The membrane progesterin receptor, mPR α , has recently been identified as an intermediary factor of the progesterin induced intracellular signaling cascades in the PR-negative breast cancer cell lines *in vitro* [6, 12]. The expression of mPR α in human breast cancer tissues, however, has not been well evaluated. With PCR assay, Dressing et al reported expression of mPR α mRNA in both normal and malignant breast tissues [7]. Using an *in vitro* hormone binding technique and a FITC conjugated BSA-progesterone, Pelekanou et al detected the “membrane- associate receptor for progesterone” in 57 of 61 breast cancers (94%) [32]. In this report, the protein expression of mPR α was detected in both human benign and malignant breasts (**Table 1**), which is quite consistent with Pelekanou’s result. The receptor was also demonstrated in all but one of triple negative breast cancers – a type of cancer that shares many common features with BPBC [33, 34]. Moreover, in the benign breasts, strong positive stain for mPR α was detected in the basal myoepithelial cells. Recently we showed that the mammary ducts of normal mice were positive for both PR and mPR α . The PR was predominantly seen in the ductal epithelium, while mPR α was mostly observed in the basal myoepithelial cells [22]. The synergistic roles of mPR α and PR in normal mammary gland remain to be explored.

The mPR α receptor has been associated with many physiological functions in vertebrates. It induces oocyte maturation, stimulates sperm hypermotility, down-regulates GnRH secretion, modulates T cell functions, and adjusts human myometrial cell contractility [6, 7, 11-13, 35]. In agreement with the earlier studies done in human myometrial cells and fish oocytes [12, 13], we found that P4 up regulated the expression of mPR α in MB468 cells (**Figure 4A**). Importantly, the P4's actions on expression of snail/EMT relevant proteins were significantly blocked by the mPR α specific siRNA (**Figure 4C**). In contrast, P4 treatment alone had no effect on snail expression in the parent MB231 cells, in which mPR α protein is undetectable by Western blot assay (**Figure 4B**). We thought that the exogenous mPR α cDNA stable transfection would cause the cell EMT responding to the P4 treatment. Unexpectedly, the expression of snail/ EMT relevant markers remained unchanged after P4 treatments, indicating other factors in the P4/mPR α signaling pathway was still blocked.

The mesenchymal phenotype of MB231 cells under normoxic culture conditions has been associated with high levels of uPA and uPAR expression and silencing uPA expression decreased expression of vimentin and Snail and induced epithelial-like transition in the cells [16]. In the current study, we showed that the P4 repressed EMT in MB231 cells is correlated to the mutant *pten* and activation of PI3K/Akt signaling pathway. PTEN is a major inhibitor of the PI3K/Akt signaling pathway. Loss of PTEN protein expression occurs commonly in breast cancer, which has been associated with loss of estrogen receptor [36] and resistance to cancer therapies [37]. The PTEN-deficient cell lines displayed greater sensitivity to the growth inhibitory effects of the PI3K inhibitor, LY294002, as compared with the PTEN-positive cell lines [38]. Recently major differences have been reported in the status of PI3K/Akt pathway and function of PTEN between MB468 and MB231 cells [39] [15] . It was assumed that the

activation of PI3K/Akt pathway, resulting from a dysfunctional PTEN, is essential for the P4-repressed EMT. In further study, we demonstrated that the expression of snail/EMT relevant proteins in the mPR α expressing MB231 cells was significantly modulated after incubating the cells with P4 plus PTEN inhibitor - bpV(phen) (**Figure 4D**). However, activation of PI3K/Akt seems not to be essential for the P4-repressed cell proliferation since the growth reduction of the mPR α -expressing MB231 cells could be induced by P4 treatment alone. It is assumed that the P4 inhibited cell proliferation may go through other pathways, e.g. the secondary messenger pathway through activation of pertussis toxin-sensitive inhibitory G proteins and mitogen activated protein kinases (MAPK/Erk1/2) [12, 20].

When exploring the intermediate pathways that regulate snail/EMT in P4 signaling, we showed that the P4's actions on EMT were significantly blocked in the late passage MB468 cells by AG1478 (an EGFR inhibitor) and wortmannin (PI3K inhibitor), suggesting EGFR and PI3K/Akt pathways are involved in the P4 repressed EMT events. Studies have shown that along with other signaling molecules e.g., PDGFR, Ha-ras, c-Src, both EGFR and PI3K are distributed in the caveolar vesicles in which caveolin-1 (Cav-1) serves as a main structure component [40]. Cav-1 usually functions as a negative regulator of other caveolar bound signaling molecules [26-29]. Existing data has shown that basal phenotype breast cancer is associated with high expression of Cav-1 [41, 42] and EMT of cancer cells is dependent upon the presence of Cav-1 [40]. Okamoto et al showed that long-term EGF treatment reduced expression of Cav-1 in cancer cells; and subsequently up-regulated snail and down-regulated E-cadherin expression [40]. Lu et al demonstrated that EGF treatment of human tumor cells that over express EGFR caused a dramatic alteration in cell-cell contacts and internalization of E-cadherin [43]. It was assumed that upon binding to EGF, EGFR forms homodimers or heterodimers which result in the

activation of their intrinsic kinases and autophosphorylation of specific tyrosine residues within their cytoplasmic domains [44]. The activated EGFR may recruit other molecular signaling complexes e.g. PI3K via several potential paths. For examples, EGFR may bind to and recruit PI3K directly since the canonical binding sites for the regulatory subunit of PI3K are not found on EGFR [45]; it may also employ the docking protein Gab1 to recruit PI3K [46]. In addition, the EGFR adapter (Shc) may recruit PI3K by assembly of a Shc–Grb2–Gab2–PI3K complex [47]. The role of PI3K/Akt pathway in cancer EMT has been well documented in various human malignancies [48-50].

The essential roles of c-Src pathway in the P4/PR signaling pathways have been demonstrated in human breast cancer cells *i.e.* T47D cells. The cell anchorage-independent growth was stimulated by progestin and blocked by inhibition of Erk1/2, c-Src, EGFR, or RNA interference of Wnt-1 [51]. Recently Lester and Jo et al reported that when MB468 breast cancer cells were cultured in hypoxia condition, expression of uPAR was increased, cell-cell junctions were disrupted, vimentin expression was increased, and E-cadherin was lost from cell surfaces, indicating enhancement of EMT [16, 52]. Jo et al proposed a model in which Src family kinases may concert with other cell signaling factors, including PI3K and ERK1/2 and play essential role in the regulation of uPA and uPAR and EMT [16]. In this report, we found that in the late passage MB468 cells, the Src family kinases inhibitor (PP1) did not block the P4's action on snail and fibronectin (one of the mesenchymal phenotypes), but it blocked the P4's action on expression of occludin and E-cadherin (epithelial phenotypes). The roles of Src family kinases on the P4-repressed EMT remain to be explored.

