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Progesterone inhibits proliferation and modulates expression of proliferation—**R**elated genes in classical progesterone receptor-negative human BxPC3 pancreatic adenocarcinoma cells

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ABSTRACT

Recent studies suggest that progesterone may possess anti-tumorigenic properties. However, a growth-modulatory role of progestins in human cancer cells remains obscure. With the discovery of a new class of membrane progesterone receptors (mPRs) belonging to the progestin and adipoQ receptor gene family, it becomes important to study the effect of this hormone on proliferation of tumor cells that do not express classical nuclear progesterone receptors (nPRs). To identify a cell line expressing high levels of mPRs and lacking nPRs, we examined mRNA levels of nPRs and three forms of mPRs in sixteen human tumor cell lines of different origin. High expression of mPR mRNA has been found in pancreatic adenocarcinoma BxPC3 cells, while nPR mRNA has not been detected in these cells. Western blot analysis confirmed these findings at the protein level. We revealed specific binding of labeled progesterone in these cells with affinity constant similar to that of human mPR expressed in yeast cells. Progesterone at high concentration of 20 μM significantly reduced the mRNA levels of proliferation markers Ki67 and PCNA, as well as of cyclin D1, and increased the mRNA levels of cyclin dependent kinase inhibitors p21 and p27. Progesterone (1 μM and 20 μM) significantly inhibited proliferative activity of BxPC3 cells. These results point to anti-proliferative effects of the progesterone high concentrations on BxPC3 cells and suggest that activation of mPRs may mediate this action. Our data are a starting point for further investigations regarding the application of progesterone in pancreatic cancer.

1. Introduction

Emerging data indicate that progesterone has a number of diverse effects not only on the reproductive function of mammals, but also on the immune system, cardiomyocytes, vascular cells, brain, skin, bone, lung and other organs and tissues[1–9]. Progesterone may act through at least two types of receptors: classical nuclear receptors (nPRs) that exist in two major isoforms, nPR-A and nPR-B (arising from the same gene), and the so-called membrane progesterone receptors (mPRs). The nPRs are ligand-activated transcription factors that bind to progesterone with Kd of 1–5 nM and regulate expression of progesterone–dependent genes [10]. The mPRs were identified in 2003 [11] and were classified into three subtypes, mPRα, mPRβ, and mPRγ. They belong to the class II Progestin- and Adiponectin-Q receptor (PAQR) family—mPRα (encoded by the *PAQR7* gene), mPRβ (*PAQR8*), mPRγ (*PAQR5*). Human mPRα, mPRβ, mPRγ have progesterone-binding affinities with Kd varying from 4-8 nM [12] to 28-39 nM [11]. To date, the membrane receptors of other two subtypes of the same family (mPRδ, mPRε) were characterized as membrane progesterone receptors. These mPRs bind to progesterone with somewhat higher

Abbreviations: nPRs, nuclear progesterone receptors; mPRs, membrane progesterone receptors; PGRMC1, progesterone receptor membrane component 1; GR, glucocorticoid receptors; QPCR, quantitative real-time PCR; Kd, the equilibrium dissociation constant; RBA, relative binding affinity; MR, mineralocorticoid receptors; Ki67, marker of proliferation; PCNA, proliferating cell nuclear antigen; cyclin D1, a cyclin-dependent kinases activator; p21, a cyclin-dependent kinase inhibitor 1A; p27, a cyclin-dependent kinase inhibitor 1B.

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affinity (Kd approximately 3 nM) than that reported for the first three forms [13]. The members of another protein family, containing a heme/steroid-binding domain, are also nonclassical progesterone sensors. However, among them, only the progesterone receptor membrane component 1 (PGRMC1) was found to bind to progesterone with high affinity (Kd 11–35 nM), depending on the cell type [14]. There is the evidence that PGRMC1 can act as an adapter protein for mPRα, transporting it to the cell surface [15]. As a result, activation of various types of progesterone receptors and sensors triggers off crisscrossing of numerous signal transduction pathways, changes the gene expression profile, affects post-translational modification of proteins, regulates intracellular Ca^{2+} levels and ion fluxes [16].

All these changes determine the multidirectional effects of progesterone on differentiation, proliferation and cell death [1,5,7,8,16,17]. Since progesterone was reported to have antiproliferative and apoptotic effects on numerous cancer cell lines, it can be used in anticancer therapy. The participation of nPRs in the action of progesterone on proliferation has been actively studied for many years and was demonstrated on different objects [1,2,17,18]. The presence and composition of mPRs in tumor cells, as well as their role in carcinogenesis has practically not been studied. Progestins affect tumor cells without expression of nPRs [7,19–21]. It was shown, that low and high concentration of progesterone could demonstrate opposite effects on the same cells, possibly acting through different forms of PRs. Low concentrations showed a proliferative effect in glioblastoma (U87MG and U118MG) [22], neuroblastoma (SK-N-AS) [23], and melanoma (A375 and A875) [7] cell lines, while high concentrations decreased tumor cell viability in all these cell lines. Only high concentration of progesterone (10^{-4} mol/L) inhibited the survival of ovarian cancer cells after progesterone treatment [24]. It is suggested that high progesterone concentrations act through mPRs, having lower affinity for steroids. There are very few studies demonstrating the role of mPRs in the action of progesterone on cell proliferation, apoptosis, and the epithelial–mesenchymal transition (EMT) in tumor cells. This is the inhibition of the migration and invasion of cancer cells in A549 human lung adenocarcinoma cells [25]; suppression of cell proliferation and EMT in MDA-MB-468 and MDA-MB-231 breast cancer cell lines with additional mPR expression [20]; upregulation of proapoptotic protein BAX mRNA expression in SKOV-3 ovarian cancer cells [21]. However, there are the studies focusing mainly on breast cancer cells, where it was demonstrated that progesterone could act through mPRs (specifically, through mPRα) to inhibit apoptosis of MDA-MB-468 and SKBR3 breast cancer cell lines [26]. Moreover, mPRα was reported to be a major marker of worse prognosis and it promoted expression of MMP-9 during invasion to local lymph nodes through the PI3K/Akt pathway in breast cancer [27]. Nevertheless, there is the reason to suggest that progesterone, acting through mPRs, can have an inhibitory effect on tumor cells originating from many tissues. Therefore, we aimed to find a suitable model for studying the progesterone effects through membrane receptors by examining nPRs, mPRα, mPRβ, and mPRγ mRNA expression levels in 16 tumor cell lines of different origin. Some lines have already been characterized in several studies, especially those originating from tissues of the reproductive organs and we selected them for the method verification [19,21,28–30]. To our knowledge, in other cell lines (A-172 and LN229—glioblastoma cells, BxPC3—pancreatic adenocarcinoma cells, NCI-H1299—non-small cell lung carcinoma, SK-UT1B—endometrial leiomyosarcoma cells) the expression of mPRs have been examined for the first time in this study. MCF-7 and T47D breast cancer cell lines were chosen as positive control of the nPRs mRNA content, while MDA-MB-231 and HeLa S3 cells served as negative control [28,31]. The mPR mRNA levels in the cell lines are scarcely studied. In this study, we used the SKOV-3 and Jurkat cells to compare the data on the mPR mRNA levels with those known from the literature [21,29]. In some glioblastoma cell lines (except LN 229) progesterone at high concentrations showed significant anti-proliferative effect [22]. That's why we also included glioblastoma cell lines (U87MG, A-172, LN 229) to compare expression of different PRs types in cells that are sensitive to the action of progesterone and in the LN 229 cells in parallel. In human prostate cell lines, Du145, PC-3, and LNCaP, the mPR composition has not been examined previously, while the role of nPRs in the prostate cancer cells proliferation, migration, and invasion has been actively studied [32,33]. Furthermore, nPRs were detected in stromal but not epithelial cells of the prostate, so epithelial lines could match the desired model. Since the effect of progesterone on proliferation was demonstrated for a number of cancer cells of non-reproductive origin (non–small cell lung cancer [34], osteosarcoma [35], colon carcinoma, and low- and intermediate-grade pancreatic neuroendocrine tumors [36,37]), we examined the progesterone receptor composition in the cells of non-small cell lung carcinoma (NCI-H1299), osteosarcoma (U-2OS), and in pancreatic adenocarcinoma (BxPC3) cells. As a result of screening of 16 cell lines, we were able to find the appropriate object (BxPC3), containing high levels of mPR RNA and no detectable nPR mRNA, to investigate the effect of progesterone on proliferative activity of tumor cells mediated through membrane receptors. The presence of mPRs in BxPC3 cells was confirmed by immunoblotting assay and specific progesterone binding. We examined the effect of two progesterone concentrations on expression of proliferation marker Ki67, PCNA (proliferating cell nuclear antigen), proliferation activator cyclin D1 (a regulatory subunit of cyclin-dependent kinase 4 or 6, the activity of which is required for the cell cycle G1/S transition), proliferation inhibitors p21 and p27 (these proteins bind to and inhibit the activity of cyclin-CDK2 or −CDK4 complexes, and thus control the cell cycle progression at G1). We also studied the effect of two progesterone concentrations on viability of selected cell line in proliferation assay (MTT assay).

