Progesterone suppresses triple-negative breast cancer growth and metastasis to the brain via membrane progesterone receptor α

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Abstract. Progesterone plays an important role in mammary epithelial cell proliferation and differentiation. Evidence from experimental and clinical studies indicates that progesterone is a risk factor for breast cancer under certain conditions through binding nuclear progesterone receptor (PR). These mechanisms, however, are not applicable to triple-negative breast cancer (TNBC) due to the lack of PR in these cancers. In this study, we demonstrate that membrane progesterone receptor α (mPRα) is expressed in TNBC tissues and the expression level of mPRα is negatively associated with the TNM stage. We found that progesterone suppressed the growth, migration and invasion of mPRα$^+$ human TNBC cells *in vitro*, which was neither mediated by PR nor by PR membrane component 1 (PGRMCl). Notably, these effects exerted by progesterone were significantly blocked by shRNA specific to mPRα. Moreover, the knockdown of mPRα expression impaired the inhibitory effects of progesterone on mPRα$^+$ tumor growth and metastasis *in vivo*. These data collectively indicate that progesterone suppresses TNCB growth and metastasis via mPRα, which provides evidence of the anti-neoplastic effects of progesterone-mPRα pathway in the treatment of human TNBC.

Introduction

Triple-negative breast cancer (TNBC), accounting for approximately 15-25% of all breast cancer cases, is characterized by the lack of estrogen receptor (ER$^-$), progesterone receptor (PR$^-$) and HER2 amplification (HER2$^-$) (1,2). Although systematic therapeutic approaches have reduced the mortality rate, TNBC is still associated with high rates of cancer recurrence, frequent metastasis to the brain and poor outcomes (2,3). Therefore, an enhanced understanding of the molecular pathways involved in the progression of TNBC may be helpful in the prevention of metastasis and the design of effective therapeutic strategies for this disease.

Progesterone plays an important role in mammary epithelial cell proliferation and differentiation (4). Studies using human breast cancer cell lines and patient tumor samples, as well as clinical studies have indicated that progesterone is a risk factor for breast cancer under certain conditions (5,6; and refs therein). Classically, the effects of progesterone on cancer cells are attributed to the binding of nuclear PR, the translocation of the progesterone/PR complex into the nucleus and the subsequent activation of target genes over the course of several hours (7,8). Breast cancer is a heterogeneous disease and several distinct subtypes exist, of which the triple-negative subtype has the most severe clinical prognosis (3,9,10). In TNBC, these mechanisms described above are not applicable due to the lack of PR in these cancers. Thus, the role of progesterone in the pathogenesis of TNBC remains controversial, and whether progesterone is a promoter or inhibitor of TNBC has not yet been fully elucidated.

During the past decade, the discovery of membrane progesterone receptor α (mPRα), unrelated to the classical PR, in fish and its subsequent identification in mammals, suggests a potential mediator of non-traditional progestin actions (11,12), particularly in tissues in which PR is absent. The broad distribution of mPRα mRNAs in reproductive and non-reproductive tissues suggests they have diverse physiological functions in vertebrates (13-17). Recently, changes have been observed in the mRNA expression of mPRα in malignant human breast tissues, and mPRα has been identified as an intermediary factor of the progestin-induced intracellular signaling cascades in the PR-breast cancer cell lines *in vitro* (15,18,19). However, the function and molecular mechanisms of action of mPRα in mediating the effects of progesterone on TNBC cells remain unknown.

In the present study, we demonstrate that mPRα is expressed in TNBC tissues and that the expression level of mPRα is negatively associated the TNM stage. Progesterone suppressed the growth, migration and invasion of mPRα$^+$ human

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TNBC cells. Notably, the inhibitory effects of progesterone on mPRα$^+$ human TNBC cells were not mediated by PR or by PR membrane component 1 (PGRMCl). Moreover, the knockdown of mPRα expression impaired the inhibitory effects of progesterone on mPRα$^+$ tumor growth and metastasis *in vivo*. Our data collectively indicate that progesterone suppresses TNBC growth and metastasis to the brain via mPRα, which provides evidence of the anti-neoplastic effects of the progesterone-mPRα pathway in the treatment of human TNBC.

Materials and methods

Cell lines and cancer tissues. Two human TNBC cell lines [MDA-MB-231 (designated as MB231) and MDA-MB-231-BR (brain-seeking cells; designated as MB231br); Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China] were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37˚C with 5% CO_2 in a humidified incubator. Primary TNBC tissues and matched non-tumour tissues were collected from 55 patients with TNBC undergoing modified radical mastectomy between 2005-2010 at the Department of Oncology, Shanghai Seventh People's Hospital, Shanghai, China. All the tissue samples were collected after obtaining patient informed consent and ethics approval (this study was approved by the Institutional Review Board of Shanghai Seventh People's Hospital; approval no. 2015011602) and confirmed by pathological examination.