Conclusions

In summary, using two human basal phenotype breast cancer cell lines as models, we identified a PR-independent pathway that involves the signaling cascade of EMT through a caveolae bound signaling complex namely mPR α , Cav-1, EGFR, and PI3K/Akt (Figure 8). It is assumed that mPR α receptor is the key modulator of EMT located on the caveolar membrane of BPBC cells. Through the receptor mediated mechanisms, P4 directly inactivates the PI3K-snail-EMT pathway or interacts with Cav-1 and modulates the activities of the EGFR pathway, which then cross inhibit PI3K pathway, and eventually suppresses the cell EMT. The proposed pathway is attractive for further understanding the molecular mechanisms of EMT and for developing novel therapeutic strategies against BPBC.

Abbreviations

BPBC: basal phenotype breast cancers; P4: progesterone; MB468: MDA-MB468 cells; EMT: epithelial-mechenchymal transition; mPR α : membrane progesterone receptor α ; MB231: MDA-MB231 cells; PRs: progesterone receptors; ATCC: the American Type Culture Collection; IHC: immunohistochemistry; TNBC: triple negative cancers.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LZ functioned as a main researcher in this study and her works covered most of the assays; WL functioned as a main researcher in this study whose contribution was mainly focused on the protein expression; SY proposed the hypothesis and designed the study. He functioned as the supervisor and PI. He also composed the manuscript.

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Table 1. Positivity of mPR α expression in human breast cancers

mPR α reactivity	TNBC	Non TNBC	Total
Positive (%)	13 (92.9)	81 (89)	94(89.5)
Negative	1	10	11
Total	14	91	105

No significant difference was found between the positive rates of mPR α (TNBC vs. non TNBC groups, $P=0.312$, Fisher's exact test). mPR α : membrane progesterone receptor α ; TNBC: triple negative cancers.

Figure legends

Figure 1. Dose curve and time course of P4's action on EMT relevant events of MB468 and MB231 cells. The growth-arrested MB468 cells were treated with different doses of P4 for 24 hours (**A and C**) or with P4 at 30 ng/ml for various times as indicated (**B**). Western blot analyses were performed with diverse antibodies as indicated. The expression of snail was inhibited by P4 treatments in dose and time dependent manners (figure 1A and 1B); and the expression of fibronectin, E-cadherin, and occludin was also modulated by P4 in dose dependent manners. (**D**) Morphological changes of MB468 cells treated with/without P4 at 30ng/ml for 48 hrs. Photos (DIC images) were taken by using confocal microscopy at 400× magnification. (**E**) The growth-arrested MB231 cells were treated with different doses of P4 for 24 hours as indicated. Western blot analyses were performed with anti-snail and anti-GAPDH antibodies. Equivalent levels of snail expression were shown after the P4 treatment at different doses as indicated. All data were collected and averaged from three individual experiments and the graphs are expressed as fold change over basal. * $P < 0.05$ for difference of protein expression induced by P4 vs. vehicle alone.

Figure 2. Effects of P4 on cell proliferation of MB468 (A), parent MB231 and mPR α stably-expressing MB231 cells (B). The data are representative for three individual experiments and the graphs are expressed as fold change over the basal. The cells treated by E2 (30pg/ml for 24 hours) and vehicle alone were used as controls. * $P < 0.05$ for difference of cell proliferation induced by P4 vs. vehicle alone.

Figure 3. The role of PR in the P4-repressed EMT and cell proliferation. (A) Growth-arrested MB468 cells were treated with P4 (30ng/ml) or E2 (30pg/ml) for 24 and 48 hours as

indicated. **(B)** Growth-arrested MB468 cells were treated with R5020 (10 nM) or RU486 (10 nM) for 2 hours, followed by P4 treatment at 30 ng/ml for 24 hours. Western blot analyses were performed with anti-snail, anti-PR and anti- α -tubulin antibodies. **(C)** MB468 cells were treated with R5020 (10 nM) or RU486 (10 nM) for 2 hours, followed by P4 treatment at 30 ng/ml for 24 hours. Cell proliferation assays were then performed. As shown in the Figure 3A-3C, the P4 treatment inhibited snail expression and cell proliferation at equivalent levels. The results are representative for three separate experiments.

Figure 4. The role of mPR α in the P4-repressed EMT of MB468 and MB231 cells. **(A)** The growth-arrested MB468 cells were treated with diverse concentrations of P4 for 24 hours. (PC: HEK293 cell lysate as a positive control for mPR α expression.) **(B)** MB231 cells and MB468 cells were treated with/without P4 at 30 ng/ml for 24 hours. Western blot analyses were performed with anti-mPR α , anti-snail, and anti- α -tubulin antibodies. **(C)** MB468 cells were transfected with 50 nM of mPR α siRNA and 50 nM of scramble siRNA; and then treated with P4 at 30 ng/ml for 24 hours. The Western blot analyses showed similar patterns of snail/EMT protein expressions in the cells transfected with scramble siRNA and equivalent levels of snail/EMT proteins in the cells transfected by mPR α siRNA, indicating the roles of specifically knocking down mPR α in MB231 cells. **(D)** The parent MB231 and mPR α stably-expressing MB231 cells were treated with/without bpV(phen) at 1 μ M for 1 hour, followed by P4 treatment at 30 ng/ml. Western blot analyses were performed with anti-snail, anti- mPR α , and anti- α -tubulin antibodies. The data are representative for three individual experiments.

Figure 5. The molecular pathways involved in the P4-repressed EMT of MB468 cells.

Growth-arrested MB468 cells were treated with AG1478 (1 μ M) or PP1 (10 μ M) (**A**) and wortmannin (0.1 μ M) or PD98059 (50 μ M) (**B**) for 1h, followed by P4 treatment at 30 ng/ml for 24 hours. Western blot analyses were performed with diverse anti-snail and/or EMT relevant antibodies as indicated. The data are representative for three individual experiments.

Figure 6. Co-localization of mPR α and EGFR in caveolar membrane of MB468 cells.

Caveolar fractions were isolated and immunoblotted for detecting Cav-1, mPR α , and EGFR after the MB468 cells were treated without (**A**) with P4 at 30 ng/ml for 24 h (**B**). The figure 6A and 6B show that Cav-1, mPR α , and EGFR are co-localized in the caveolar fraction #3 component regardless of P4 treatments. The results are representative for two separate experiments. PC – mPR α positive cells (HEK293).

Figure 7. Expression of mPR α in human breast cancer tissues.

The Figure 7A and 7B show the low to intermediate intensities of mPR α stains in the cytoplasm of most human breast cancer cells. Apparent cytoplasm - membrane stains were observed in some of the cancer cells (black arrows). The Figure 7C shows the immunostain of mPR α in normal human breast. EP-ductal epithelium, MEP- myoepithelium.

Figure 8. Schematic illustration of molecular pathway initiated by P4 signaling in BPBC cells.

MB468 cells usually maintain the activities of PI3K/Akt at high levels since these cells contain a mutant *pten* gene and over expressed EGFR. Activation of PI3K/Akt pathway is a vital signal for cell survival and epithelial to mesenchymal transition (EMT). It is assumed that the

binding of P4 to mPR α in the caveolar membrane of the cells inhibits EMT relevant events either directly through Cav-1 activation or through a cross interaction with EGFR and trans-activation of Cav-1 which subsequently inactivate PI3K/Akt pathway. Inactivation of PI3K/Akt pathway subsequently inhibits the nuclear translocation of snail and then modulates expression of other EMT relevant proteins.