2. Experimental

2.1. Materials

Reagents were purchased from Sigma—Aldrich Corporation (St. Louis, MO, USA); RPMI-1640 and DMEM/F-12 media, fetal calf serum, charcoal–dextran-treated fetal bovine serum, and 100x antibiotic-antimycotic solution—from Gibco (Carlsbad,CA, USA); TRIzol reagent—from Invitrogen (Carlsbad, CA,USA); RQ1 Dnase and ImProm-IITM Reverse Transcription System—from Promega (Madison, WI, USA), a kit of reagents for real time PCR including the intercalating dye SYBR Green I—from Syntol (Moscow, Russia); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)—from Sigma (M2128, USA); cortisol, estradiol-17β, testosterone, progesterone—from Sigma Chemical Co (St. Louis,MO, USA); DPBS Ca^{2+}/Mg^{2+}—from Corning (USA) and PanEco (Moscow, Russia). [1,2-^{3}H] progesterone (specific activity 40 Ci/mmol) was synthesized by VP Shevchenko at the Institute of Molecular Genetics, Russian Academy of Sciences.

2.2. Cell culture

Human MDA-MB-231, MCF-7 (breast adenocarcinoma cells), T47D (intraductal breast carcinoma cells), HeLa S3 (cervix adenocarcinoma cells), Du145, PC-3, LNCaP (prostate carcinoma cells), SKOV-3 (ovarian adenocarcinoma cells), BxPC3 (pancreatic adenocarcinoma cells) were from the American Type Culture. Collection (ATCC, Manassas, VA). A-172, LN 229, U87MG (glioblastoma), SK-UT1B (endometrial leiomyosarcoma cells), NCI-H1299

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(non-small cell lung carcinoma), U-2OS (osteosarcoma), Jurkat (T-cell leukemia) were from Cell culture collection of vertebrates (Institute of Cytology, Russian Academy of Sciences, St. Petersburg) and were kindly provided by our collaborators from Blokhin Russian Cancer Research Center (Moscow). The cell lines were cultured in standard conditions (37 °C with 5% CO_2 in humidified atmosphere). The MCF-7, DU145, PC-3, SK-UT1B, A-172, LN229, U87MG, U-2OS cells were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS) and 1x antibiotic–antimycotic solution mixtures. The BxPC3, T47D, LNCaP, SKOV-3, MDA-MB-231, NCI-H1299, Jurkat, HeLa S3 cells were cultured in RPMI-1640 supplemented with 10% FBS and 1x antibiotic–antimycotic solution mixtures. The fourth passage of the cultures after thawing was transferred to phenol-red-free medium, supplemented with 10% charcoal– dextran-treated fetal bovine serum (DFBS) and antibiotics. Then, after three passages, the cells were used for RNA extraction, immunoblotting and [^{3}H]-progesterone binding assays.

2.3. Progesterone treatment

For incubation with progesterone, the BxPC3 cells (the third passage in phenol-red-free medium with supplements) were placed in 3.5-cm culture dishes at initial concentration of 1×10^5 and cultured to 70% confluence. Then, fresh phenol-red-free medium containing progesterone or ethanol alone (0.2% volume of medium) was added. Cells were treated with 0, 1, 20 μM progesterone for 24, 48, and 72 h. In each experiment, the control sample "control—0 h" was included. At the end of the incubation, the culture supernatant was removed, cells were lysed in 0.5 ml of Trizol reagent and the lysates were stored at −80 °C.

2.4. RNA preparation and quantitative real-time PCR (QPCR)

Total cellular RNA was isolated with the TRIzol reagent according to the manufacturer's protocol and treated with DNase RQ1. After phenol-chloroform extraction and precipitation, RNA was used for cDNA synthesis. RNA (1.0 μg) was reverse transcribed to cDNA using random hexameric oligonucleotides and ImProm-II™ Reverse Transcription System as described by Promega. The cDNA was then amplified by real time PCR using a kit of reagents including SYBR Green I intercalating dye in accordance with the manufacturer's recommendations on a Rotor Gene 3000 thermocycler («Corbett Research», Australia). Specific primers are given in a supplement Table. Primer sequences were selected by the Beacon Designer 7.51 (www.PremierBiosoft.com) and Primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) software programs, and were synthesized by "Syntol" (Moscow, Russia).

Quantitative PCR cycling conditions consisted of initial denaturation at 95 °C for 5 min; followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 17 s, extension at 72 °C for 20 s; melting 72°–95 °C. Fluorescence detection was performed at the end of the extension step at 72 °C in the FAM/Sybr channel. To check for the absence of genomic DNA amplification products, isolated RNA not treated with reverse transcriptase was used as a template. The details were described previously [38,39]. The GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase) gene was selected as a reference gene [40]. The second reference gene, GNB2L1 (guanine nucleotide-binding protein, beta-peptide 2-like 1), was used to test the stability of GAPDH expression in the experiments with progesterone cell treatment [41–43]. The ratio of GNB2L1 mRNA, normalized to GAPDH mRNA, in experimental (1 and 20 μM progesterone) and control samples were close to 1.0, in the range of 0.84 ± 0.27 to 1.24 ± 0.31 (average values ± SD) in different experimental groups. These results suggest that GAPDH could serve as a reference gene in the selected experimental conditions. The amount of particular mRNAs was normalized to GAPDH mRNA amount as an internal reference control. The normalized value of mRNA expression level was calculated by ΔΔCt method relatively to calibrator, i.e., the sample "control—0 h" [40]. The data on progesterone effect on gene expression were obtained in three or four independently performed experiments. Estimation of the nPR and mPR mRNA levels in 13 cell lines, performed in two independent experiments was considered as semi-quantitative. The amounts of particular mRNAs were normalized to the amount of GAPDH mRNA and expressed as a percentage to GAPDH mRNA.

2.5. Immunoblotting assay

The cells were lysed with RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology) supplemented with protease inhibitor cocktail, PMSF and sodium orthovanadate, provided with the kit. Protein total concentration was determined by BCA protein assay (23227, Thermo Scientific Pierce). Cell lysates (20 μg) were separated by 12.5% polyacrylamide gel electrophoresis in denaturing conditions, and the proteins were electrophoretically transferred onto 0.45 μm pore size nitrocellulose membrane for 1 h in a transfer buffer containing 20 mM Tris, 150 mM glycine, and 20% ethanol. The membrane was then blocked in PBS containing 0.1% Tween-20 and 2% ECL Advance Blocking Agent (GE Healthcare Life Sciences) overnight at 4 °C. After 1x wash with PBS containing 0.1% Tween-20 (0.1% PBST), the nitrocellulose filters were incubated with primary polyclonal rabbit antibodies against mPRα (ab75508, Abcam, Cambridge, Ma, USA) at a1:800 dilution, mPRβ (ab123693, Abcam, Cambridge, Ma, USA) at a 1:250 dilution, mPRγ (ab79517, Abcam, Cambridge, Ma, USA) at a 1:800 dilution and with primary monoclonal rabbit antibodies against nPR (ab32085, Abcam, Cambridge, Ma, USA) at a 1:5000 dilution. The primary antibodies were diluted in 1% BSA solution in 0.1% PBST and incubated overnight at 4 °C with mild agitation. After 3 × 5 min washes with 0.1% PBST, the nitrocellulose filters were incubated with blotting-grade horseradish peroxidase secondary antibody conjugate (1706515, Bio-Rad, USA) at a 1:3000 dilution for 1 h at room temperature, then washed 3 × 5 min with 0.1% PBST, 1 × 5 min PBS. Proteins of interest were detected by Clarity Western ECL Substrate on a ChemiDoc MP System (Bio-Rad, USA).

