

Original Article

Loss of progesterone receptor through epigenetic regulation is associated with poor prognosis in solid tumors

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Received February 26, 2020; Accepted May 30, 2020; Epub June 1, 2020; Published June 15, 2020

Abstract: Background: Hormonal therapy using progestins, acting through the progesterone receptor (PR), is a well-established method to treat uterine endometrial hyperplasia and carcinoma. Recent population studies indicate that progestin exposure significantly reduces the incidence of ovarian, pancreatic and lung cancers in addition to endometrial cancer in women. This unexpected differentiating function of progestin in organs outside of the reproductive system led us to hypothesize that progestins/PR are protective against cancer development and progression in many tumor types. Methods: The Cancer Genome Atlas, OncoPrint and Prognostic Databases were searched to determine the relative expression of PR in tumors from multiple sites, and clinical outcomes linked to PR expression were determined. In addition, mRNA and protein expression were evaluated using real-time PCR and Western blotting. Chromatin immunoprecipitation (ChIP) assay was performed to understand the PR downregulation mechanisms in tumor cells and patient samples. Methylation-specific PCR was conducted to survey the PR methylation status. The Student's t-test were performed to determine significance. Flow cytometry was used to quantify apoptotic cells. Results: Low PR expression levels were consistently linked to less favorable clinical outcomes in endometrial, pancreatic, ovarian and non-small cell lung cancers. Clinical specimens and cell lines from these cancers demonstrate low levels of PR, and we now report that the mechanism for loss of PR is mediated through epigenetic repression. However, PR silencing can be overcome with epigenetic modulators. Histone deacetylase inhibitor (LBH589) and hypomethylating agent (5-aza-decitabine) restored functional PR expression at both the mRNA and protein levels and promoted marked cell death through induction of apoptosis in the presence of progesterone. Conclusions: Our studies support the possibility that progestin therapy in combination with epigenetic modulators, a concept we term "molecularly enhanced progestin therapy", is an approach worthy of study for malignancies originating from tissues outside of the reproductive tract.

Keywords: Progesterone receptor, progestin, epigenetic regulation, ovarian cancer, lung cancer

Introduction

Progesterone's protective function in endometrial cancer and other cancers

The uterine endometrium is exquisitely sensitive to steroid hormones. Estrogen acting through the estrogen receptor (ER) drives proliferation, while progesterone acts through the progesterone receptor (PR: PRA and PRB) to counteract these effects by inhibition of proliferation, inducing differentiation, promoting

apoptosis, and inhibiting invasion. Both PRA and PRB are derived from the *PGR* gene. PRB is the full length of PR, while PRA is missing the first 164 amino acids. Specifically, PR-A is essential for proper uterine development and reproductive activities, while PRB has the dominant function in inhibiting proliferation in endometrial cancer cells [1-3]. Given progesterone's protective function in endometrial cancer, progesterone and its synthetic analogues (progestins) have been a traditional choice for hormonal therapy for more than 70 years [1, 2].

A recent provocative report from Finland demonstrated that use of a progestin-eluting intra-uterine device (IUD) for menorrhagia significantly reduced the risk of endometrial cancer [4]. In this study of 93,843 women, the incidence of endometrial adenocarcinoma was reduced by 54% (odds ratio =0.46, 95% CI 0.33-0.64, $P<0.001$), and women with two or more IUDs had a 75% reduction in incidence (odds ratio =0.25, 95% CI 0.05-0.73, $P<0.01$). Somewhat surprisingly, these effects were not limited to endometrial cancer, with reduced incidences of ovarian (OR=0.60), lung (OR=0.68), and pancreatic (OR=0.50) cancers also documented in this study [4]. This unexpected protective function of progestin in organs outside of the reproductive system led us to hypothesize that tumorigenesis and possibly progression in a number of solid tumors could be countered by progesterone.

Progestin therapy in multiple cancer types

Progesterone/progestin therapy has been used to treat malignancies other than endometrial cancer. Studies in ovarian cancer date back to at least 1962 [5]. Jolles reported that in 10 recurrent or refractory ovarian cancer patients, treatment with progesterone achieved a 50% clinical response rate [5]. Progestin treatment has since been used mostly in recurrent or refractory ovarian cancer patients who have failed first-line therapy. Zheng summarized 13 clinical trials with total 432 patients and reported a complete response rate of 2.3% (10/432), a partial response rate of 4.9% (21/432) and stable disease in 10.9% (47/432) [6]. When progestin was used as first-line therapy for ovarian endometrioid carcinomas, the overall response rate was 53.5%, possibly owing to positive hormone receptor expression; these tumors were 81.3% positive for ER and 72.1% positive for PR [6].

The most consistently reported preventive effects of progestins are against ovarian and endometrial carcinogenesis. Epidemiological evidence from 20 studies provides strong support that progestin-containing contraceptives reduce ovarian cancer risk by an average of 35%. Even short-term use (6 months or less) appears to be protective [7].