Additional file legends

Additional file 1: Supplementary figure S1. Morphology of early and late passage MB468 cells. The cultured MB468 cells at early passages (6 passages) appeared as oval and/or polygonal shapes; and after multiple passages (50+ passages), these cells exhibit apparent mesenchymal phenotypes with spindle and elongated shapes as indicated. Photos (DIC images) were taken by confocal microscopy at 200 \times magnification.

Additional file 2: Supplementary figure S2. Diverse intensities of mPR α immunostains in human breast cancers. (A) Strong positive stain - most of the cancer cells are stained dark brown; (B) moderate positive - most of the cancer cells are stained moderate brown; (C) weak positive - light brown; (D) negative stain - very light brown or no stain.

Additional file 3: Supplementary figure S3. Cell morphology of late passage MB468 cells at low magnification with/without P4 treatment. Photos (DIC images) were taken by confocal microscopy at 200 \times magnification. This is an enlarged view of the Figure 1D.

Additional file 4: Supplementary figure S4. Dose curve of the P4-repressed cell proliferation of MB 468 cells. The growth-arrested MB468 cells were treated with different doses of P4 as indicated. The cell proliferation was inhibited in a dose dependent manner (15 ng/ml – 20%, 30 ng/ml – 25%, 60 ng/ml – 48%). The data were averaged from three experiments and the graph represents an averaged data expressed as fold change over basal. * $P < 0.05$ for difference of cell proliferation induced by P4 vs. vehicle alone.

Additional file 5: Supplementary figure S5. Knocking down expression of mPR α by siRNA in MB468 cells. MB468 cells were transfected with indicated amount of mPR α siRNA. Western blot analysis was performed with anti-mPR α and anti- α -tubulin antibodies. As shown in the figure, more than 90% mPR α expression was inhibited by transfection of mPR α siRNA at 50nM. The data are representative for three experiments.

Additional file 6: Supplementary figure S6. A figure showing that the treatment of MB468 cells with P4 alone significantly promotes phosphorylation of EGFR (**A**), Akt (**B**), Src (**C**) and ERK1/2 (**D**); and co-treatment of the cells with P4 and the specific pathway inhibitors abolishes the P4 induced phosphorylation on diverse pathway components (i.e. EGFR, Akt, Src and ERK1/2), indicating the effectiveness of P4 treatments in activation of diverse molecular pathways.

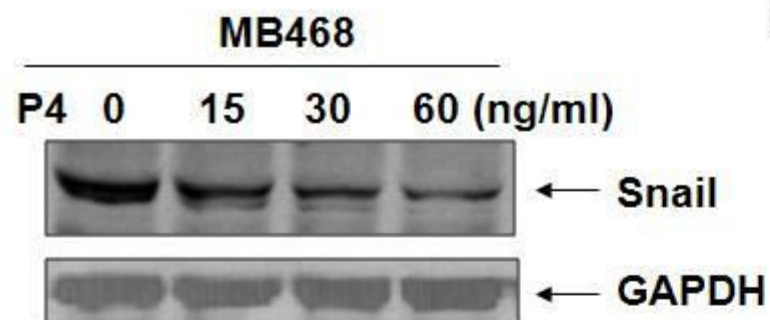
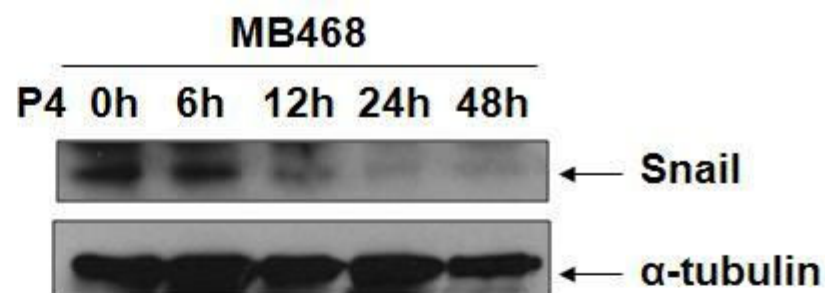
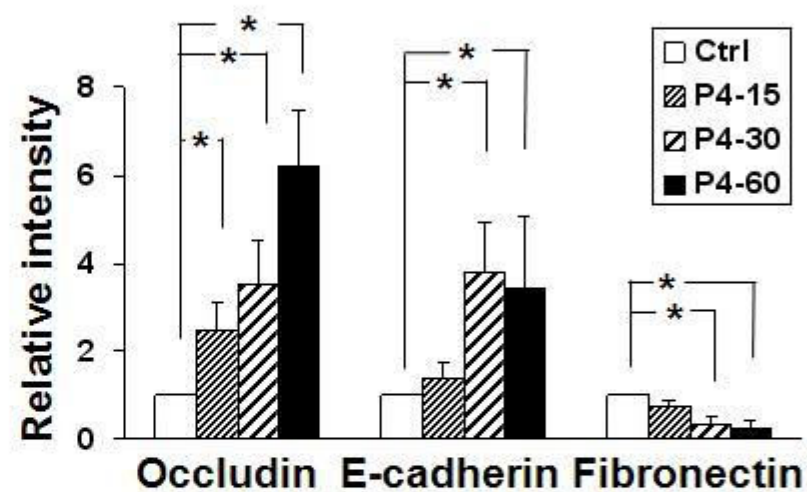
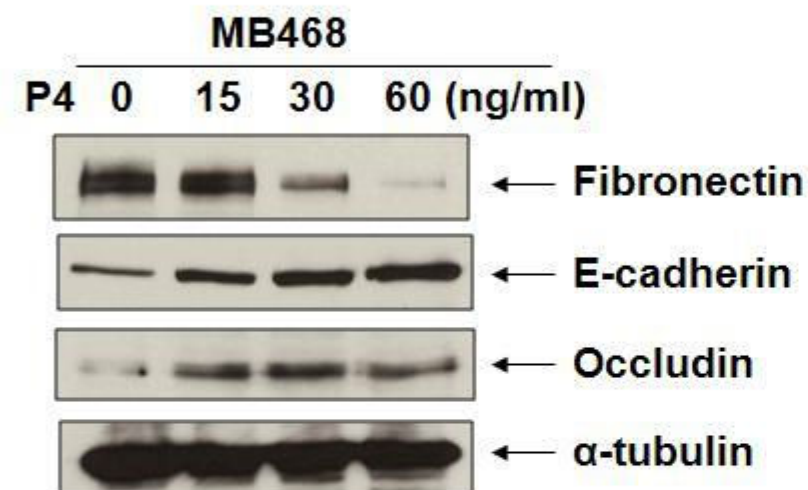
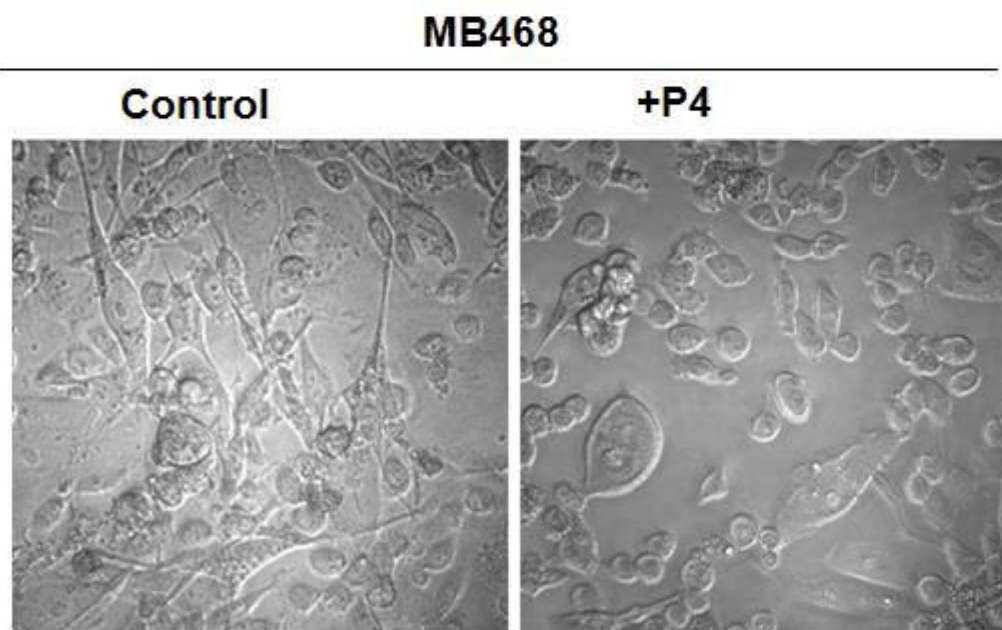
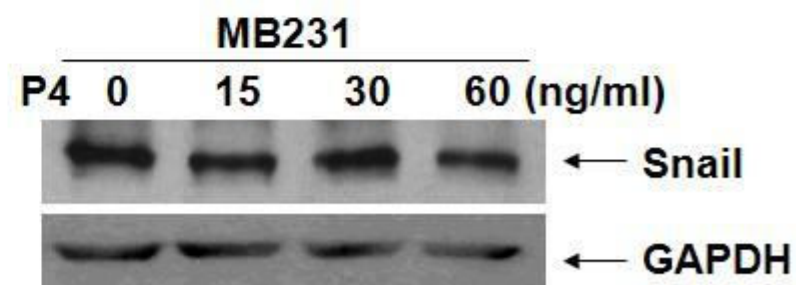
A**B****C****D****E**