Immunohistochemistry. Briefly, the tissue sections (4-μm-thick) were deparaffinized in xylene and rehydrated in graded ethanol. Following antigen retrieval, endogenous peroxidase quenching and blocking with 10% normal goat serum, the samples were incubated with primary anti-mPRα antibody (ab75508; Abcam, Cambridge, MA, USA) at 4˚C overnight and then incubated with a secondary antibody (DakoCytomation, Glostrup, Denmark). The immunostained slides were counterstained with hematoxylin. In each experiment, a negative control was included in which the primary antibody was omitting. Staining was scored by a trained research pathologist who was blinded to patient clinical data.

Plasmid construction and transfection. The full-length cDNA of mPRα was amplified using the following primers: 5'-CAT GGCGACGGTGGTGATG-3' (forward) and 5'-GGCA GCAGAAGAAATAGGCG-3' (reverse), and then subcloned into the vector, pIRES2-EGFP (BD Biosciences Clontech). The sequence for the construction of mPRα-shRNA was sense, 5'-GGAGCTGTAAGGTCTTCTTTA-3'; and antisense, 5'-TAAAGAAGACCTTACAGCTCC-3', and then subcloned into the vector, pSUPER (GenerayBiotech Co., Ltd., Shanghai, China). Sequence fidelity and reading frame accuracy of the mPRα expression or mPRα-shRNA plasmid were achieved by DNA sequencing analysis. The MB231 or MB231br cells were transfected using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with the mPRα expression plasmid or mPRα-shRNA plasmid, respectively. The pIRES2-EGFP vector or pSUPER vector was also transfected into the MB231 or MB231br cells which served as controls. The transfected cells were then cultured in medium supplemented with G418 (Promega, Madison, WI, USA) or puromycin (InvivoGen, San Diego, CA, USA) for the establishment of stably transfected cell clones.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and reverse transcribed using a reverse transcription kit (Promega) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed to determine the expression of mPRα using SYBR-Green PCR master mix. The primers for mPRα were as follows: sense, 5'-GTGGTGATGGAGCAGATTGGT-3' and antisense, 5'-TGCCAGGAGGACGATGAATAG-3'. The primers for GAPDH were as follows: sense, 5'-TTGGCATCG TTGAGGGTCT-3' and antisense, 5'-CAGTGGGAACACGG AAAGC-3'. The relative expression ratio of mPRα was calculated using the $2^{-\Delta\Delta Cq}$ method.

Western blot analysis. Cell lysates were prepared with RIPA (Beyotime Institute of Biotechnology, Haimen, China) and separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. Following incubation with blocking buffer (TBS + 0.1% Tween-20 + 5% not-fat milk), the membranes were probed with the primary antibody (anti-mPRα antibody; ab75508; Abcam) overnight at 4˚C. The membranes were then washed with TBST and incubated with an HRP-conjugated secondary antibody (horseradish peroxidase-labeled goat anti-rabbit IgG; A0208; Beyotime Institute of Biotechnology), and signals were detected using ECL reagent (Millipore, Billerica, MA, USA). mPRα, caspase-3, cleaved caspase-3 and GAPDH antibodies were from Abcam.

Cell proliferation assay. Cell proliferation was determined using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.). Briefly, $5x10^5$ cells were seeded in 24-well plates and treated with various concentration of progesterone (20 ng/ml, 40 ng/ml and 80 ng/ml; Sigma-Aldrich, St. Louis, MO, USA), RU486 (mifepristone; EMD Chemicals, Gibbstown, NJ, USA) or PGRMCl neutralizing antibody (H-46; sc-98680, Santa Cruz Biotechnology, Santa Cruz, CA, USA). CCK-8 solution was added to each well, and the absorbance at 450 nm was measured using an Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Cell migration and invasion assays. Cell migration assay was performed using Transwell chambers (8 μm, 24-well insert; Corning Inc., Corning, NY, USA). The cells were added to the upper chamber with serum-free medium and the medium of the lower chamber contained 10% FBS. Following incubation for 48 h, the migrated cells in the lower chamber were stained with 0.1% crystal violet (C0121; Beyotime Institute of Biotechnology). Invasion assays were completed under the same conditions using Transwell membranes coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA).

