Progesterone inhibits the migration and invasion of A549 lung cancer cells through membrane progesterone receptor α-mediated mechanisms

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Received December 27, 2012; Accepted February 18, 2013

DOI: 10.3892/or.2013.2336

Abstract. Lung cancer is the leading cause of cancer morbidity and mortality in the world. The incidence of lung cancer, particularly lung adenocarcinoma, is increasing in women compared to men. The role of sex hormones in the development of lung cancer has attracted substantial interest, but remains largely unknown. In this study, we demonstrated that membrane progesterone receptor α (mPRα) was expressed in a lung adenocarcinoma cell line, A549, and was located on the cell membrane. In additional experiments, we found that mPRα functioned as an essential mediator for progesterone (P4)-induced inhibitory effects on cell migration and invasion of A549 cells. Furthermore, PP1 (an Src pathway inhibitor), when co-incubated with P4, synchronously enhanced the inhibitory effects of P4 on cell migration and invasion. To explore the mechanisms of inhibition, we found that P4 and PP1 induced a cascade of molecular signaling events, such as dephosphorylation of focal adhesion kinase (FAK) and downregulation of matrix metalloproteinase 9 (MMP-9). Our study provides a mechanistic view on the effects of P4 through mPRα→Src/FAK relevant pathways in human lung adenocarcinoma cells and may aid in the development of novel therapeutic tools for the treatment of lung cancer.

Introduction

Lung cancer is the leading cause of morbidity and mortality in malignant tumors. Non-small cell lung cancer (NSCLC) accounts for 80% of lung cancer cases. Epidemic studies reveal gender differences in NSCLC patients, particularly in lung adenocarcinoma. Women are more susceptible to smoke or other environmental factors (1), in view of the fact that estrogen and progesterone are well-known prognostic factors for breast, endometrial and ovarian cancer, suggesting a possible involvement of gender-dependent factors in the pathogenesis and/or development of NSCLCs (2).

Sex hormones and their receptors have been the focus of considerable cancer research. Among sex steroids, estrogens play an important role in the development of breast and endometrial carcinoma, whereas androgens significantly contribute to the development of prostate cancer (3). By contrast, progesterone generally promotes differentiation and inhibits cellular proliferation through the nuclear progesterone receptor (nPR) (4). Previous studies showed that progesterone-mediated growth inhibition was mainly preceded by decreased expression of cyclins and/or induction of cyclin-dependent kinase inhibitors (5-7). Administration of progestins, including medroxyprogesterone acetate, is currently used as an endocrine therapy in breast and endometrial carcinoma patients (8,9). During embryogenesis, sex hormones influence the development of lung tissue, but during adulthood, the lung is not a target organ for sex hormones. Notably, female adenocarcinoma has a better prognosis than male lung cancer or other female pathologic types of lung cancer, indicating gender as an independent prognostic factor (10). It is reported that progesterone can mediate growth inhibition in PR-positive tumors in mice through decreased expression of cyclins A, D1 and E and/or induction of cyclin-dependent kinase inhibitors such as p21 and p27 (11). By contrast, PR antagonist mifepristone can inhibit spontaneous growth of lung cancer in mice (12). Combination of estrogen and progestins in NSCLC cells may cooperate in promoting expression of vascular endothelial growth factor (VEGF) which is essential for the progression of lung carcinoma, mainly by increasing proliferation of endothelial cells from neighboring blood vessels (13). Differences of progesterone actions may partly be explained by different types of progesterone receptors in various tissues.

Progesterone receptors include the nPR family, mPR family and progesterone membrane receptor components

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Key words: membrane progesterone receptor α, progesterone, lung cancer, migration, invasion

family (PGMRCs). The nPR family has been studied extensively in lung cancer, with a focus on the pathological characteristics, clinical stage and lymph node metastasis (14), while studies on the mPR family in lung cancer are few. Recently, mPRα was cloned from the ovarian tissue of spotted seatrout oocytes and recognized as the earliest and most thoroughly-studied progesterone membrane receptor by binding P4 in the membrane and subsequently inducing a series of alterations in the secondary messenger pathways through activation of the pertussis toxin-sensitive inhibitory G-proteins (15-17). Our preliminary study proved that mPRα acted as an epithelial-mesenchymal transition (EMT) negative regulatory protein, mediated progesterone's effect to reverse the EMT process and inhibited tumor development in breast cancer MDA-MB468 (MB468) cells (18). To date, there are no previous reports on whether mPRα-mediated progesterone signal plays a role in tumor invasion and metastasis. Our study examined the expression and location of mPRα in the lung adenocarcinoma cell line A549. Further research is focused on whether mPRα can mediate the effects of P4 on lung adenocarcinoma cell migration and invasion as well as its molecular pathway mechanism.