Figure 1

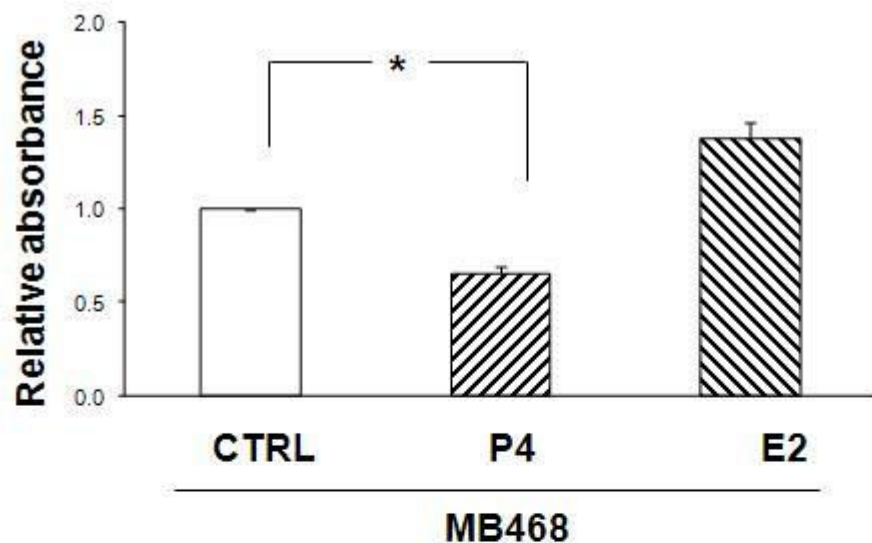
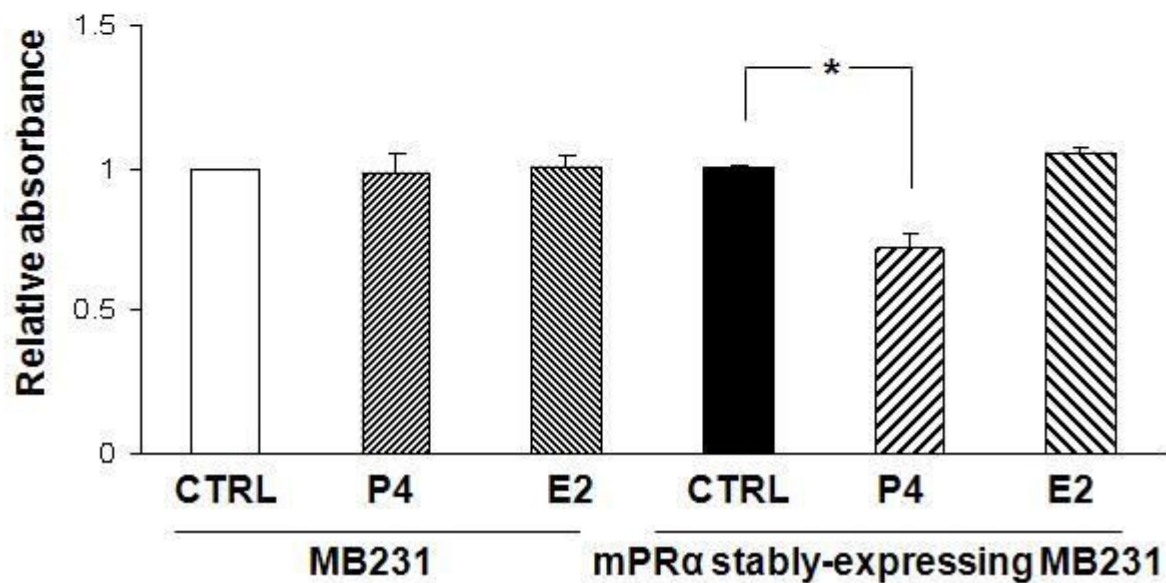
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Figure 2