In vivo tumorigenesis and metastasis assays. The cells were collected and injected into the left ventricle of the hearts of 40 female athymic nude mice (SPF grade; 4-6 weeks old, weighing 18-22 g; Institute of Zoology, Chinese Academy of Sciences, Shanghai, China) subjected to oophorectomy using

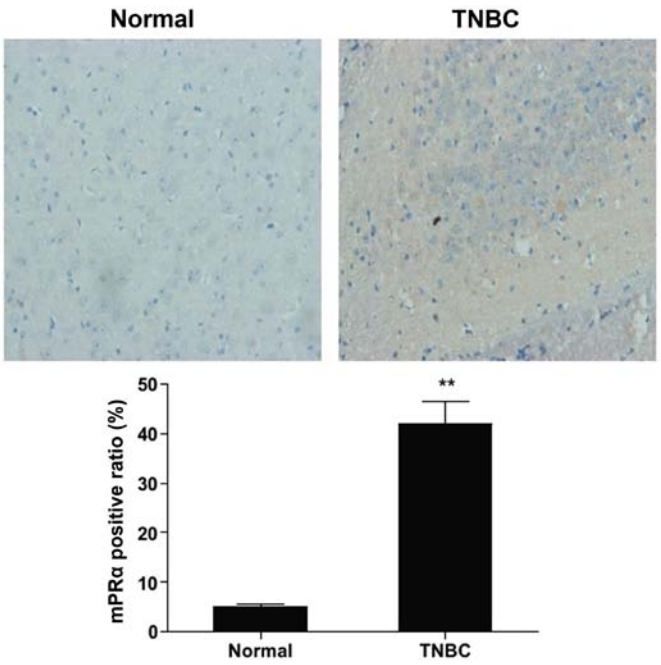

Figure 1. Membrane progesterone receptor α (mPRα) is overexpressed in triple-negative breast cancer (TNBC) tissues. The expression of mPRα was examined by immunohistochemical staining in TNBC tissues and adjacent normal breast tissues. Magnification, x200. The mPRα-positive ratio of mPRα in the TNBC tissues was significantly higher than that in the adjacent normal breast tissues (**P<0.01).

Table I. Correlation between the mPRα expression level and clinicopathological parameters in the 55 patients with TNBC.

	mPRα expression level		
Characteristics	Moderate	Strong	P-value
Age (years)			
≥50	12	13	
<50	16	14	>0.05
Menopausal status			
Pre-menopause	15	14	
Menopause	14	12	>0.05
Histological grade			
Grade I	11	7	
Grade II	8	10	>0.05
Grade III	9	10	
TNM stage			
I-II	18	20	
III-IV	7	10	<0.01

mPRα, membrane localized progestin receptor α; TNBC, triple-negative breast cancer.

standard procedures. The mice were sacrificed after 5 weeks and a CT scan was performed for the analysis of brain metastasis formation. The brains were then collected, fixed and embedded in 4% paraformaldehyde. Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay was carried out following the manufacturer's instructions of the DeadEnd™ Colorimetric TUNEL system kit (Promega). All animal experiments were carried out following the approval of the Institutional Review Board of Shanghai Seventh People's Hospital; approval no. 2015011601)

Statistical analysis. Data analyses were carried out using SPSS v17.0 software with the Student's t-test. The results are presented as the means ± SE. Statistical significance was determined at P<0.05.

Results

mPRα is overexpressed in TNBC tissues and is associated with clinicopathlogical characteristics. To evaluate the expression of mPRα in human TNBC tissues, we first determined the expression level of mPRα by immunohistochemistry. Typical immunostaining of mPRα in normal and TNBC specimens is shown in Fig. 1. The positive expression of mPRα was mainly observed in the cytoplasm and/or cell membrane. In the normal breast tissues, mPRα was detected at low levels; the ductal and alveolar epithelial cells were shown to be negative or weakly positive and the myoepithelial cells were shown to be moderately positive for mPRα. By contrast, all 55 TNBC tissues were stained moderately to strongly positive for mPRα antibody. The expression of mPRα tended to decrease with the increasing TNM stage (P<0.05), while no correlation was observed between mPRα expression and the patient age, menopausal state or histological grade (Table I).