Materials and methods

Antibodies and inhibitors. Mifepristone (MIF) and pyrazolopyrimidine compound (PP1) were purchased from EMD Chemicals (Gibbstown, NJ, USA). Anti-mPRα goat polyclonal IgG, anti-MMP-9 goat polyclonal IgG, anti-GAPDH goat polyclonal IgG, anti-mPRα blocking peptide, donkey anti-goat IgG-HRP, goat anti-rabbit IgG-HRP and anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FAK rabbit polyclonal and anti-p-FAK rabbit polyclonal IgG were from Cell Signaling (Danvers, MA, USA). P4-BSA-FITC conjugate and anti-α-tubulin mouse monoclonal IgM were purchased from Sigma (St. Louis, MO, USA).

Cell culture. The human lung adenocarcinoma cancer cell line A549 and the breast cancer cell line MDA-MB231 (MB231) were obtained from the American Type Culture Collection (Rockville, MD, USA). The MB231 cell line is negative for mPRα. MB231 w/mPRα cells are derived from MB231 cells transfected with mPRα cDNA plasmid, with mPRα mRNA and protein strong expression. These cancer cells were cultured in DMEM (Mediatech, VA, USA) containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Carlsbad, CA, USA) in a humidified incubator at 37˚C with 5% CO_2.

RT-PCR assay. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and concentrations of RNA were determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). Reverse transcription for synthesizing cDNA was carried out using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA). PCR amplification (35 cycles of 95˚C for 20 sec, 58˚C for 30 sec and 72˚C for 20 sec) was conducted in a total volume of 25 μl using the GoTaq Hot Start Green Master Mix (Promega, Madison, WI, USA). Following PCR amplification, 25 μl of the samples were separated via electrophoresis on a 1.5% agarose gel. The primers used for PCR amplification were: mPRα: 5'-CCTGCTGT GTGATCTTAG-3' and 5'-CGGAAATAGAAGCGCCAG-3' (19), 18-S: 5'-GTTGGTTTTCGGAACTGAGGC-3' and 5'-GTC GGCATCGTTTATGGTCG-3' (20).

Immunoblotting assay. Western blot assays were performed as previously described (18). Following treatment with or without P4 and/or diverse pathway inhibitors, the growth-arrested cells were lysed with 500 μl ice-cold lysis buffer (50 mM HEPES, 5 mM EDTA, 50 mM sodium chloride, pH 7.4), 1% Triton X-100, protease inhibitors (10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin) and phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate). Cell lysates (30 μg) were separated using SDS-PAGE and transferred to nitrocellulose membranes, blocked for 1 h in TBS buffer containing 5% non-fat dry milk and 0.1% Tween-20 and incubated overnight with primary antibodies at proper dilutions. Following incubation with secondary antibodies, proteins of interest were detected by ECL chemiluminescence. Image J (http://rsb.info.nih.gov/ij/) was used for image analysis and quantitative data were normalized with the reference proteins (i.e., GAPDH or α-tubulin) or calculated as ratios of phosphor protein/total protein when the reference proteins were the same.

Localization of P4-BSA-FITC binding sites. Cells were cultured in chamber slides and exposed to 100 nM P4-3-(o-carboxymethyl) oxime-BSA-FITC (P4-BSA-FITC) for 30 min in serum-depleted medium. Cells were then washed with PBS buffer, fixed with 10% buffered formalin, counterstained with DAPI and observed under a confocal microscope (Olympus FV1000, Tokyo, Japan) using an oil objective lens (x60).

Wound closure migration assay. Cells ($5x10^5$/well) were seeded in a 24-well plate, cultured to reach confluence and then scraped with a sterile micropipette tip to create a denuded zone (gap) with a constant width (W_0). After removing cell debris with repeated PBS rinses, fresh serum-free DMEM medium with or without P4 (30 ng/ml) and/or other testing reagents was added. Anti-mPRα antibody (1:200) and/or anti-mPRα blocking peptide (1:100) was added 2 h prior to P4 treatment. PP1 (10 μM) was added 1 h prior to P4 treatment. The cells migrated at various speeds toward the middle axis from both edges of the scraped gaps, depending on the treatment of the aforementioned testing reagents, when they were incubated continually for 16 h. Following incubation, the width of the gap (T_{16h}) was measured by Image J. The rate of wound closure (WC) was calculated by the following equation: $WC = 1 - (W_{16h} / W_0) \times 100\%$ (21); regarding control cells, migration inhibiting rate of treated cells (MIR) = $1 - (WC_{treatmen}/WC_{control}) \times 100\%$.