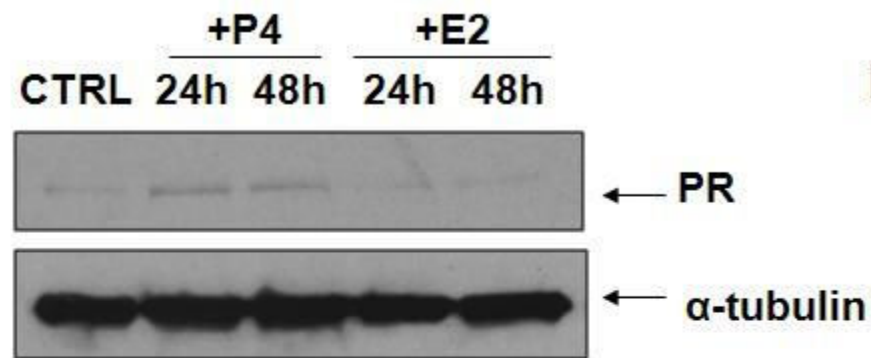
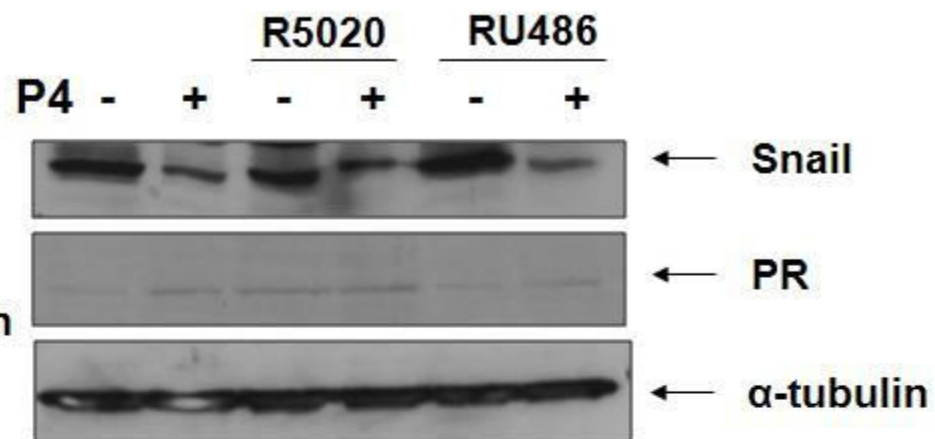
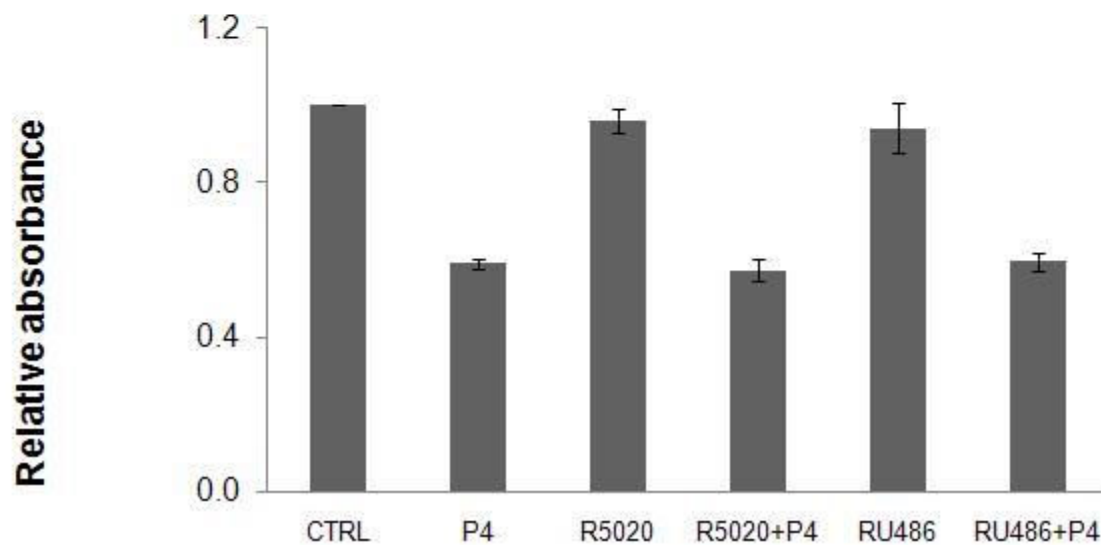
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Figure 3