Progesterone suppresses the growth of mPRα⁺ human TNBC cells. To determine whether progesterone affects the growth of TNBC cells with a different mPRα status, we first established stably transfected TNBC cell lines with different mPRα expression levels. The MB231 and MB231br cells are TNBC cells that do not express ER, PR and HER2; mPRα expression was detected at higher levels in the MB231br cells compared with the MB231 cells (Fig. 2A and B). Subsequently, mPRα full-length expression vector or mPRα-shRNA were transfected into the MB231 and MB231br cells, respectively. The overexpression or knockdown of mPRα in the MB231 or MB231br cells was confirmed by RT-qPCR (Fig. 2A) and western blot analysis (Fig. 2B). Cell proliferation was analyzed by CCK-8 assay in the presence of various concentrations of progesterone (20 ng/ml, 40 ng/ml and 80 ng/ml). As shown in Fig. 2C, the proliferation of the MB231br (MB231br-shNC) and MB231-mPRα (MB231 cells transfected with mPRα overexpression vector) cells, but not that of the MB231br-shmPRα (MB231br cells transfected with shRNA targeting mPRα) and MB231 (MB231-vector) cells was inhibited by progesterone treatment in a dose-dependent manner. We then analyzed cell apoptosis by examining the expression of cleaved caspase-3 by western blot analysis following treatment with progesterone. As shown in Fig. 2D, the expression of cleaved caspase-3 was upregulated in both the MB231br and MB231-mPRα cells, but not in the MB231br-shmPRα or MB231 cells following treatment with progesterone. Thus, these results indicate that progesterone suppresses the growth of mPRα⁺ TNBC cells by inhibiting cell proliferation and inducing cell apoptosis.

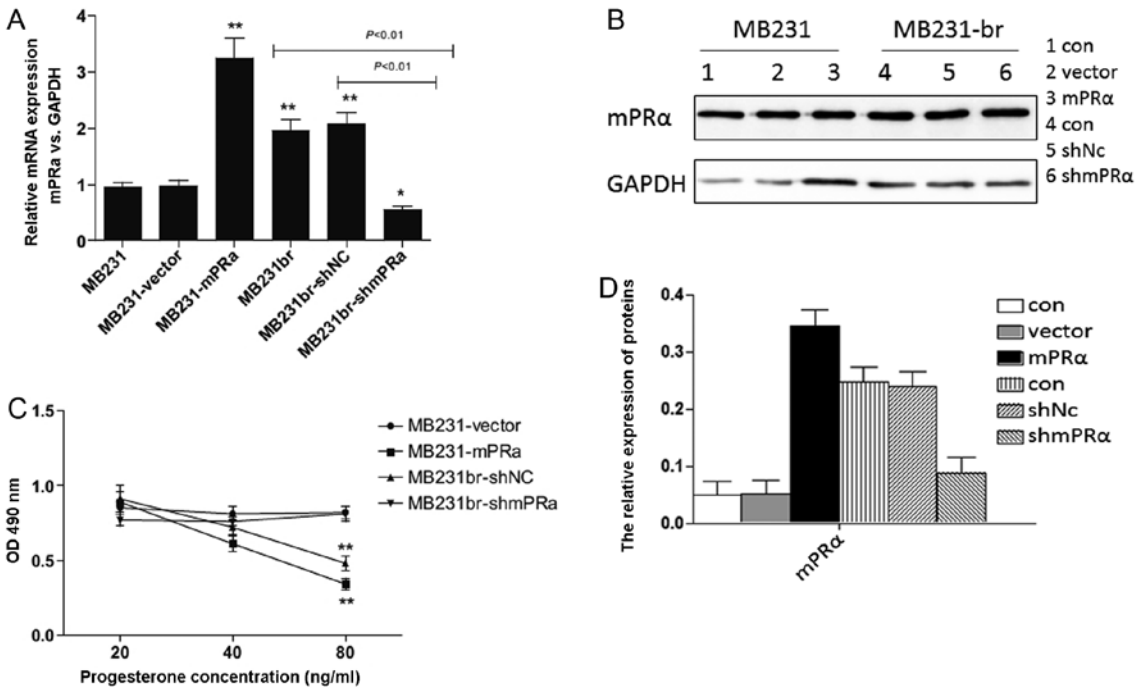

Figure 2. Progesterone suppresses the growth of membrane progesterone receptor α (mPRα$^+$) human triple-negative breast cancer (TNBC) cells. (A) The mRNA expression levels of mPRα in TNBC cells were detected by RT-qPCR following transfection with mPRα full-length expression vector or mPRα-shRNA. *P<0.05, **P<0.01. (B) The protein levels of mPRα in breast cancer cells were analyzed by western blot analysis following transfection with mPRα full-length expression vector or mPRα-shRNA. GAPDH protein was used as the loading control. (C) Proliferation of TNBC cells with different mPRα expression status was evaluated by CCK-8 assay following treatment with indicated concentrations of progesterone. Data are shown as the means ± SD that represents as least 3 independent experiments. *P<0.05. (D) The expression of cleaved caspase-3 in TNBC cells with different mPRα expression status was analyzed by western blot analysis following treatment with 40 ng/ml progesterone for 48 h. The mPRα-positive ratio of mPRα in TNBC tissues was significantly higher than that in the adjacent normal breast tissues (**P<0.01).