2.6. Measurement and analysis of [^{3}H] progesterone binding

Aliquots of BxPC3 cells suspension in DPBS Ca^{2+}/Mg^{2+} (120–150 thousand cells in 100 μl) were incubated with 110 μl of steroid solution in the same buffer at room temperature (20–22 °C) for two hours at constant agitation. The steroid solution included 10 μl of [1.2—^{3}H] progesterone (final concentration 4 – 6.5 nM), 90 μl of unlabeled progesterone or competitor (final concentration 0—6.3 μM) and 10 μl containing 400 ng unlabeled cortisol (final concentration 5.2 μM). After the incubation, the cells were precipitated by centrifugation (Jouan CR 3i) at 500g for 7 min at room temperature. The cell pellets were washed with 700 μl of DPBS Ca^{2+}/Mg^{2+} and centrifuged at 500g for 7 min at room temperature. The cells were resuspended in 250 μl of distilled water, transferred to the vials for counting the radioactivity content on the RackBeta 1217 liquid scintillation counter (LKB WALLAC, Finland). Specific binding of [^{3}H]- progesterone was measured in duplicate in 11 independent experiments. The equilibrium dissociation constant Kd and Bmax were determined as implemented in the GraphPad Prism Program 6 (San Diego, CA, USA) or according to the formula

$$Kd = \frac{[U]\{Ns - [Bs]\}}{[Bs]},$$

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where U, unbound progesterone, Bs, specifically bound progesterone, Ns (Bmax), the value, characterizing binding capacity of the protein to a ligand, which is determined by extrapolation of Scatchard plot to the X-axis. Extrapolation of Scatchard plot to the Y-axis gives the Ns/Kd value.

2.7. MTT cell proliferation assay

BxPC3 cells were grown in RPMI1640 complete medium without phenol red, harvested by trypsinization, seeded at a density of 8000 cells/well onto 96-well cultural plate and left to attach for 24 h. The medium was discarded and substituted with 150 μl/well of fresh medium containing 1 μM, 20 μM progesterone and ethanol control at the same percentage 0.2% (v/v), and the cells were left to grow for 72 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) stock solution in DPBS was added to a final concentration of 0.45 mg/ml to each well and left for 2 h. The medium was discarded and the formazan crystals were dissolved in DMSO 150 μl/well by vigorous shaking (600 rpm) in a thermoshaker at room temperature. The absorbance at 595 nm was measured in the Zenyth 3100 multimode plate reader (Anthos). The proliferation was calculated as a percentage of ethanol control (which was taken as 100% of proliferative activity). The data were measured in 5 independent experiments and were represented as mean ± SD.

2.8. Statistical analysis of the results

All values are mean ± SD. The data shown in Figs. 5–11 are representative of at least three independent experiments. Data were compared using one-way (for results of the MTT assay) and two-way analyses of variance (ANOVA) and significant differences were obtained using the Tukey multiple variances post hoc test (GraphPad Prism version 6, GraphPad Software Inc., San Diego, CA, USA). Correlations of the mRNA levels of different receptor types and subtypes were evaluated using the Pearson test. For all tests, statistical significance was considered when p was <0.05.

3. Results

3.1. nPR and mPRs expression in human tumor cell lines

Using RT-qPCR technique, we detected the presence of nPR mRNA in the breast cancer cell lines MCF-7 and T47D (positive control) but not in MDA-MB-231 and HeLa S3 (negative control) (Fig. 1).

The mRNAs for mPR α,β,γ subtypes were detected in MCF-7, T47D, MDA-MB-231 and HeLa S3 cells in moderate amounts, lower than the amount of GAPDH mRNA (Figs. 2 and 3). In Jurkat cells, derived from T-lymphocytes, we did not detect visible expression of nPR and mPRγ mRNA.

In SKOV-3 ovarian adenocarcinoma cells, we found trace amounts of nPR mRNA, high level of mPRγ mRNA (144% relative to the GAPDH mRNA level), moderate amounts of the other two mPRs mRNA subtypes. In prostate carcinoma epithelial cells (Du145, PC-3, LNCaP), nPR mRNA was barely detected. We have found high levels of mPRs mRNA, in particular mPRα mRNA in two prostate cell lines, Du145 and PC-3 (163% and 152% relative to the GAPDH mRNA levels, respectively). LNCaP has been revealed as a progesterone receptor- negative cell line. We practically did not detect progesterone receptor mRNAs, including mPRs mRNAs, in LNCaP cells (Figs. 1 and 3).

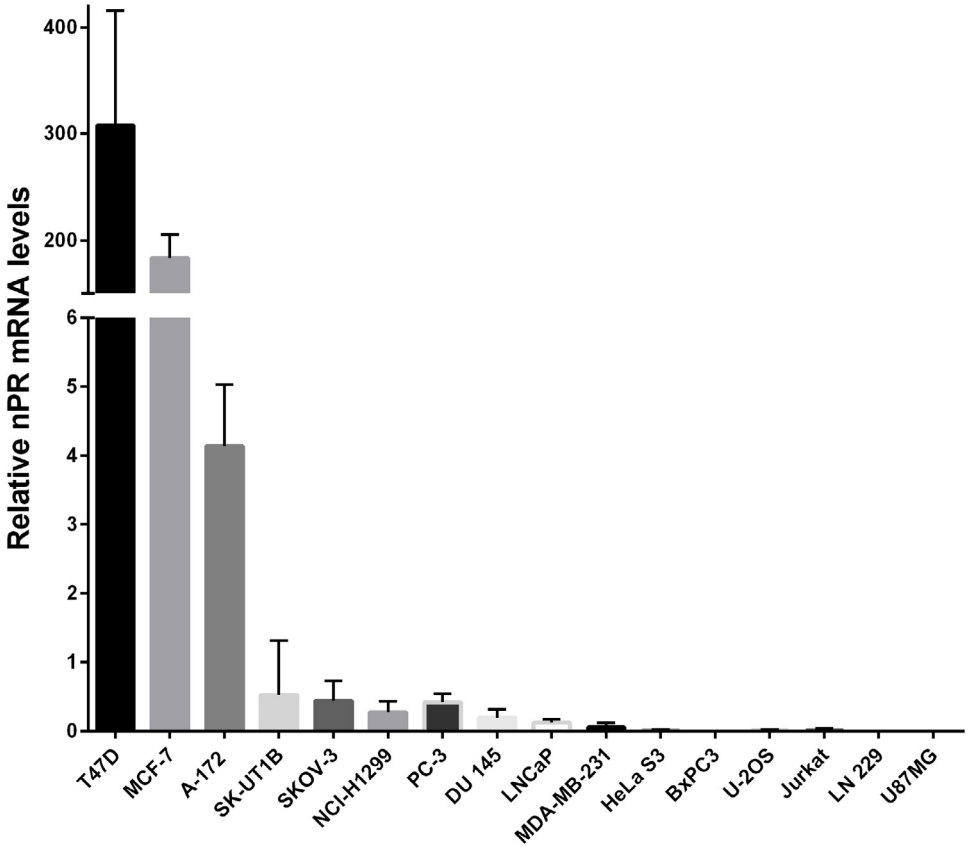

Fig. 1. Relative nPR mRNA levels in human cell lines.
mRNAs of interest were normalized to GAPDH mRNA and expressed as a percentage. Values are mean ± SD of three (for BxPC3, SKOV-3 and Jurkat cell lines) or two (for the other cell lines) independent experiments, each done in triplicate.