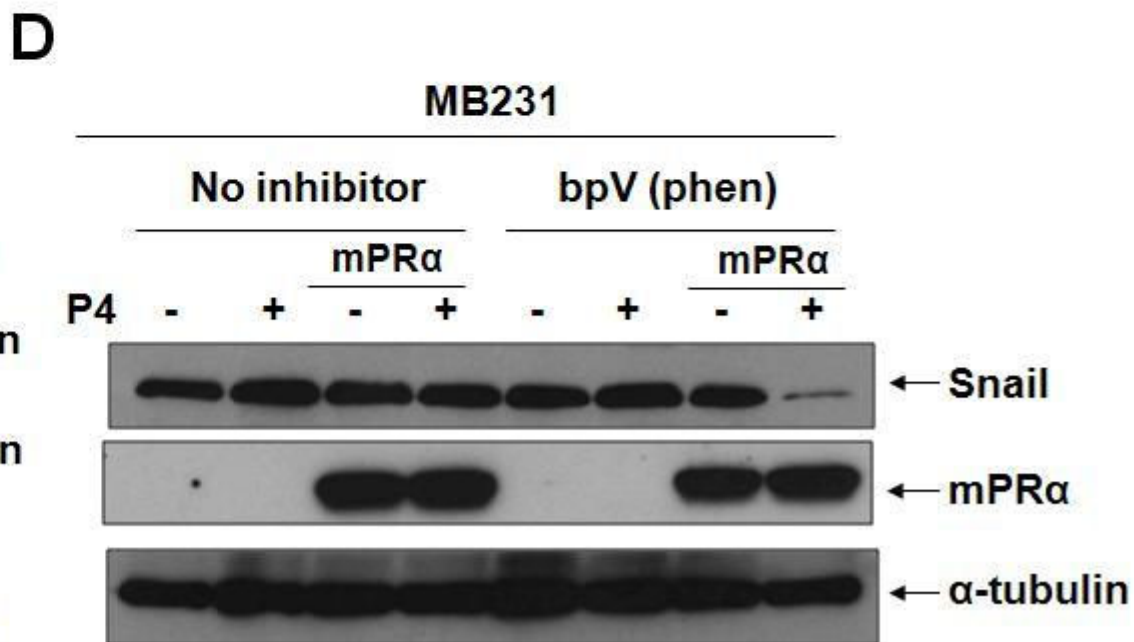
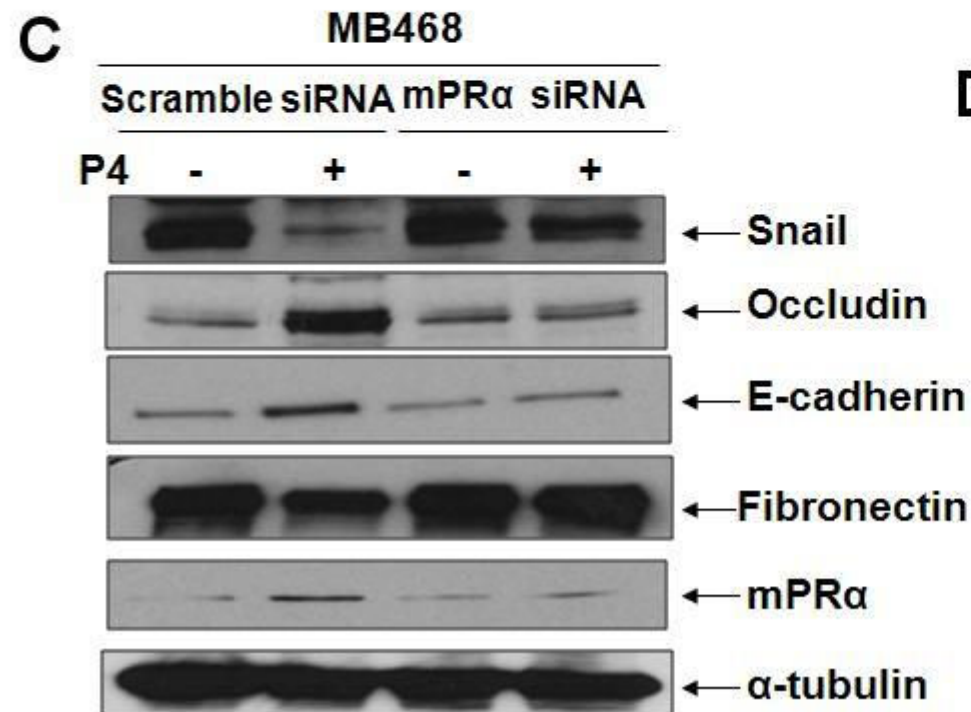
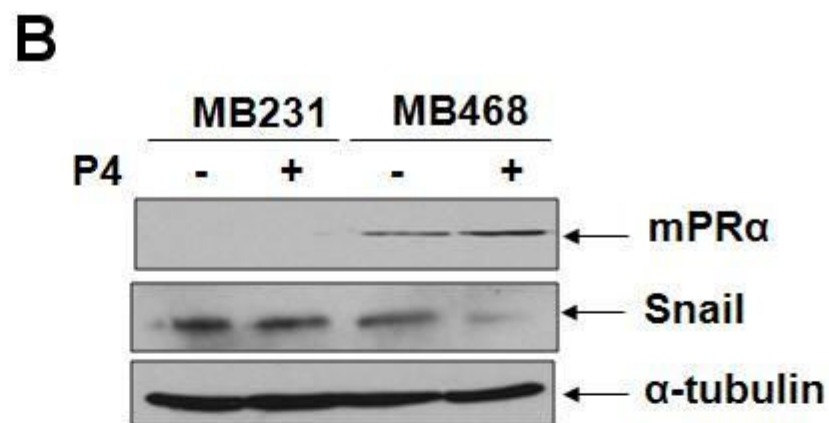