Progesterone suppresses the migration and invasion of mPRα$^+$ human TNBC cells. We then examined the effects of progesterone on the migration and invasion of TNBC cells with a different mPRα expression status. As shown in Fig. 3A, following treatment with progesterone, the MB231br cells (untreated group, 168±29 cells/field vs. treated group 95±12 cells/field, P<0.05), but not the MB231 cells (untreated group, 97±19 cells/field vs. treated group 89±12 cells/field) exhibited decreased migration. This inhibition was blocked by the knockdown of mPRα expression in the MB231br cells (untreated group, 166±25 cells/field vs. treated MB231br-shmPRα group, 176±30 cells/field) (Fig. 3A). Moreover, the introduction of exogenous mPRα cDNA into the MB231 cells enhanced the responsiveness of the cells to progesterone treatment, decreasing migration (untreated group, 103±22 cells/field vs. treated MB231-mPRα group 62±14 cells/field) (Fig. 3A). Similarly, in the presence of progesterone, the MB231br cells (untreated group, 95±16 cells/field vs. treated group 58±10 cells/field, P<0.05) and MB231-mPRα cells (untreated group, 74±14 cells/field vs. treated group 42±9 cells/field, P<0.05), but not the MB231 (untreated group, 72±12 cells/field vs. treated group 68±15 cells/field) or MB231br-shmPRα cells (untreated group, 88±12 cells/field vs. treated group 93±9 cells/field) exhibited a decreased invasion (Fig. 3B). Thus, these results indicate that progesterone suppresses the migration and invasion of mPRα$^+$ TNBC cells.

The inhibitory effects of progesterone in mPRPα$^+$ human TNBC cells are not mediated by either PR or PGRMCl. Although the MB231br and MB231 cells are basically negative for nuclear PR expression, it has been reported that cancer cells may repress PR in response to sex hormone treatments (20). In this study, in order to exclude the role of PR in the above-mentioned effects of progesterone on TNBC cells, the MB231br-shNC, MB231br-shmPRα, MB231-vector and MB231-mPRα cells were co-incubated with progesterone plus RU486 (mifepristone), a PR-specific blocker. As expected, RU486 had no effects on the inhibitory effects of progesterone on cell proliferation, migration and invasion (Fig. 4A, B and C). PGRMCl is the other type of progesterone membrane receptor that mediates non-classical progestins actions (21). In this study, in order to exclude the possible role of PGRMCl in the inhibitory effects of progesterone on TNBC cells, the MB231br cells and MB231-mPRα cells were then co-incubated with progesterone plus PGRMCl neutralizing antibody. As shown in Fig. 4, PGRMCl neutralizing antibody had no effects on the inhibitory effects of progesterone on cell proliferation, migration and invasion. Thus, these results suggested that the inhibitory effects of progesterone on mPRα$^+$ human TNBC cells are not mediated by either PR or PGRMCl.

Knockdown of mPRa expression impairs the inhibitory effects of progesterone on mPRα$^+$ tumor growth and metastasis in vivo. We further examined whether the knockdown of mPRα expression in TNBC cells can reverse the inhibitory effects of progesterone on tumor growth and metastasis *in vivo*. The MB231br-NC cells and MB231br-shmPRα cells were injected into the left ventricle of the hearts of athymic nude mice subjected to oophorectomy. Five weeks later, the