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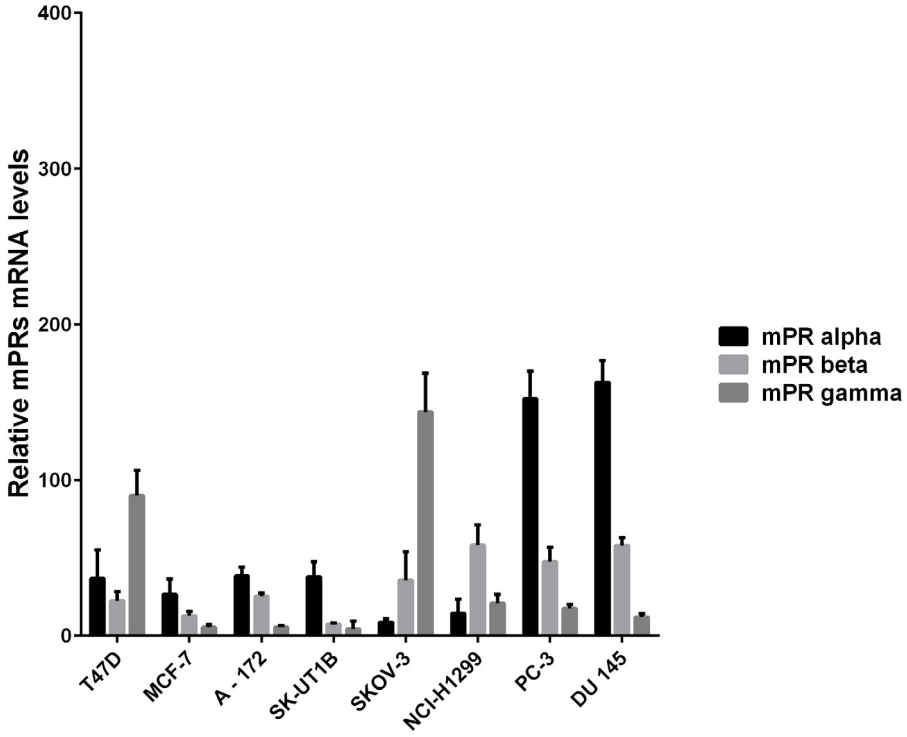

Fig. 2. Relative levels of mPR α, β, and γ mRNAs in human cell lines, expressing detectable amount of nPR mRNA.
mRNAs of interest were normalized to GAPDH mRNA and expressed as a percentage. Values are mean ± SD of three (for SKOV-3 cell line) or two (for the other cell lines) independent experiments, each done in triplicate.

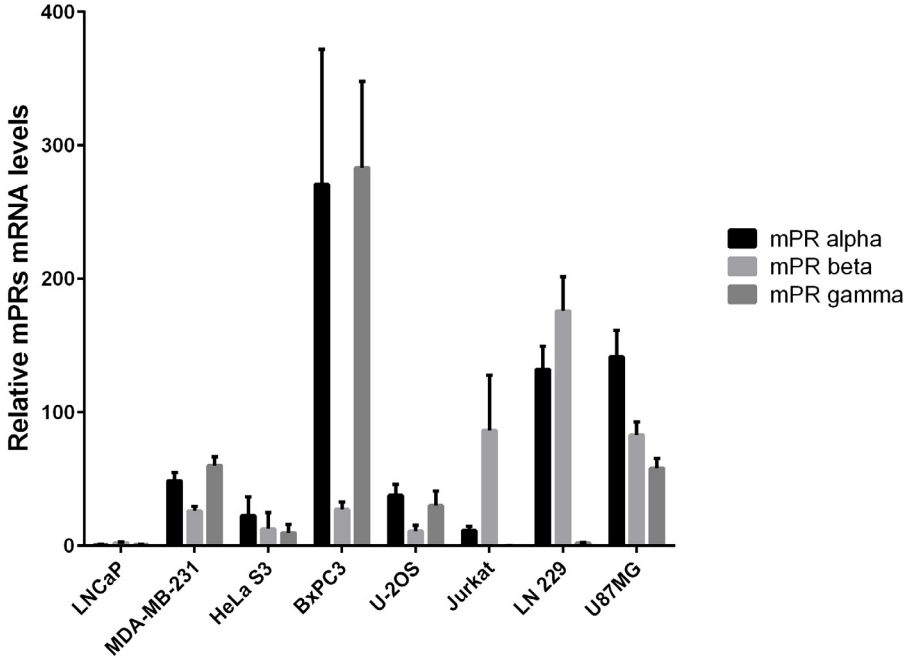

Fig. 3. Relative levels of mPR α, β, and γ mRNAs in human nPR-negative cell lines.
mRNAs of interest were normalized to GAPDH mRNA and expressed as a percentage. Values are mean ± SD of three (for BxPC3 and Jurkat cell lines) or two (for the other cell lines) independent experiments, each done in triplicate.

We did not detect nPR mRNAs in U87MG and U-2OS cells. The mRNAs for the three types of mPRs were detected in these cells at moderate amounts except for the high level of mPRα mRNA in U87MG cells (141% relative to the GAPDH mRNA level). The next step was to examine expression of nPR and mPRs mRNAs in A-172, LN 229, NCI-H1299 and BxPC3 cell lines which, to our knowledge, was still unknown. Our data showed that three glioblastoma cell lines differed significantly in the composition of progesterone

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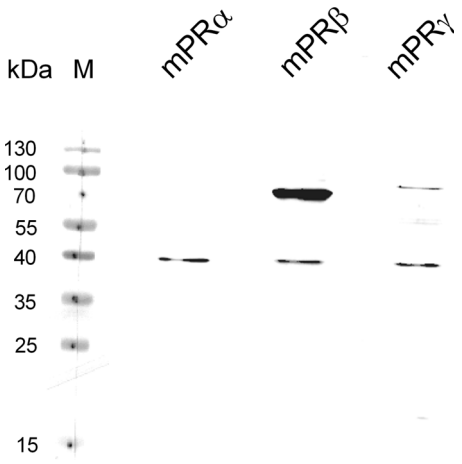

Fig. 4. Immunoblotting of mPRα, mPRβ, mPRγ proteins.
Expression of mPRα, mPRβ, mPRγ proteins in BxPC3 cells. M—molecular weight marker.

receptors. While the A-172 cells contained well-defined levels of nPR mRNA (4% relative to the GAPDH mRNA level) and moderate amounts of mPRs mRNA (38% for mPRα and lower, for other subtypes relative to the GAPDH mRNA level), two other cell lines of glioblastoma demonstrated a lack of nPR and rather high level of expression of mPRs mRNA in LN 229 cells (132% for mPRα and 176% for mPRβ relative to the GAPDH mRNA level) (Figs. 1 and 3). In NCI-H1299 cells, nPR mRNA was barely detected, while mPRs mRNAs were at moderate levels. According to our results, nPR mRNAs are not expressed in BxPC3 cells. The highest expression of mPRs, especially of α (270% relative to the GAPDH mRNA level) and γ (283% relative to the GAPDH mRNA level) types, was observed in pancreatic adenocarcinoma BxPC3 cells (Figs. 1 and 3). Therefore, this cell line is suitable for the study of progesterone effect mediated by mPRs on the transcription of several genes related to proliferation and in proliferation assay. In the studied cell lines, there is no correlation between the expression of nPR and mPRs subtypes. A moderate positive correlation was only observed between the expression of mPRα and mPRγ ($r = 0.54$, $p = 0.03$).

3.2. Immunoblotting

Immunoblotting using specific mPRα, mPRβ, mPRγ polyclonal antibodies demonstrated the presence of all three mPRs in BxPC3 cell lysates with molecular weights of approximately 40 kDa. An additional 80- kDa bands of mPRβ and mPRγ have also been detected in cell lysates (Fig. 4). This molecular weight is double that predicted from the deduced amino acid sequences of the mPRs and is likely indicative of the formation of sodium dodecyl sulfate (SDS)-resistant homodimers. The monoclonal antibodies against nPR detected the protein in T47D cell lysate used as positive control but not in BxPC3 cell lysate (data not shown).

3.3. Characterization of the receptor composition in BxPC3 cells

Since the action of progesterone on BxPC3 cells was studied at time intervals of 24 h, 48 h, and 72 h after addition of the hormone, it was important to evaluate the levels of mPRα, -β, and -γ mRNAs under basal conditions without addition of steroids and within the same time frame. Relative level of mPRβ mRNA remained virtually unchanged during the cultivation, while mRNA mPRγ increased significantly by 48 h (from 1.00 ± 0.13 to 1.90 ± 0.2 in relative values) and by 72 h (from 1.00 ± 0.13 to 1.89 ± 1.12), comparing to the "control-0 h" sample (supplement figure). The level of mPRα mRNA increased insignificantly comparing to the same control sample (from 1.00 ± 0.05 to 1.57 ± 0.12 by 48 h; to 1.58 ± 0.2 by 72 h in relative values). In the studied cells, the levels of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNA were also determined. The level of GR mRNA was $412\% \pm 82\%$; MR mRNA was $0.5\% \pm 0.3\%$ relative to GAPDH mRNA level.