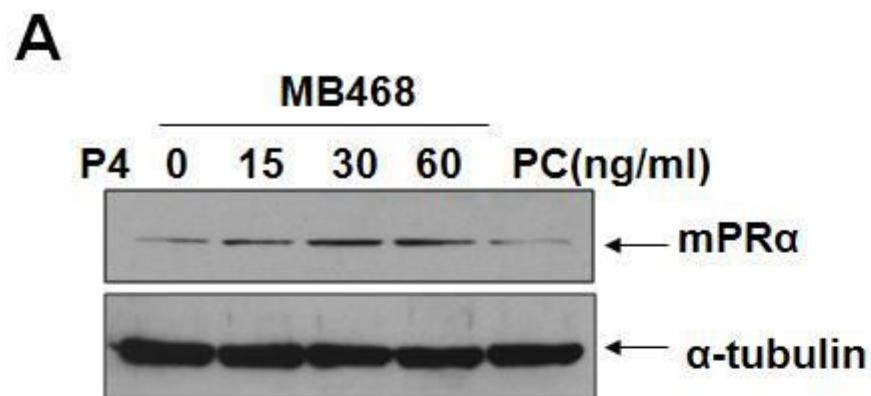


Figure 4

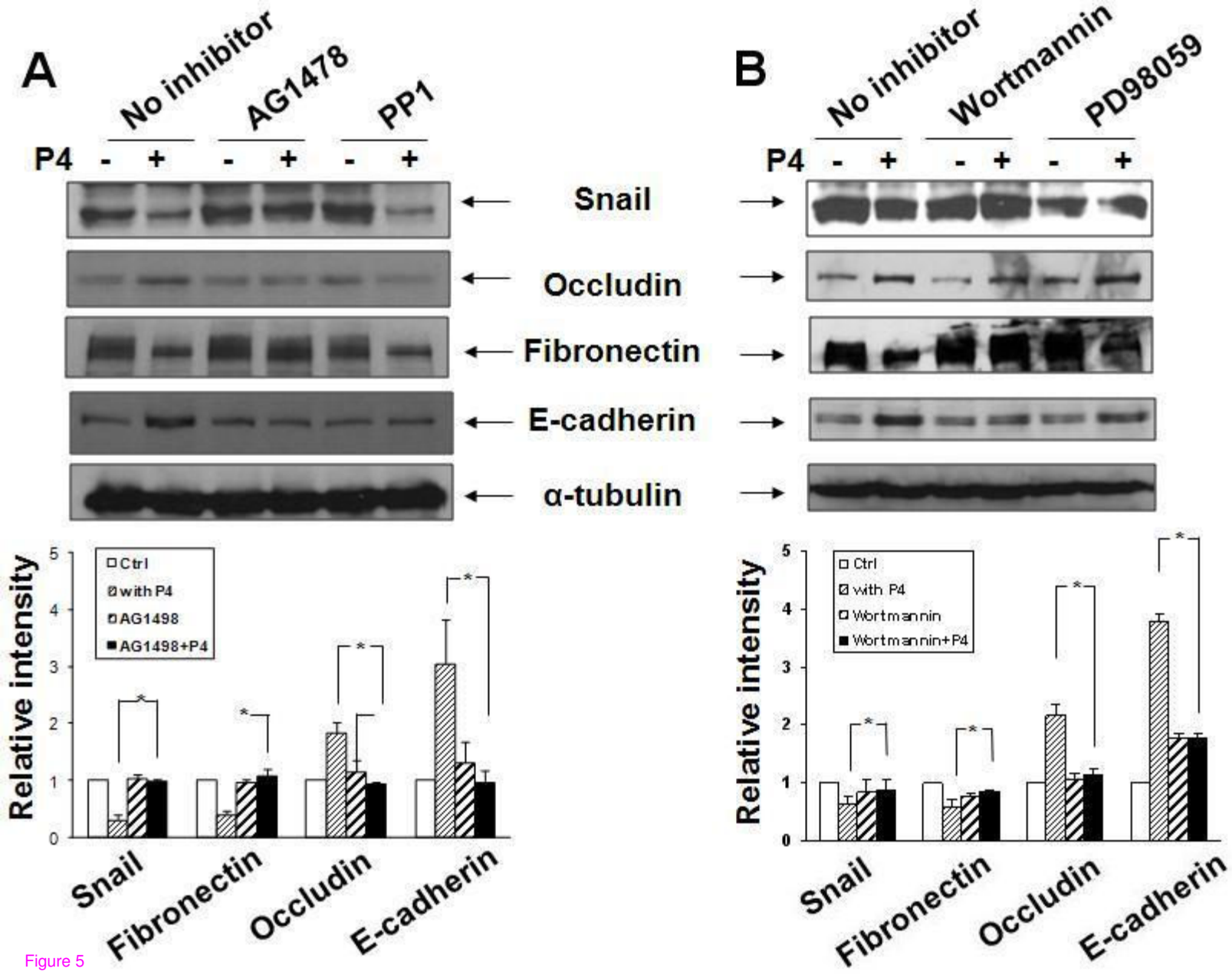
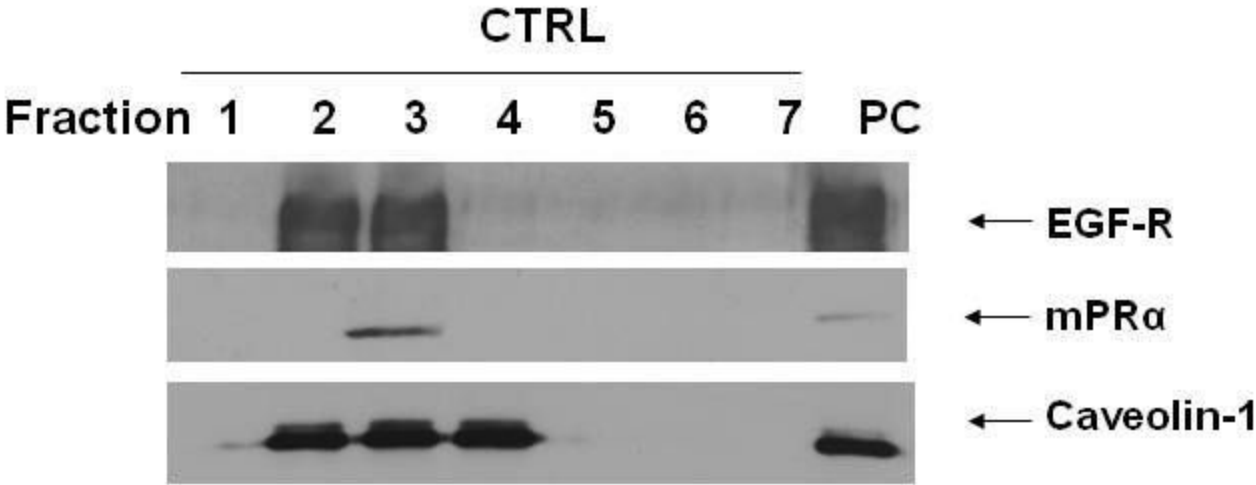