Invasion assay. Cell invasion was assayed using the BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences, MD, USA) (22). Cells ($4x10^4$ cells/well) were seeded in the upper chamber of a 24-well BD transwell coated with Matrigel and cultured with DMEM medium containing 1% FBS. Following treatment with P4 at 30 ng/ml for 24 h with or without PP1 treatment at 10 μM for 1 h, the complete medium was applied to the lower chamber as chemoattractant. Cells were then incubated for an additional 16 h and the cells in the upper surface

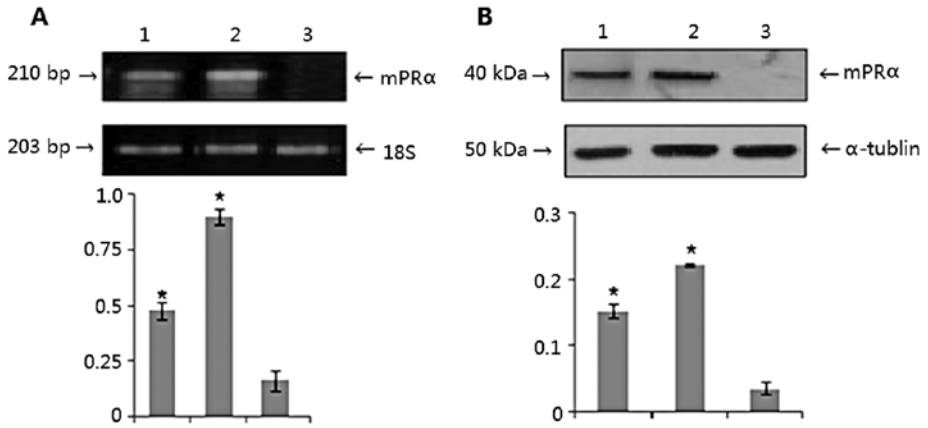

Figure 1. Expression of mPRα mRNA and protein in A549 cells. Lane 1, A549 cells; lane 2, MB231 w/mPRα cells; lane 3, MB231 cells. Reference, 18S. *P<0.05.

of the chamber membrane were then carefully removed with a cotton swab. Cells that invaded to the lower surface of the membrane were fixed with 10% buffered formalin and stained with hematoxylin solution. The number of invaded cells (IC) from 20 random microscopic fields (magnification, x200) was counted. Invasion inhibition rate (IIR) was calculated as follow: IIR = 1 - ($IC_{treatment}/IC_{control}$) x 100%.

Statistical analysis. Data are expressed as the means ± standard error (SE) and statistical differences between mean values were determined by the Student's paired two-tailed t-test, followed by the Fisher's protected least significance difference (PLSD). P<0.05 was considered to indicate statistically significant differences.

Results

mPRα expression in lung adenocarcinoma A549 cells. We set 18-S mRNA and α-tublin as internal references, and breast cancer MB231 and MB231 w/mPRα cells as negative and positive control. We found that expression of mPRα was positive both at the transcriptional and translational levels in lung adenocarcinoma A549 cells. As shown in Fig. 1A, the designated PCR band for mPRα in A549 cells was clearly observed at a moderate level (lane 1), distinct bands were found for the mPRα cDNA transfected MB231 w/mPRα cells (lane 2), however no band for parental MB231 cells was identified (lane 3; P<0.05). Using cell lysates isolated from those cells, an identical pattern of mPRα protein expression was documented by western blot assays (Fig. 1B) (P<0.05).

Positioning of mPRα on A549 cells and its binding characteristics. To determine if the mPRα protein is translocated to the membrane compartment of A549 cells, we performed *in vitro* binding tests using a cell-impermeable P4 conjugate (P4-BSA-FITC). After a 30-min incubation, we observed clear fluorescent signals in the membrane of A549 cells (Fig. 2, white arrows). Similar fluorescent signals were also found in the membrane of MB231 w/mPRα cells, but not in parent MB231 cells. To further demonstrate the binding specificity, we co-incubated A549 cells with P4-BSA-FITC conjugate and excessive un-conjugated free P4. As shown in Fig. 2, no fluorescent signals were observed in A549 cells.