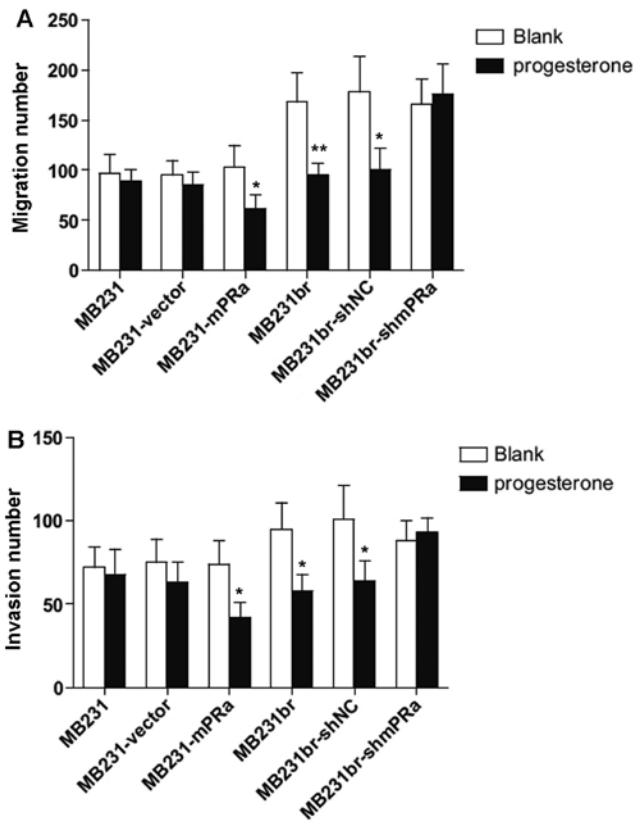

Figure 3. Progesterone suppresses migration and invasion of membrane progesterone receptor α (mPRα$^+$) in human triple-negative breast cancer (TNBC) cells. (A) Transwell migration assay of the effects of progesterone on cell migration. (B) Transwell invasion assay of the effects of progesterone on cell migration. Data are shown as the mean ± SD that represents as least 3 independent experiments. *P<0.05, **P< 0.01.

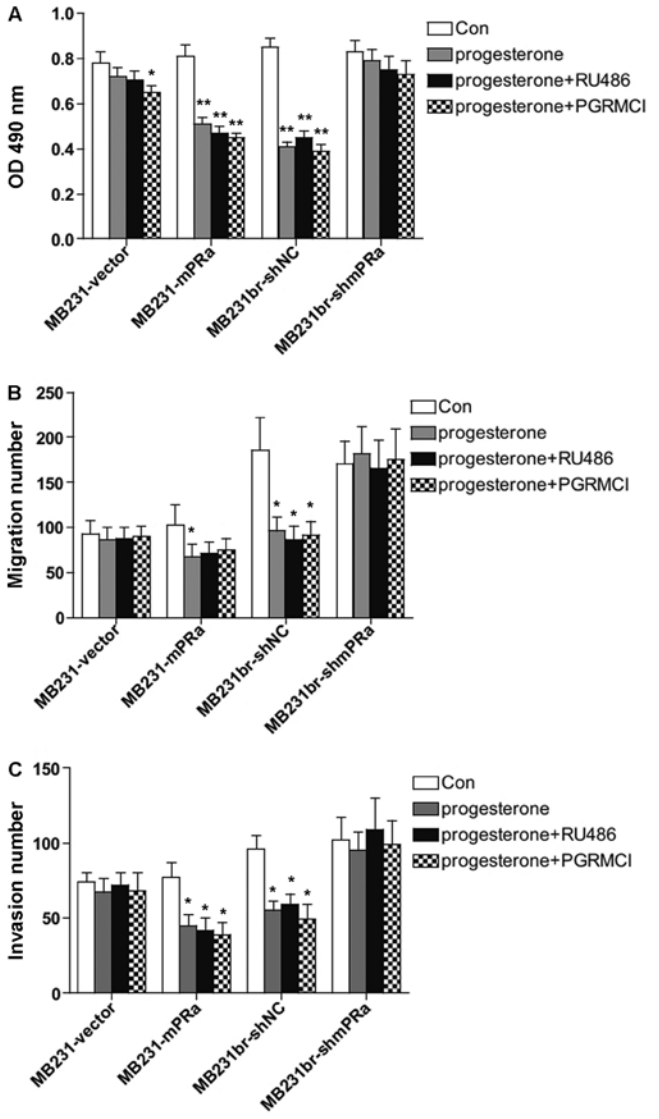

Figure 4. Inhibitory effects of progesterone on membrane progesterone receptor α (mPRα$^+$) in human triple-negative breast cancer (TNBC) cells are not mediated by either PR or by PR membrane component 1 (PGRMCl). (A) Proliferation of MB231br and MB231-mPRα was evaluated by CCK-8 assay following treatment with progesterone plus RU486 (mifepristone) or PGRMCl neutralizing antibody. (B and C) Inhibitory effects of progesterone on the migration and invasion of MB231br and MB231-mPRα. Data are the means ± SD that represents as least 3 independent experiments. *P<0.05 **P<0.01.