3.4. Progesterone-binding to BxPC3 cells

Fig. 5 shows the representative competition curves of steroid binding to the protein(s) of BxPC3 cells and Scatchard plot for interaction between BxPC3 cells' protein(s) and progesterone.

As a result of 11 experiments, Kd of mPRs to progesterone was found to be 162.4 ± 57.6 nM and Bmax = 7.9 ± 4.9 nM. Testosterone also bound to mPRs with the RBA $11.7 \pm 3.3\%$ that of progesterone, whereas no binding of estradiol-17β to the receptor was detected

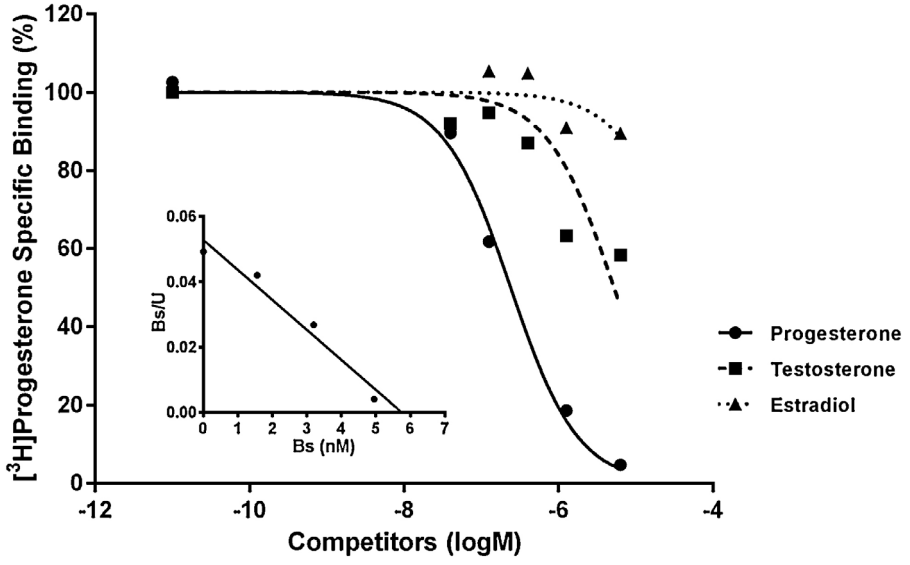

Fig. 5. Representative competition curves of steroid binding to the protein(s) of BxPC3 cells. The inset shows Scatchard analysis of the same data for progesterone.
Binding of [^{3}H]-progesterone was measured in the absence or in the presence of increasing concentrations of nonradioactive steroids and expressed as a percentage of maximum specific [^{3}H]- progesterone binding. It was measured in duplicate in 11 independent experiments. Bs—specifically bound progesterone, U—unbound progesterone.

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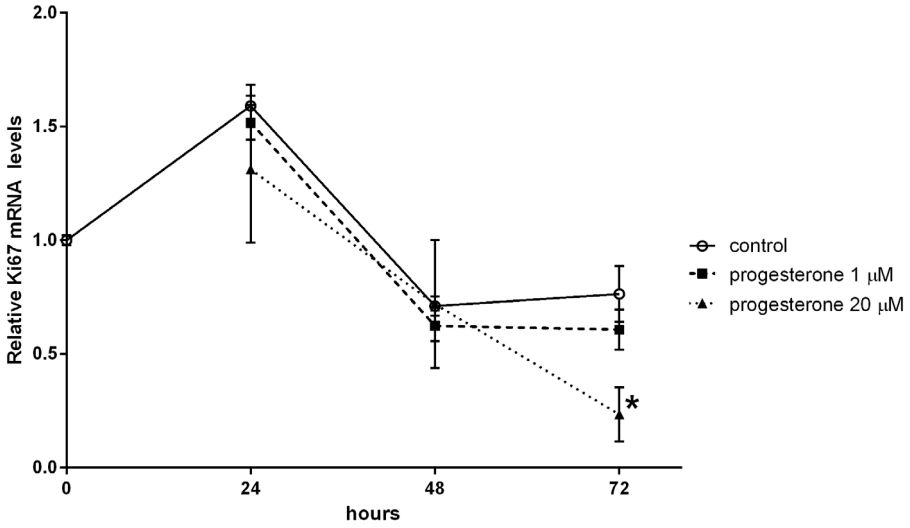

Fig. 6. Profiles of Ki67 mRNA expression in BxPC3 cells treated with progesterone for 24, 48, and 72 h.
The *MKI-67* transcription level in BxPC3 cells was quantified by QPCR. Transcripts were normalized to that of GAPDH. The mRNA level in the "control—0 h" sample was set at 1.0. Values are mean ± SD of three or four independently performed experiments, each done in triplicate. Only progesterone at concentration of 20 μM significantly inhibited the *MKI-67* expression after 72 h of exposure. *The difference from control at the same time interval, $p < 0.0001$ by two-way ANOVA, Tukey's multiple comparisons test

at concentrations up to 1 μM. The RBA of estradiol-17 was 1.3 ± 0.9% that of progesterone.

3.5. The effect of progesterone on the expression of proliferation-associated genes

The effect of progesterone on gene transcription was studied at two concentrations—1 and 20 μM and at three time intervals of 24, 48, and 72 h. The effect of progesterone on the expression of Ki67 proliferation marker (*MKI-67* gene) mRNA is shown on Fig. 6.

The levels of Ki67 mRNA in control samples increased to 24 h (from 1.00 ± 0.10 to 1.59 ± 0.28 in relative values), indicating that the peak of cell proliferation was within the first day of cultivation. Then, proliferation decreased and stabilized between 48 and 72 h (0.71 ± 0.16 and 0.76 ± 0.46 relative values). It was possibly due to the decrease in the content of nutrients and growth factors. Only 20 μM of progesterone significantly inhibited the *MKI-67* expression after 72 h of exposure (from 0.76 ± 0.46 in control to 0.23 ± 0.12, in relative values). Fig. 7 shows the changes in the expression of another proliferation marker, PCNA, after the progesterone treatment.

PCNA expression was more stable in control, but more sensitive to the influence of progesterone. The PCNA mRNA level was significantly reduced after exposure to 20 μM of progesterone for 48 h (from 0.97 ± 0.19 to 0.68 ± 0.16 in relative values). After 72 h, even 1 μM of this hormone significantly inhibited the PCNA mRNA expression (from 1.03 ± 0.23 to 0.76 ± 0.21), not only 20 μM (from 1.03 ± 0.23 to 0.27 ± 0.11 in relative values). In this case, the progesterone effect was dose—dependent. The level of cyclin-dependent kinases activator—cyclin D1 (gene *CCND1*) mRNA also

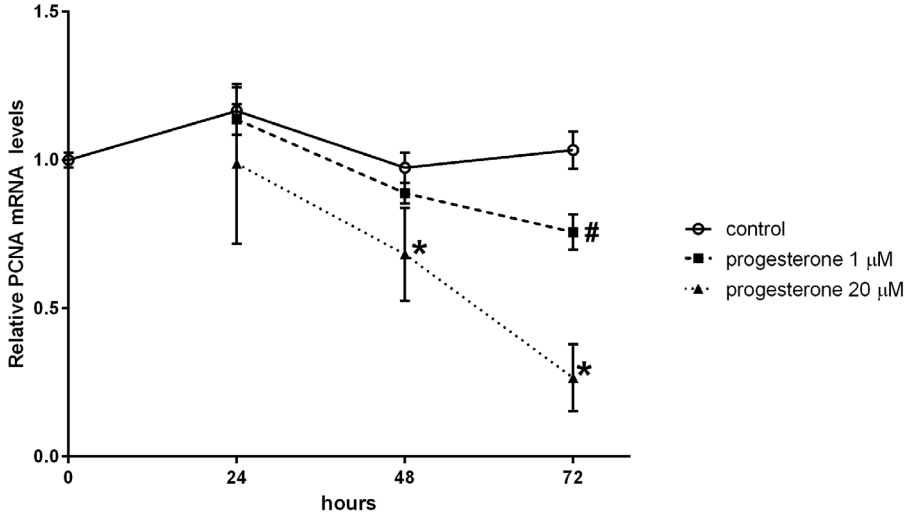

Fig. 7. Profiles of PCNA mRNA expression in BxPC3 cells treated with progesterone for 24, 48, and 72 h.
The *PCNA* transcription level in BxPC3 cells was quantified by QPCR. Transcripts were normalized to that of GAPDH. The mRNA level in the "control—0 h" sample was set at 1.0. Values are mean ± SD of three or four independently performed experiments, each done in triplicate. The PCNA mRNA level was significantly reduced by the action of 20 μM of progesterone after 48 h of incubation. *The difference from control at the same time interval, $p = 0.0002$. After 72 h, even 1 μM of this hormone significantly inhibited expression of the PCNA proliferative marker. #The difference from control at the same time interval, $p = 0.0001$. Progesterone at concentration 20 μM significantly inhibited the *PCNA* expression after 72 h of exposure. *The difference from control at the same time interval, $p < 0.0001$ by two-way ANOVA, Tukey's multiple comparisons test.