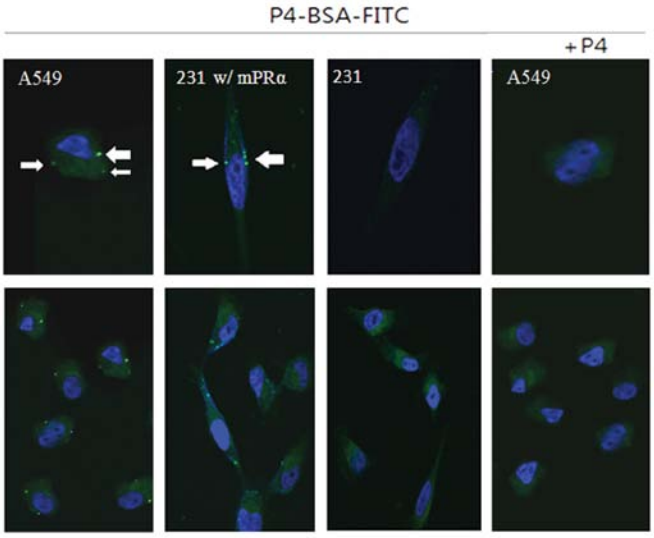

Figure 2. Positioning of mPRα on A549 cells and its binding characteristics. Upper lane images are typical cells from lower whole view field.

Cell migration of A549 cells in response to treatment of P4 and/or PP1. Further experiments were performed to determine the effect of P4 treatment on cell migration. Using a WC assay, we found that the WC of A549 cells was slower, although it was only marginally significant, when the cells were treated with P4 (30 ng/ml) for 16 h as compared to the cells without P4 treatment (45.4±1.8 vs. 47.3±1.7%, MIR 4.0%, P_{WC}=0.51). The WC rate for cells treated by PP1 alone was minimally inhibited (45.6±0.7 vs. 47.3±1.7%, MIR 3.4%, P_{WC}=0.63), which was comparable to cells treated with P4 (P4 vs. PP1 P=0.46). Co-incubation with P4 and PP1 resulted in a WC rate that was significantly slower in A549 cells, compared to control (23.0±1.1 vs. 47.3±1.7%, MIR 51.3%, P_{WC}<0.001) or to P4 or PP1 treatment alone (P_{WC}=0.02 and 0.02). These results indicated a synchronous inhibitory effect of P4+PP1 in the cell migration of mPRα+A549 cells (Fig. 3A).

In order to further clarify the role of P4→mPRα signaling in cell migration, we pre-incubated A549 cells with anti-mPRα antibody to block the binding of P4 to mPRα receptor 1 h prior to P4+PP1 treatment. The inhibitory effects of P4+PP1 on cell migration were abrogated (WC 45.8±1.9 vs. 46.5±2.2%,

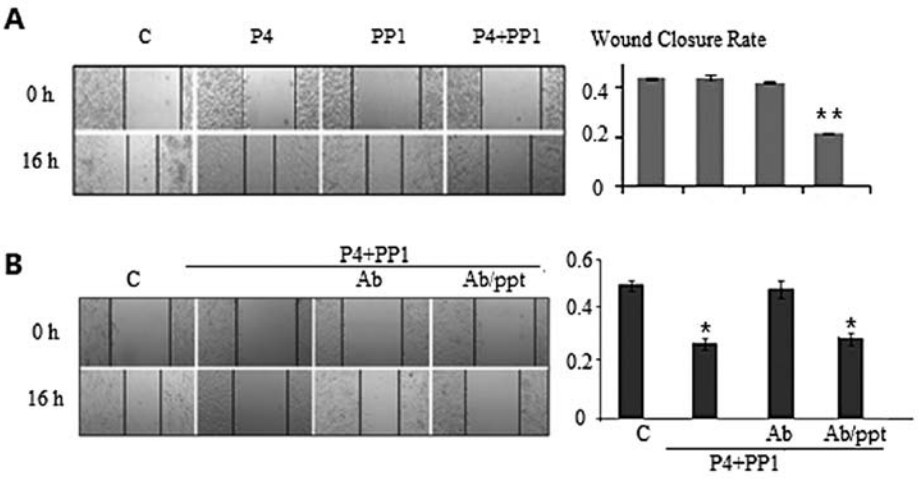

Figure 3. mPRα mediates cell migration inhibition of A549 cells in response to treatment of P4 and/or PP1. *P<0.05; **P<0.001.