mice were euthanized, and H&E staining and a CT scan were then performed for the analysis of brain metastasis formation. As shown in Fig. 5A and B, the MB231br-NC cells and MB231br-shmPRα cells formed brain metastases in the nude mice with oophorectomy. However, following treatment with progesterone, the MB231br-NC cells, but not the MB231br-shmPRα cells, did not form brain metastases in the nude mice with oophorectomy. In addition, TUNEL assays of the brain tissues were performed. As shown in Fig. 5C, the MB231br-shmPRα cells exhibited less tumor cell-positive staining and a significantly lower apoptotic index than the MB231br-NC cells (P<0.01). These results suggested that the knockdown of mPRa expression impairs the inhibitory effects of progesterone on mPRα$^+$ tumor growth and metastasis *in vivo*.

Discussion

Progesterone plays an important role in mammary gland development in females and also appears to be involved in the development of breast cancer (5,6). There is evidence to indicate that progesterone promotes rodent mammary carcinogenesis under certain conditions, in which PR is necessary for murine mammary gland tumorigenesis (7,8). Breast cancer is a heterogeneous disease and several distinct subtypes exist, of which the triple-negative subtype has the worst clinical prognosis (9,10). However, the role of progesterone as either a promoter or inhibitor of TNBC that lacks the expression of PR has not yet been fully elucidated. In this study, we demonstrated that mPRα was overexpressed in human TNBC tissues and the expression level of mPRα was negatively associated with the TNM stage. We found that mPRα mediates the inhibitory effects of progesterone on TNBC cell growth, migration and invasion *in vitro*, as well as growth and metastasis *in vivo*. These results provide evidence of a novel mechanism mediated by the progesterone-mPRα axis in the development and progression of TNBC.

The mPRα receptor has been associated with manyphysiologic functions in vertebrates. It induces oocyte maturation, stimulates sperm hypermotility, modulates immune function, downregulates gonadotropin-releasing hormone (GnRH) secretion and adjusts human myometrial