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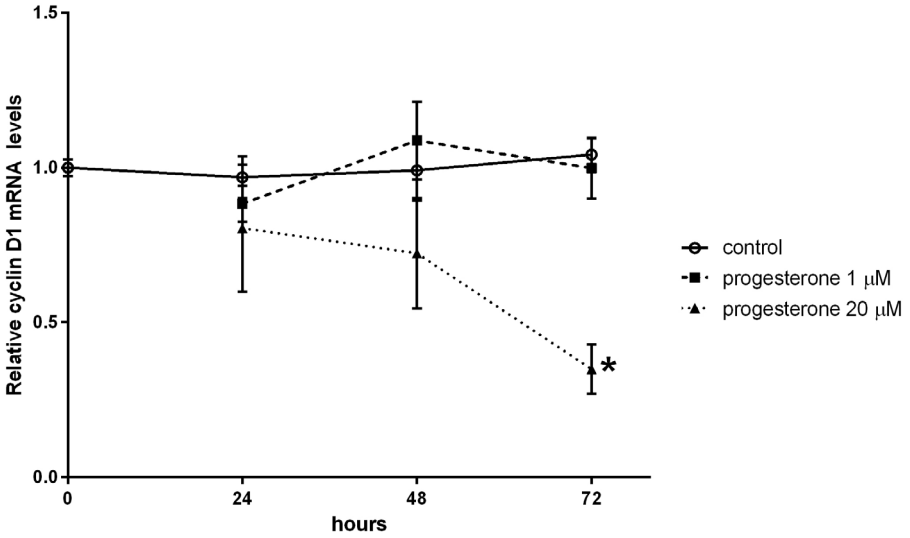

Fig. 8. Profiles of cyclin D1 mRNA expression in BxPC3 cells treated with progesterone for 24, 48, and 72 h.
The *CCND1* transcription level in BxPC3 cells was quantified by QPCR. Transcripts were normalized to that of GAPDH. The mRNA level in the "control—0 h" sample was set at 1.0. Values are mean ± SD of three or four independently performed experiments, each done in triplicate. Only progesterone at concentration of 20 μM significantly inhibited the *CCND1* expression after 72 h of exposure. * The difference from control at the same time interval, $p < 0.0001$ by two-way ANOVA, Tukey's multiple comparisons test

significantly reduced after 72 h of exposure to 20 μM of progesterone, from 1.04 ± 0.20 to 0.35 ± 0.08 in relative values (Fig. 8).

We also studied the effects of progesterone on the mRNA levels of two major inhibitors of cyclin-dependent kinases—p21 (*CDKN1A* gene) and p27 (*CDKN1B* gene). As can be seen from figures (Figs. 9 and 10), 20 μM of progesterone significantly induces expression of these mRNAs after 48 h of incubation with the hormone: p21 from 1.21 ± 0.29 to 1.90 ± 0.54 in relative values and p27 from 0.87 ± 0.46 to 1.57 ± 0.41 in relative values.

3.6. The effect of different progesterone concentrations on viability of BxPC3 cells

The BxPC3 cell viability was evaluated by MTT assay. We observed significant ($p < 0.0001$) anti-proliferative effect after 72 h of progesterone exposure at both concentrations (1 μM from 100 ± 2.4% to 81.5 ± 2.3%; and 20 μM to 78.6 ± 1.3%). At 20 μM of progesterone this anti-proliferative effect was slightly more pronounced than at 1 μM, but not significantly (Fig. 11).

4. Discussion

4.1. The choice of cell line

In this paper, we aimed to find a suitable model for studying the progesterone effects through membrane receptors, because their role in cancer progression remains unclear. Expression of nPR and three mPR subtypes was studied in a number of human tumor cell lines. The goal of the first part of the experiments was to detect the presence or absence of particular receptor mRNAs and roughly compare their relative amounts in different cell lines. In some cell lines, the expression of mPRs has been evaluated for the first time. The MCF-7 and T47D cells were included as positive controls for

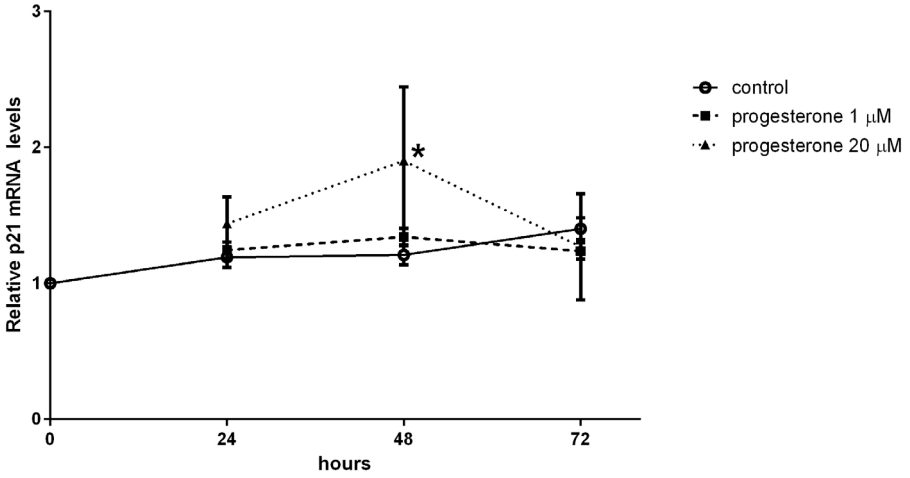

Fig. 9. Profiles of p21 mRNA expression in BxPC3 cells treated with progesterone for 24, 48, and 72 h.
The *CDKN1A* transcription level in BxPC3 cells was quantified by QPCR. Transcripts were normalized to that of GAPDH. The mRNA level in the "control—0 h" sample was set at 1.0. Values are mean ± SD of three or four independently performed experiments, each done in triplicate. Only progesterone at concentration of 20 μM significantly induced the *CDKN1A* expression after 48 h of exposure. *The difference from control at the same time interval, $p < 0.0001$ by two-way ANOVA, Tukey's multiple comparisons test.

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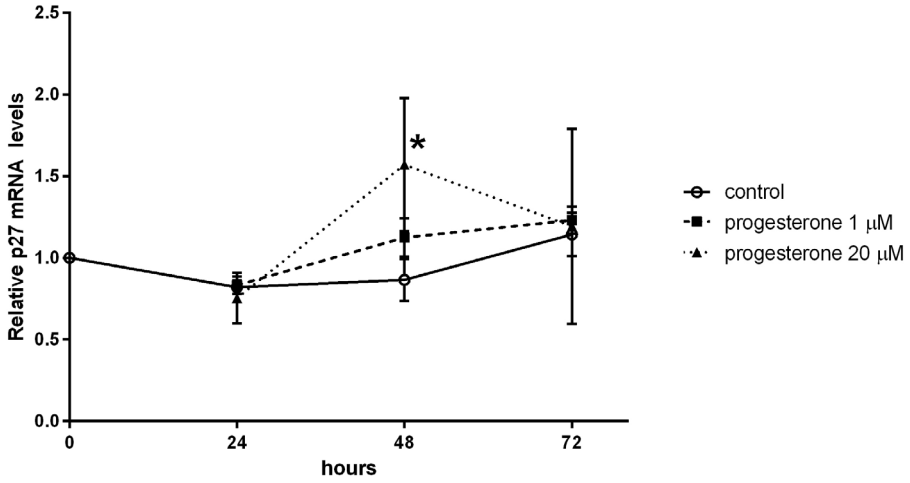