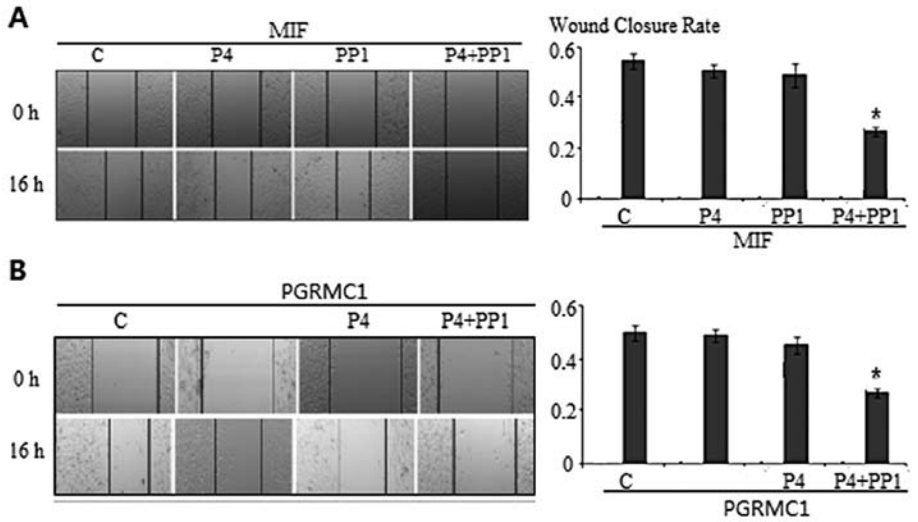

Figure 4. Neither nPR nor PGRMC1 plays a key role in the mediation of P4+PP1 inhibitory effects on cell migration of A549 cells. *P<0.05.

MIR 1.5%, P_{WC}=0.82), indicating the mPR receptor plays a key role in P4+PP1-induced cell migratory inhibition. When the cells were pre-incubated with anti-mPRα antibody and excess anti-mPRα blocking peptide, the inhibitory effects of P4+PP1 on the cell migration of A549 cells were restored (25.8±1.4 vs. 46.5±2.2%, MIR 44.5%, P_{WC}<0.001) (Fig. 3B).

Neither nPR nor PGRMC1 plays a key role in the mediation of P4+PP1 inhibitory effects on the cell migration of A549 cells. The expression of PR in PR negative cancer cells may be induced by P4 treatment, although the extent of induction is very low. To clarify if induction of endogenous PR has a role in P4-induced cell migration inhibition, we pre-incubated A549 cells with MIF, an nPR antagonist, prior to P4 and/or PP1 treatment. WC rates were not affected (P4 vs. MIF+P4, PP1 vs. MIF+PP1, P4+PP1 vs. MIF+P4+PP1 were 47.4±0.2 vs. 46.0±1.4%, 45.6±0.6 vs. 44.9±2.8% and 23.0±0.2 vs. 24.0±1.1%, respectively, all P_{WC} values >0.05) (Fig. 4A).

In addition to mPRα, PGRMC1 has been implicated in membrane-initiated progesterone signaling. It is unclear whether mPRα functions alone or if it requires PGRMC1. We then pre-incubated A549 cells with PGRMC1 antibody to block or interfere with the function of PGRMC1 receptor 1 h prior to P4 and/or PP1 treatment. The WC rates, in the presence of anti-PGRMC1 antibody, demonstrated no change on the cell migration pattern as compared to those induced by P4+PP1 (WC 26.0±0.1 vs. 47.4±1.3%, P=0.02). Treatment of anti-PGRMC1 antibody alone had no effect on cell migration (47.0±1.1 vs. 47.4±1.3%, P=0.78) (Fig. 4B).

Cell invasion of A549 cells in response to treatment of P4 and/or PP1. As cancer invasion *in vivo* is a three dimensional process involving transendothelial migration and penetration through extracellular matrix, we considered that a 3D cell invasion model would further delineate the role of P4 and/or PP1 on the metastatic potential of A549 cells. To confirm the role of P4 and PP1 on the cell migration of A549 cells, a cell invasion assay was performed. Following P4 and/or PP1 treatment for 16 h, the number of cells that invaded into the lower chamber of Matrigel (IC) was decreased as compared to control (53±2 vs.