Figure 5

Figure 6

A



B

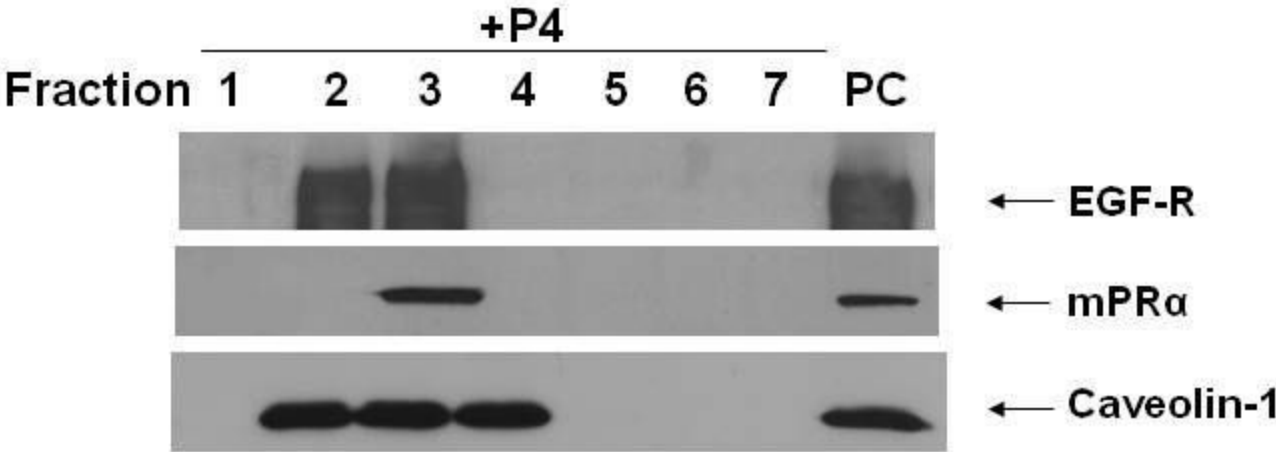


Figure 6

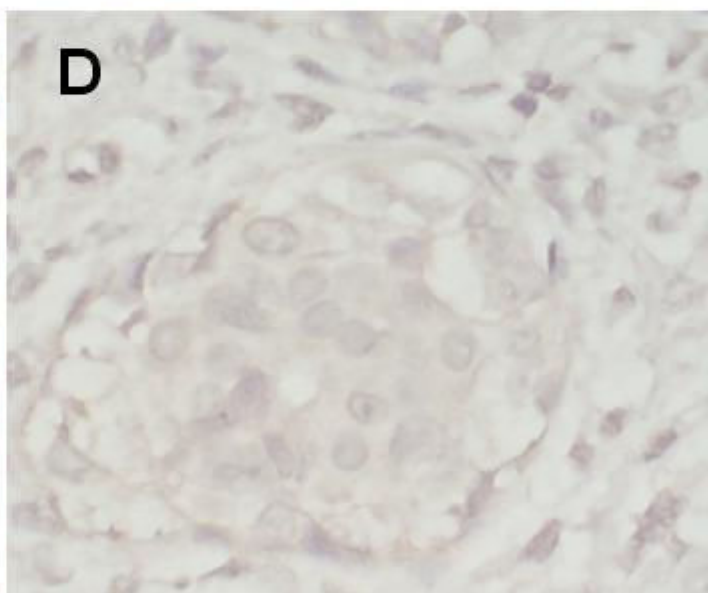
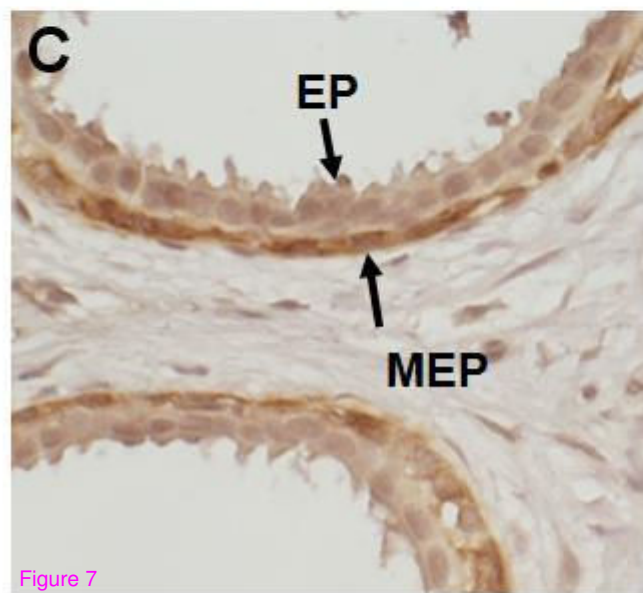
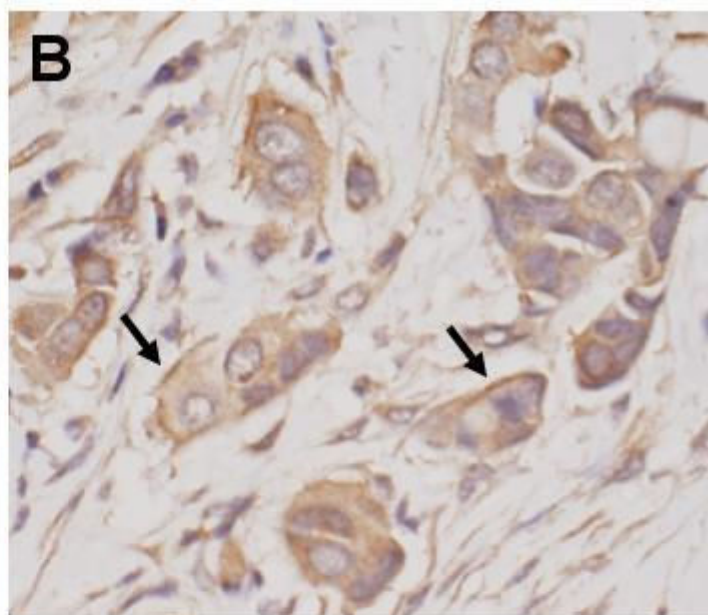
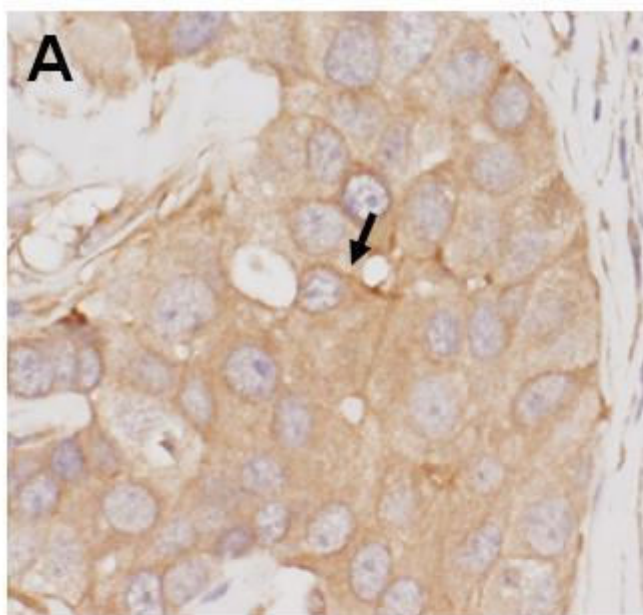


Figure 7

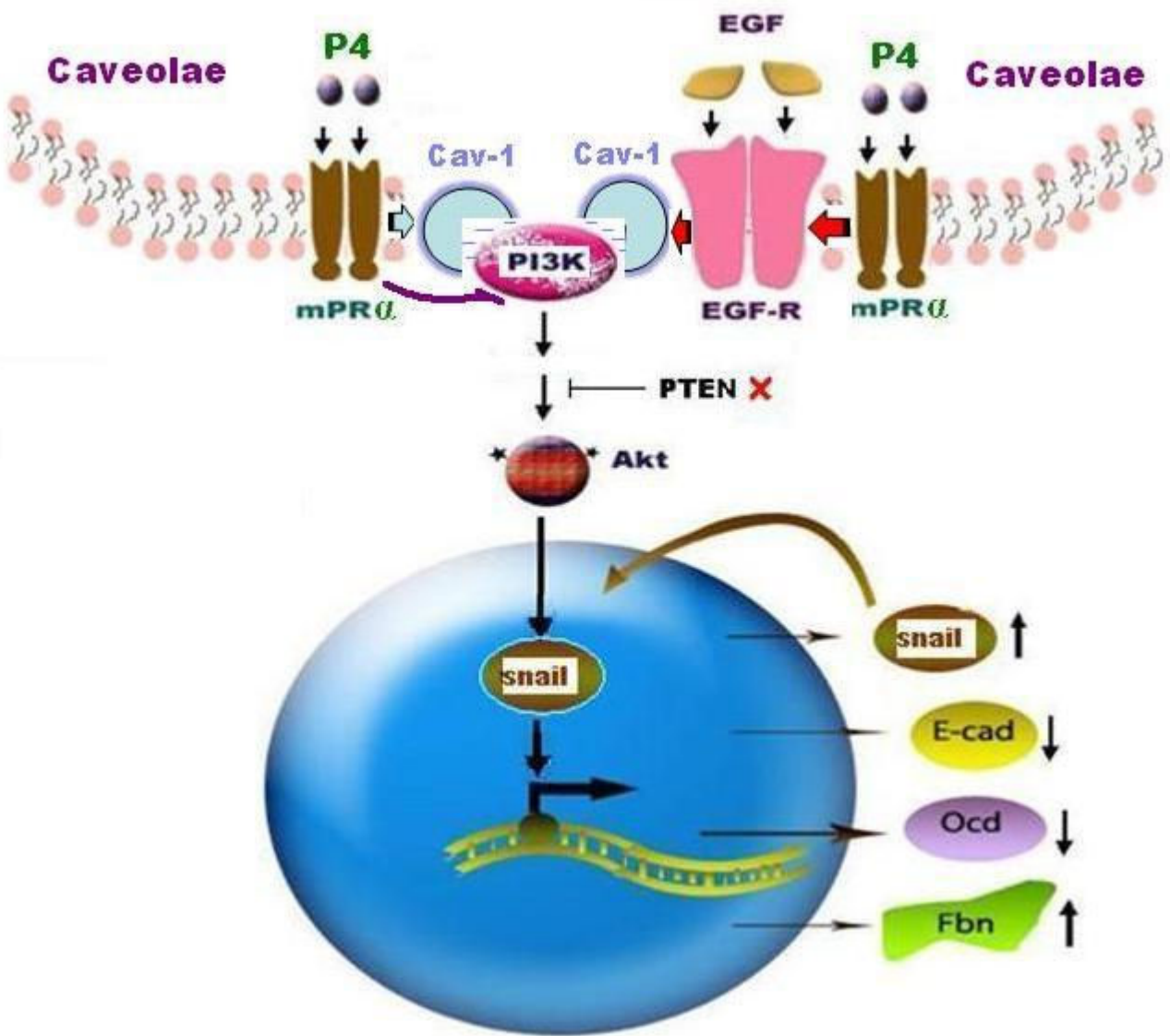


Figure 8

Additional files provided with this submission:

Additional file 1: Supplemental Figure 1.JPG, 61K

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Additional file 2: Supplemental Figure 2.JPG, 100K

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