Fig. 10. Profiles of p27 mRNA expression in BxPC3 cells treated with progesterone for 24, 48, and 72 h.
The *CDKN1B* transcription level in BxPC3 cells was quantified by QPCR. Transcripts were normalized to that of GAPDH. The mRNA levels in "control—0 h" were set at 1.0. Values are mean ± SD of three or four independently performed experiments, each done in triplicate. Only progesterone at concentration of 20 μM significantly induced the expression of the gene *CDKN1B* after 48 h of exposure. *The difference from control at the same time interval, $p < 0.0001$ by two-way ANOVA, Tukey's multiple comparisons test.

nPRs mRNA expression, HeLa S3 and MDA-MB- 231 cells were used as negative controls. MCF-7 and T47D cell lines of breast cancer belong to differentiated subtype "luminal A", containing high level of progesterone nPRs. The MDA-MB-231 line of breast cancer belongs to the "claudin-low" subtype. The claudin-low subtype initially clustered with the basal subtype due to the lack of ERα, PR and HER2 expression and expression of the features associated with mammary cancer stem cells [28]. As it was expected, we found high levels of nPR mRNAs in the first two cultures and observed almost no expression of these mRNAs in the latter two cultures. Similar results were obtained by several laboratories, where the presence of nPRs was shown both at the mRNA and protein levels in MCF-7 and T47D cells, and their almost complete absence was demonstrated in MDA-MB-231, HeLa [30,31,44,45]. There are few studies on the mRNA quantification for the mPRs of α,β,γ subtypes in cell lines. The composition of different types of PR, including mPRs, was examined in Jurkat cells, derived from T-lymphocytes, since there was significant evidence on the role of progesterone as an immunomodulator. In Jurkat cells, we only detected the expression of mPRα, and -β. Similar results were obtained for human peripheral blood mononuclear cells from donors of both sexes (data not shown). Our data are consistent with those obtained by Dosiou and colleagues [29], but contrary to the data of Chien and colleagues, who have detected mPRγ mRNA in human male T lymphocytes [46]. There are the data on expression of mPRα, -β, -γ mRNAs and nPR mRNA in SKOV-3 ovarian adenocarcinoma cells. Our results also coincide with the data of Dressing and Goldberg [47]. In the previous studies, only nPR mRNA expression was examined in prostate carcinoma cell lines originating from epithelium (Du145, PC-3, LNCaP). Most of the researchers found both nPR isoforms exclusively in the stroma of the prostate, namely, in fibroblasts and smooth muscle cells, but not in the cells of basal and luminal epithelium of the prostate [32]. The reported level of nPR expression in prostate cancer cell lines is not without controversy. LNCaP and Du145 cells were reported to express PR-A and PR-B mRNA, while PC-3 did not express nPRs [33]. However, Tieszen and colleagues were unable to detect nPR proteins in LNCaP cell line [30]. Our data confirm the above-mentioned results obtained by mRNA measurement. Since expression of mPRs in prostate has not been studied previously, we examined three prostate cell lines, Du145, PC-3, and LNCaP, and found, that the latter line differed from the other lines examined in our studies in almost complete absence of nPR-, as well as of mPRs mRNA expression. Similarly to the reported absence of nPR expression in U87MG and U-2 OS cells [30], we did not detect nPR mRNA in these cell lines. The mRNA levels for the three types of

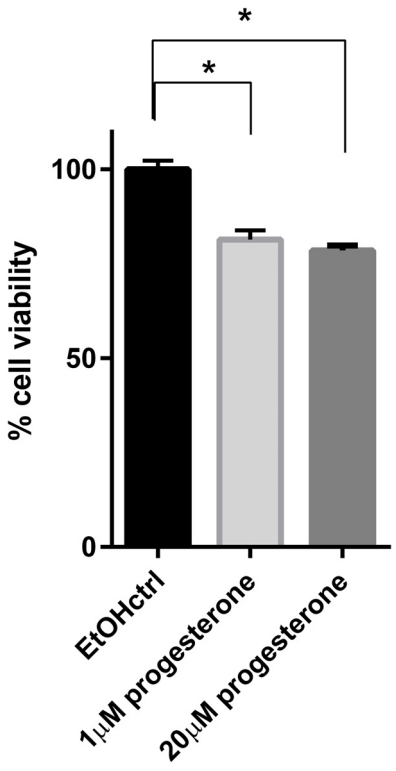

Fig. 11. Progesterone induces anti-proliferative effect in BxPC3 cells.
Cells were exposed to 1 μM and 20 μM and ethanol control at the same percentage (EtOHctrl) for 72 h. The cell proliferation rate was measured by MTT assay. The data are expressed as mean ± SD of five independent experiments. Significant difference: $p < 0.0001$ compared with vehicle group.

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mPR in these lines have been determined for the first time in our study, as well as the expression of nPR- and mPRs mRNA in A-172, LN 229, NCI-H1299, and BxPC3 cell lines. Among all cell lines, the highest levels of mPRs mRNA expression have been revealed in human pancreatic adenocarcinoma BxPC3 cells, while nPR mRNA could not be detected in these cells. Therefore, this cell line was selected to study the binding affinity of progesterone and its effects mediated by mPRs on the expression of proliferation markers, inhibitors and activator of proliferation in the absence of interference with nPR. Interestingly, there is moderate, but significant positive correlation in the content of mPRα mRNA and mPRγ mRNA in different cell lines ($r = 0.54$, $p = 0.03$). In the literature, we found no information on coordinated regulation of mRNA levels of these 2 subtypes of mPR. On the contrary, there is evidence on their opposite changes. The expression profiles of mPR transcripts were examined in human endometrium during ovarian cycle. It was demonstrated that an increase in progesterone in the luteal phase was accompanied by substantial upregulation of mPRα mRNA and downregulation of mPRγ mRNA, while the level of mPRβ mRNA remained unchanged [48]. A study of mPRs mRNA expression in the rat corpus luteum showed that expression of the relevant genes is controlled by prolactin, which upregulates mPRα, mPRβ and downregulates mPRγ [49]. It seems likely, that some common regulatory mechanism of the mPRα mRNA and mPRγ mRNA expression appears during malignant transformation of the cells.

4.2. Characterization of the receptor composition in BxPC3 cells and their dynamics during the cultivation

We showed that BxPC3 were characterized by very high levels of mPRs mRNA. Western blot experiments designed to examine nPR and mPRα,β,γ proteins expression showed the absence of the nPR protein and the presence of the mPR proteins of about 40 kDa in BxPC3 cell lysates. In addition, the second bands of approximately 80 kDa were observed for mPRβ and mPRγ, that probably are the SDS-resistant homodimers of the 40 kDa mPR proteins described previously [12,29,50]. The relative abundance of the 40 and 80 kDa bands in different preparations varied, possibly as a result of minor differences in preparation, storage, and solubilization conditions [12]. Furthermore, BxPC3 cells contained the protein(s), specifically binding the progesterone. The Kd of mPRs-progesterone in BxPC3 cells was found to be 162.4 ± 57.6 nM and $Bmax = 7.9 \pm 4.9$ nM. Estradiol-17β failed to inhibit binding of [^{3}H]-progesterone to BxPC3 cells. Testosterone displayed moderate affinity for the receptors ($RBA = 11.7 \pm 3.3\%$ that of progesterone). These findings are in agreement with the data on mPRα, where the RBA of the testosterone was 22.4% of that of progesterone [12]. According to the literature data, Kd values for human α, -β, -γ mPR—progesterone interactions varied from 4-8 to 28-39 nM [11,12]. However, in our previous study, human mPRα expressed in heterologous yeast cells, had the Kd for the progesterone interaction of 143 ± 43 nM [51]. Since binding characteristics of mPRα, -β, and -γ were approximately the same [11], these data are in good agreement with our previous results. This finding suggests the presence of functional mPRs in the studied cell culture. Similar results have been reported for ovine mPRα, expressed in CHO cells with Kd for progesterone of 122 ± 50 nM [52]. In rat corpora lutea, containing the mPRα homolog, an apparent Kd for progesterone was found to be 162 nM [49]. The dissociation constant value constituting in our experiments 162 nM is well within the range of values reported for other membrane progesterone-binding proteins, including those found in bovine microsomal membrane fractions of corpora lutea—197 nM [53], rat liver—170 nM [54], porcine liver 11–286 nM [55] and rat brain – 160 nM [56]. Since the Kd of progesterone affinity for nPR constitutes 1–5 nM [10], binding affinity of progesterone to the human nPR is at least 30-fold higher than that to mPRs (5-fold higher, according to Thomas et al.) [12]. These apparent differences in binding affinities result in differential activation of the two progestin receptor systems, with the mPRs only being continuously activated where progestin levels are high near their sites of synthesis and intermittently activated at other target tissues when plasma progestin levels are elevated. This may provide clues of the physiological roles of mPRs during the reproductive cycle, pregnancy, and the use of contraceptives.