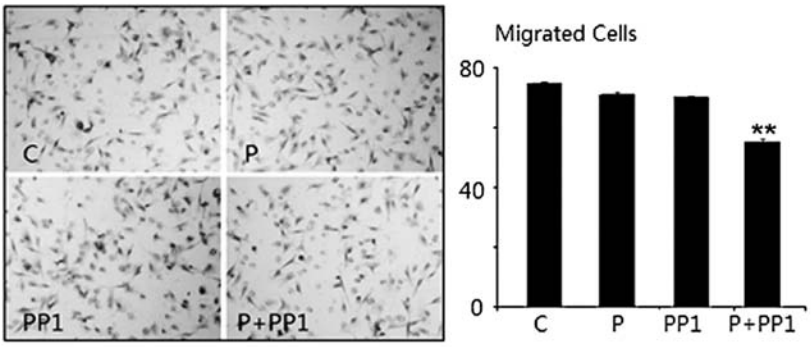

Figure 5. Cell invasion of A549 cells in response to treatment of P4 and/or PP1. **P<0.001.

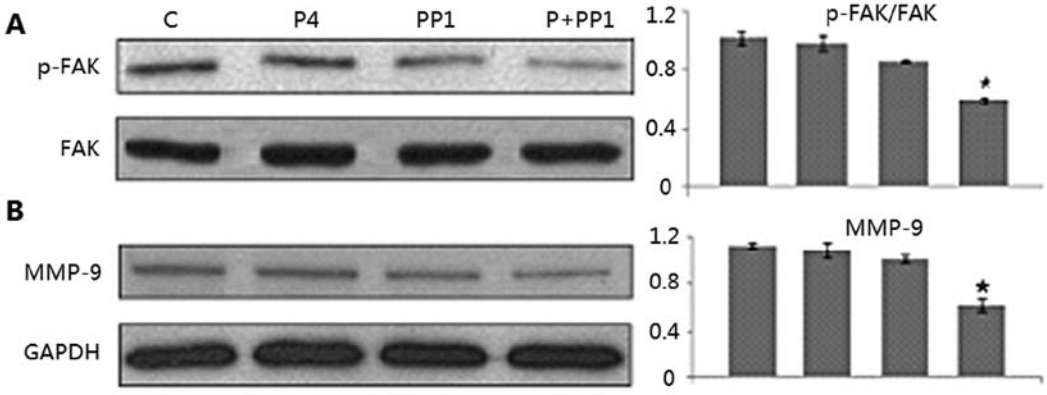

Figure 6. Molecular pathways involved in the P4+PP1-induced cell migration inhibition of A549 cells. *P<0.05.

78±1 cells, IIR 32.1%, P<0.001), but treatment with either P4 or PP1 alone was ineffective (76±2 and 74±3 cells, IIR were 3.2 and 7.2%, P_{IC} values were 0.83 and 0.92) (Fig. 5).

Molecular pathways involved in the P4+PP1-induced cell migration inhibition of A549 cells. Based on the results of the cell migration assays, a synergistic effect of P4 and PP1 on cell migration and invasion of A549 cells was suggested. Moreover, P4 has been reported to signal via Src family kinases for the formation of focal adhesion complex via focal adhesion kinase (FAK, a key component for tumor metastasis) phosphorylation at Tyr (397). To confirm the molecular mechanisms underlying this action, we evaluated the phosphorylation of FAK using western blot assay. It was found that the level of phosphor-FAK in A549 cells was significantly inhibited by P4+PP1 treatment (54.2 vs. 100%, P=0.01), but not by P4 or PP1 treatment alone (97.3, 88.9 vs. 100%, all P-values >0.05). We also investigated the effect of P4 and/or PP1 on the expression of other selected cancer metastasis relevant proteins such as MMP-9. The expression levels of MMP-9 (58.3 vs. 100%, P=0.01) were markedly reduced by the P4+PP1 combination treatment in A549 cells, but again not by P4 or PP1 individual treatments as compared to controls (all P-values >0.05) (Fig. 6).