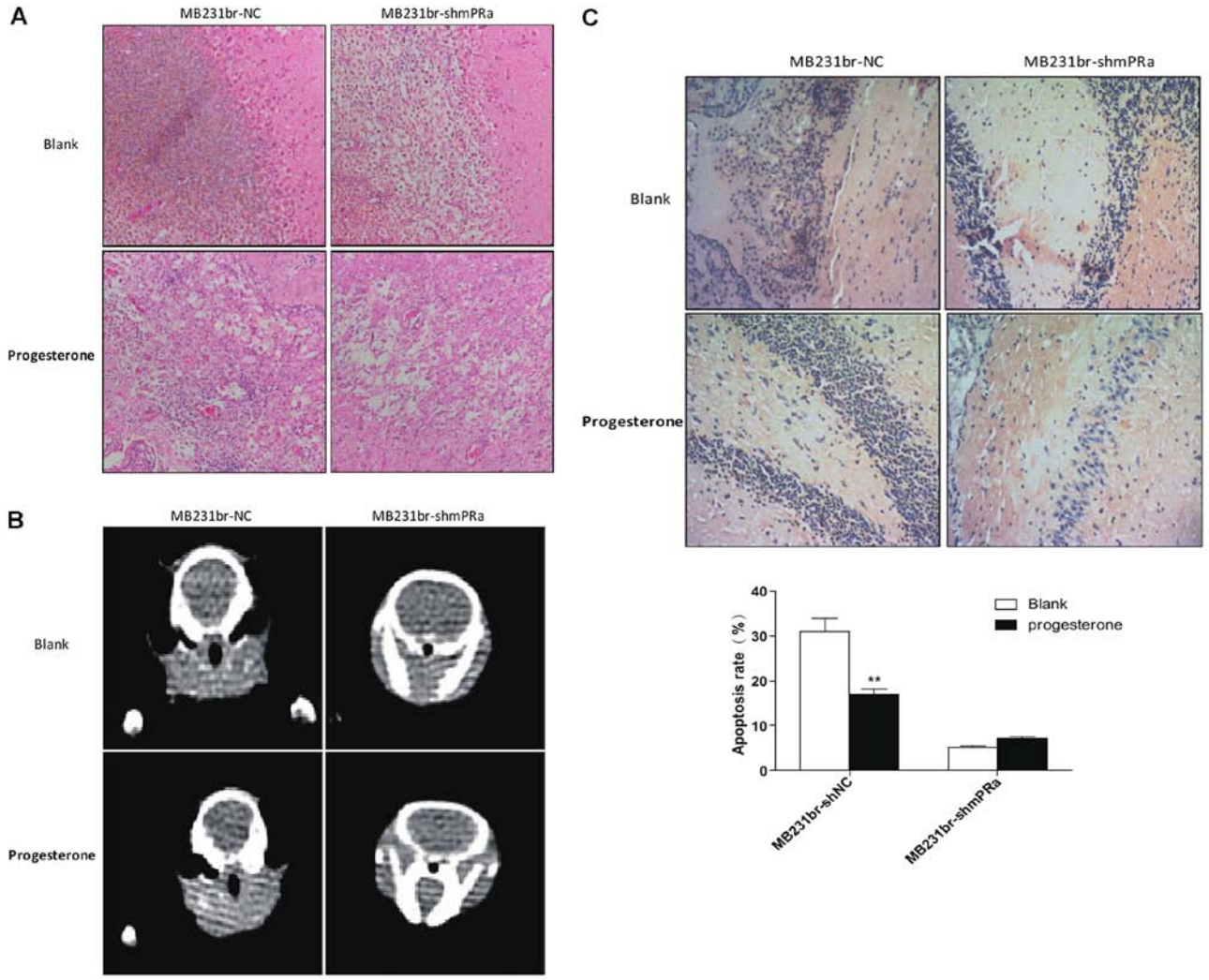

Figure 5. Knockdown of membrane progesterone receptor α (mPRα) expression impairs the inhibitory effects of progesterone on mPRα$^+$ tumor growth and metastasis *in vivo*. (A) MB231br-NC cells and MB231br-shmPRα cells were injected into the left ventricle of the heart ofathymic nude mice with oophorectomy. Five weeks later, the mice were euthanized and H&E staining was performed for the analysis of brain metastasis formation. (B) Five weeks later, mice were euthanized and a CT scan was performed for the analysis of brain metastasis formation. (C) TUNEL assays were performed in brain tissues from nude mice injected with MB231br-NC cells and MB231br-shmPRα cells and treated with progesterone. **P<0.01

cell contractility (11,17,21-23). mPRα was first identified in human breast cancer biopsies and epithelial-derived breast cancer cell lines by Dressing *et al* (18). Recently, it has been identified as an intermediary factor of the progestin-induced intracellular signaling cascades in PR$^-$ breast cancer cell lines *in vitro* (15,18,19). mPRα mediates epithelial-mesenchymal transition (EMT) through the activation of the PI3K/Akt pathway (19). In this study, the expression of mPRα was detected in both normal and malignant breast tissues and its expression level was negatively associated with the TNM stage of TNBC, which is consistent with previous results (18,19,24). However, knowledge of the aberrant expression and potential role of mPRα in TNBC remains largely unknown. In this study, we demonstrated that progesterone suppressed the growth, migration and invasion of human TNBC cells via mPRα *in vitro* and *in vivo*. Importantly, these effects induced by progesterone treatment were significantly blocked by transfection with mPRα-specific shRNA. Therefore, mPRα may contribute to cancer development, proliferation and metastasis in TNBC.

Classically, progesterone exerts its effects through the binding of nuclear PR and subsequently activates downstream pathways (7,8). A previous study demonstrated that cancer progenitor cells may proliferate and express PR in response to sex hormone treatments (6); thus, the classical nuclear PR was first considered as a molecular mediator of the progesterone's inhibitory effects on TNBC cells even though they are basically negative for nuclear PR expression in normal culture condition. PGRMCl is the other type of progesterone membrane receptor that mediated the non-classical progestins actions (21). To exclude the possible role of PR or PGRMCl in the inhibitory effects of progesterone on TNBC cells, we introduced RU486 or PGRMCl neutralizing antibody into the culture system; neither of these had an effect on the inhibitory effects of progesterone. Thus, our data demonstrate that the status of mPRα in TNBC cells play an essential role in determining the cell biological behavior of TNBC in responding to progesterone treatment. However, the detailed molecular mechanisms need to be further explored in the future.

In conclusion, our study has provided experimental evidence indicating a role for progesterone in inhibiting TNBC development and progression. Although the detailed mechanisms require further investigation, a strong link between mPRα expression and the effects of progesterone may provide a possible explanation as to how progesterone suppresses TNBC progression. Progesterone may play a duel role in breast cancer; therefore, a better understanding of the role of the progesterone-mPRα axis in various subtypes of breast cancer may help to provide insight into its complex function. In conclusion, our study indicates that progesterone suppresses TNBC cell growth and metastasis to the brain via mPRα, and may therefore serve as a potential target in the treatment of TNBC.

Acknowledgements

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