It is important to ensure that expression of mPRs in the cells was stable when cultivated throughout the experiment. Therefore, we determined the levels of mPRs mRNA every 24 h of cultivation and revealed that the expression did not decrease. On the contrary, we have found a reliable rise of mPRγ mRNA levels and insignificant increase of mPRα mRNA after 48 and 72 h of cultivation. The effect of the hormone after 48 h appears to be more pronounced. Indeed, we were not able to find any significant effects after 24 h of incubation with the hormone, which probably can be explained by complicated and multistep mechanisms of progesterone effects on transcription in BxPC3 cells. It is interesting, that increase of mPRγ mRNA in conjunction with mPRα mRNA was detected in accordance with the correlation of their levels in different cell lines. It can be suggested that expression of these two mPR subtypes, but not of mPRβ, in the cell lines is regulated by some common factor.

4.3. The effect of progesterone on the expression of genes associated with proliferation and on viability of BxPC3 cells

Recent reports suggest that mPRs participate in the development of cancer by regulating the processes, such as cell proliferation and apoptosis. Known facts point to more likely antitumor effect of progesterone upon mPRs activation [20,21,25]. The observed biphasic effect of high and low concentrations of progesterone in certain tissues may be associated with the action through different types of receptors. Since the affinity of the hormone to the membrane receptors is lower, many authors suggest that high concentrations act through mPRs, thus providing anti-proliferative effect. Significant cell death was observed in 3 human glioblastoma cell lines (including U87MG) and in SK-N-AS neuroblastoma cells after 3 days of progesterone exposure at high concentrations (20–80 μM), whereas lower concentrations (0.1–5 μM) did not induce any cell death. After 6 days of exposure, this cell death-inducing effect was more pronounced than after 3 days. It was demonstrated that progesterone at concentration of 20–80 μM was not toxic for healthy cells. High doses of progesterone did not induce cell death of healthy primary cortical neurons and human fibroblasts in either single or repeated exposures [22,23]. However, not all forms of tumor cell lines may be amenable to the salutary effects of the high—dose progesterone treatment. For example, progesterone did not show any cell death-inducing effect in LN-229 cells even at very high concentrations after 3 and 6 days of exposure. Thus, the hormone can differentiate between healthy cells and tumor cells and specifically induces cell death in certain cell types [22,23]. According to our data, U87MG and LN299 cells are similar in the absence of nPR and expression of mPRα, but significantly differ in the expression of mPRβ and -γ mRNA. How much this difference impacts on the progesterone effects on cell death in these two lines is unknown. Maybe it is not a matter of receptors composition, but disturbance in signaling pathways downstream of PRs in LN-299 cells. In the primary culture of ovarian cancer cells, progesterone inhibited the proliferation of cancer cells at a concentration of 100 μM after 48 and 72 h of progesterone treatment. Low concentrations of progesterone (1 μM and below) did not affect the survival rate of ovarian cancer cells [24]. This study was the first to examine the effect of

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progesterone on the expression levels of Ki67 and PCNA proliferation markers, cyclin D1 proliferation activator, and p21 and p27 proliferation inhibitors in BxPC3 cells. We were also the first to examine the effect of progesterone on viability of the chosen cell line in the proliferation assay (MTT test). After 48 h of exposure, progesterone induced the changes of PCNA, p21 and p27 mRNA levels, but the most significant effect was observed on transcription of the three genes (*MKI-67*, *PCNA* and *CCND1*) associated with active proliferation after 72 h of incubation with progesterone. After progesterone exposure at a concentration of 20 μM, the decrease of the mRNA levels of Ki67 (from 0.76 ± 0.46 to 0.23 ± 0.12, $p < 0.0001$) and PCNA (from 1.03 ± 0.23 to 0.27 ± 0.11, $p < 0.0001$) proliferation markers, as well as of cyclin D1 activator of proliferation (from 1.04 ± 0.20 to 0.35 ± 0.08, $p < 0.0001$) were observed. Vice versa, mRNA levels of inhibitors of proliferation increased (p21 from 1.21 ± 0.29 to 1.90 ± 0.54, $p < 0.0001$; p27 from 0.87 ± 0.46 to 1.57 ± 0.41, $p < 0.0001$). These findings suggest that high concentrations of progesterone have anti-proliferative effect on the BxPC3 cells at 48 and 72 h after exposure. This was confirmed by the results of MTT test. Interestingly, 1 μM of progesterone was almost as effective in inhibiting the cell viability, as 20 μM of progesterone after 72 h of exposure ($p < 0.0001$). As shown, a significant change in *PCNA* gene expression alone was detected at this concentration of hormone, but a tendency to the change of *MKI-67* and *CDKN1B* genes' transcription at 1 μM progesterone was also observed. The effect of progesterone on cell proliferation seems to be additive, so even the slight but combined changes in particular genes' expression at 1 μM hormone may result in significant alteration of proliferation rate at the cellular level. At 20 μM progesterone, although particular gene expression changes are more pronounced, the proliferation rate doesn't differ significantly from that at low-dose of hormone. Furthermore, at post-transcriptional and post-translational level, progesterone can affect the Ki67, PCNA, cyclin D1, p21, and p27 proteins, and regulate other proliferation-related genes and proteins [1,16,21–23,47]. In our study, human BxPC3 pancreatic adenocarcinoma cells were found to be more sensitive to anti-proliferative action of progesterone than glioblastoma and neuroblastoma cells [22,23], probably, due to high levels of mPRs expression. Based on our results, we have confirmed the anti-proliferative effect of high physiological progesterone concentrations on BxPC3 cells. Perhaps, the same effect can be realized in vivo against some types of pancreatic tumors. Pancreatic cancer accounts for 277,000 new cases diagnosed each year in the world [57]. While currently pancreatic cancer represents the fourth leading cause of cancer death, the search for new effective cure is an actual problem because of the relative poor efficacy of systemic chemotherapy treatments. More basic researches are needed to clarify the role of mPRs in mediating the action of progesterone. It might help to extend the scope of steroid hormone application for therapy of tumors of non-reproductive organs. A probable limitation for using progesterone as antineoplastic therapy is the fact that oral progesterone must be applied at very high dosages, since the metabolism rate is very high. This work is the first stage of the research of the progestin effects in pancreatic tumor cells. In the further, selective progesterone analogs may be used which act only through mPRs and are more stable in blood stream. The steroid specificities of human mPRs differ markedly from those of nPRs. These marked differences in binding affinities to various ligands suggest that selective mPRs modulators can be developed to independently explore progestin actions in target tissues mediated by each of these two receptor systems. The next stage of the research will include the confirmation of the ex vivo data on animal models with the use of selective agonists for mPRs and the investigation of the signaling pathways activated by membrane receptors in BxPC3 cells after interaction with progesterone.

4.4. Conclusions

This study was the first to investigate the mPRα, -β, -γ mRNA and nPR mRNA expression in BxPC3 cells. An absence of nPR and the presence of mPR of α, β, γ subtypes were shown both at the mRNA and protein levels. The data obtained clearly demonstrate that BxPC3 cells are the convenient object to study the effects of progesterone through membrane receptors. Importantly, the characteristics of [^{3}H]-progesterone—binding to BxPC3 cells showed high affinity similar to that we observed for human mPRα in the heterologous yeast expression system. Using the BxPC3 cell line as an experimental model, we confirmed that progesterone at concentrations of 1 μM and 20 μM negatively regulated cell proliferation through mPRs. Our findings represent a modest first step in evaluating progesterone as an adjunct therapy for pancreatic adenocarcinoma.

Conflicts of interest

None of the authors had a conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2016.07.007.

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