Discussion

mPRα expression and positioning in the lung adenocarcinoma A549 cell line. In 1985, Beattie *et al* reported that hormone receptors were significantly higher in lung tissue than in normal tissue and proposed that lung cancer is a hormone-dependent tumor (23). Several studies have shown that estrogen and progesterone receptors are expressed in normal lung tissue, lung cancer and paraneoplastic tissues, as well as in lung cancer cell lines. However, nPR expression rates in lung cancer fluctuated in various laboratories, its association between pathological characteristics, clinical stage, lymph node metastasis or clinical characteristics did not reach a unanimous conclusion (24-26). mPRα is a new protein found in cancer and has not yet been widely investigated in lung cancer. Thomas *et al* also detected cell surface positioning of mPRα using antibodies against the extracellular amino-terminal of mPRα in mPRα-transfected MB231 cells; subsequently, flow cytometry and immunofluorescence staining showed the same cell membrane location (27). Our study is the first to detect mPRα mRNA and protein expressions in the lung adenocarcinoma A549 cells. Using the progestin binding experiment, we detected clear fluorescent signals from P4-BSA-FITC in the cell membrane of A549 cells, but not in the cytoplasm and nucleus. This suggests that the mPRα is positioned on the cell membrane of A549 cells and that mPRα is combined with progesterone.

In order to further explore the binding of mPRα with progesterone, we co-cultured P4-BSA-FITC with excessive free P4 in A549 cells, considering that excessive free P4 can replace P4-BSA-FITC to bind with P4. At this time, no green fluorescent signals were detected in the cell membrane, cytoplasm or

nucleus of A549 cells, suggesting that the original cell binding P4-BSA-FITC was competitively replaced by excessive free P4 and eluted. These results confirmed the specific binding of mPRα with P4-BSA-FITC in the membrane. Therefore, the fluorescence signal in the cell membrane is neither non-specific fluorescence, nor is it due to binding with BSA; it is the specific binding of P4 with mPRα occurring in the cell surface. Pang and Thomas used an mPRα small interfering RNA (mPRα siRNA) to interfere with mPRα expression in MB468 cells and found reduced radioactive [^{3}H]-labeled progesterone binding to the cell membrane by a laser microscope (28). These studies further support the specific binding of mPRα and P4 in the plasma membrane, instead of combined with progesterone nuclear receptor or other steroid hormone receptors, which is consistent with our study. However, Krietsch *et al* reported that recombinant mPRα of several vertebrate species was not present on the plasma membranes of transfected cells, but was localized in the endoplasmic reticulum, which is inconsistent with our findings (29). Foster *et al* later confirmed the membrane localization of mPRα using immuno-gold transmission electron microscopy. Stimulation of M11 cells with P4 (100 nM) resulted in internalization of mPRα from the plasma membrane to the cytoplasm in 10 min and subsequent partial translocation back to the cell surface in 20 min using RT-PCR, immunofluorescence and immuno-gold electron transmission microscopy (30). This internalization and recycling of mPRα may provide an explanation of mPRα inside the cell plasma. The accurate positioning of mPRα requires further exploration.

mPRα and its role in P4+PP1-enhanced cell migration and invasion inhibition. Cell migration plays a vital role in several biological processes, such as immune response, wound healing, embryogenesis and cancer metastasis. During cell migration, a series of cellular events, such as substrate sensing, adhesion formation, dynamic cytoskeletal reorganization and cell membrane rearrangements, occur in a strictly regulated manner (31). Limited knowledge, however, is available on the mechanisms by which P4 modulates cancer cell migration and invasion. Our study demonstrated that P4 inhibited, rather than enhanced, cell migration of mPRα-positive A549 cells slightly, and, notably, when co-incubating with P4+PP1, cell migration was inhibited significantly. Since PP1 treatment alone inhibited cell migration only at a moderate level which was comparable to P4, we hypothesized that combination treatment with both can synchronize the molecular signal magnitude and vigorously inhibit cell migration *in vitro*. Similarly, synchronizing results were also obtained from assays in which cell invasion was inhibited by P4+PP1, but not by P4 or PP1 treatment alone.

The role of P4 in cancer development has attracted substantial interest, but the mechanisms remain controversial. It is believed that the physiological action of P4 is mediated through either nuclear PR or membrane-bound receptors. A549 cells were reported to be nPR positive in previous research (32). To exclude the possibility that nPR could mediate the effect of P4 in A549 cells, we applied a pre-incubation of MIF, a P4 antagonist. This procedure had no impact on the effects of P4 and/or PP1 on A549 cell migration, indicating nPR is not involved in inhibiting cell migration. Additionally, 2 h prior to P4 treatment, the addition of specificity mPRα antibodies eliminated inhibition of A549 cell migration; adding a further specific binding mPRα antibody blocking peptide could restore P4+PP1's synergetic inhibition of the migration of lung adenocarcinoma A549 cells. PGRMC1 is required for some aspects of P4 signaling in estrogen receptor-negative breast tumors through an unidentified mechanism (33,34). In this study, we demonstrated that the cell migration patterns were not affected by incubating A549 cells with P4 and/or PP1 in the presence or absence of anti-PGRMC1 antibody, which suggested that PGRMC1 and its signaling pathways are not involved in the role of P4 and PP1 on cell migration. These data indicated that mPRα served as a key mediator of P4 in regulating migration of A549 lung cancer cells.

Molecular pathways involved in P4+PP1/mPRα signaling. Src has been reported to be a starting point for a number of biochemical cascades and exerts a profound effect on focal adhesion systems and cytoskeleton reorganization, thereby influencing cancer cell migration and invasion as well as other tumor progression-related events such as EMT (35). PP1 has been identified as a powerful inhibitor of Src family members, which binds to the ATP domain of Src and does not affect Src expression (36,37). PP1 inhibits Src-mediated tumor cell migration, invasion and metastasis. However, Src inhibitor alone does not appear to achieve therapeutic effects in clinical trials. Finn *et al* demonstrated that the Src family inhibitor dasatinib alone showed only limited anticancer effects in a phase II clinical trial of triple negative breast cancer patients (38); combination with chemotherapy medication may improve the therapeutic effects (39). Our results also suggested that P4+PP1 combined treatment significantly inhibited the migration and invasion of lung adenocarcinoma A549 cells; P4 or PP1 alone showed limited effects. Therefore, we consider that P4 and PP1 collaborate and synergistically expand molecular signaling cascade in the inhibition of lung adenocarcinoma A549 cell migration and invasion.

FAK is a downstream signaling component to control cell motility. Through multifaceted and diverse molecular connections, FAK regulates cell movement by influencing the cytoskeleton, structures of cell adhesion sites and membrane protrusions (40). FAK is highly expressed in lung cancer. All metastatic tumor tissues were found with high levels of FAK expression (41,42). Further research is being performed to investigate PR-mediated P4 impact on FAK. Hsu *et al* found PR can mediate P4's rapid nongenomic effect to inhibit Src/FAK phosphorylation in mice aortic smooth muscle cells, strengthen RhoA degradation, thus inhibiting the migration of smooth muscle cells (43). In this A549 cell model, which is depleted of nPR but has expressed mPRα, P4 and PP1 treatment alone affected the status of FAK minimally; however, combination treatment with both induced significant dephosphorylation of FAK. These results indicated that individual treatment with P4 or PP1 might not be powerful enough to inhibit cell migration and inactivate FAK, and combination treatment with both is essential for FAK inactivation.

Matrix metalloproteinases (MMPs) have been implicated in several aspects of tumor progression, such as invasion through basement membrane and interstitial matrices, angiogenesis and tumor cell growth. Expression of MMP-9 is strictly monitored in physical conditions. In malignant cells,

the balance is destroyed which results in elevated expression of MMP-9 and enhanced metastatic abilities. Studies showed that MMP-9 is highly expressed in NSCLC and serves as a pivotal step in the process of cancer metastasis (44-46). Hung *et al* found that Skp2 stable transfectants from A549 cells exhibited increased migratory and invasive abilities by upregulated expression of MMP-9 (47). By contrast, Xu *et al* demonstrated that osthole suppresses migration and invasion of A549 human lung cancer cells through inhibition of MMP-9 (48). In the present study, we found that in response to P4 or PP1 treatment alone, the expression of MMP-9 in A549 cells exhibited minimal changes; however, treatment with both induced significant reduction in MMP-9 expression, a similar pattern as that of FAK dephosphorylation, supporting this pro-metastatic protein as the downstream effector of P4→Src/FAK pathway mediated by mPRα.

In conclusion, our study first detected the expression and positioning of mPRα in the cell membrane of lung adenocarcinoma A549 cells. We also identified an mPRα-mediated pathway that involves Src/FAK and a downstream cell signaling component MMP-9. This cascade of molecular pathways can be inhibited by the concurrent use of P4 and PP1. Our results provide insight into the combinational use of an Src inhibitor and hormone agonist for the treatment of lung cancer and metastatic lung adenocarcinoma in particular.

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