There are no reports of progestin therapy for patients with pancreatic or lung cancer. One

study reported that for women with non-small-cell-lung cancer (NSCLC, $n=485$), hormone therapy containing estrogen plus progestin significantly increased survival. The median survival time was 80 months for women receiving hormone therapy versus 37.5 months for women not receiving hormone therapy [8]. Progesterone treatment has been tested in pancreatic and lung tumor xenograft models and cell lines. In NSCLC, progesterone treatment led to growth inhibition of PR positive tumor xenografts and induction of apoptosis [9], in agreement with clinical data that the presence of PR was correlated with longer survival in NSCLC patients [9]. Progesterone also has been shown to inhibit migration and invasion of lung cancer cell lines [10]. There is increasing evidence that ER, the androgen receptor (AR) and PR are expressed in the pancreas, which suggests that the pancreas is a sex steroid-dependent tissue [11]. Progesterone treatment in pancreatic cancer xenograft models was tested as early as 1995 [12]. Progesterone inhibited pancreatic tumor cell growth in a PR-dependent manner [12]. Other studies confirm that progestin treatment results in induction of apoptosis in human pancreatic carcinoma cells and a delay in tumor growth [13].

Progesterone receptor expression

Response to progestin therapy positively correlates with the expression of PR [9, 14, 15]. PR expression is a marker for favorable outcomes for ovarian cancer patients [16-18]. The outcome is even better for patients with combined ER+ and PR+ tumors [19], and loss of PR expression is correlated with increasing ovarian cancer grade [20]. Lee et al studied 322 cases of ovarian tumors and found that 73.8% were PR negative. Those patients with low PR expression had worse overall and disease-specific survival [18]. In another larger study, Sieh et al studied 2933 cases of ovarian tumors from the Ovarian Tumor Tissue Analysis Consortium and found that PR expression correlated with favorable disease-specific survival in endometrioid ovarian carcinoma ($n=484$) and high-grade serous ovarian carcinoma ($n=1742$) [17]. However, the mechanisms underlying PR down-regulation in patients with ovarian cancer remain unclear. PR expression has also been studied in non-small-cell lung cancer. As early

as 1996, Kaiser et al reported that PR expression was low in 13 of 17 NSCLC cell lines [21]. Ishibashi et al studied 228 cases of NSCLC tumors and found that more than 50% of tumors were negative for PR expression. Patients with low PR expression experienced higher rates of lymph node metastasis, less histologic differentiation and worse clinical outcomes [9]. Skjefstad et al collected 335 NSCLC tumors and reported negative PR expression in 220 (66.7%) of the tumors [22].

PR expression has rarely been studied in the pancreas and pancreatic cancer, yet in one study, PR was the most consistently expressed sex steroid hormone receptor with the strongest immunoreactivity [23]. PR expression was demonstrated in multiple subtypes of pancreatic cancer, including mucinous cystadenomas and cystadenocarcinomas, papillary cystic neoplasms and endocrine pancreatic tumors. The absence of PR correlated with worse outcomes in pancreatic mucinous and endocrine neoplasms [23]. Kim et al reported that in 298 cases of pancreatic neuroendocrine tumors, loss of PR expression was associated with tumor progression, shorter survival and increased tumor size in neuroendocrine microadenomas (n=21) and Pancreatic Neuroendocrine Tumor (n=277) [24]. Abe et al reported that PR expression was high in 3 of 9 pancreatic cancer cell lines, and only PR positive cells were growth inhibited after progestin treatment [13].

Molecularly enhanced progesterone therapy for endometrial cancer and other cancers

We have reported the different mechanisms contributing to low PR expression in endometrial cancers, most notably epigenetic silencing of PR transcription [25, 26]. We also demonstrated that epigenetic modulators can restore functional PR expression, and the combination of progesterone with epigenetic modulators can sensitize endometrial cancer to progestin therapy. We termed the combination of epigenetic modulators with traditional hormonal therapy “molecularly enhanced progestin therapy”. We now hypothesize that PR expression is low in the majority of ovarian, pancreatic and lung cancer cells through epigenetic repression; therefore, molecularly enhanced progesterone therapy may reverse PR silencing and amplify

the differentiating effects of progesterone in cells from multiple tumor types. These studies have the potential to set the stage for creative drug combinations to treat solid tumors not traditionally considered to be responsive to hormonal therapy.

Material and methods

Antibodies and reagents

Progesterone (#P6149) was obtained from Sigma Aldrich and resuspended in ethanol. Panobinostat (LBH589) was purchased from Selleck Chemicals and resuspended in DMSO. Antibodies against PRA/B (#3153), PRB (#3157), FOXO1 (#2880), Myc (#13987), p21 (#2947), p27 (#3686) and cyclin D1 (#2926) were from Cell Signaling. The β -actin antibody (#A1978) was obtained from Sigma Aldrich. Charcoal-stripped serum (#12676-011) was obtained from Gibco.

Cell lines and cell culture

Seventeen cancer cell lines were collected and used for these studies. Ishikawa H, ECC1 and Hec50 are endometrial cancer cells. MiaPaca-2, Panc-1, AsPC-1 and BxPC-3 are pancreatic cancer cell lines. A549, H1299, H292 and H358 are non-small-cell lung cancer cells. OV-90, HEY, TOV-112D, Ovar-8, ES-2 and Ovar-3 are ovarian cancer cell lines. The growth media and the resources are listed in [Table S1](#). All cell lines have been authenticated using STR analysis by BioSynthesis.

TCGA, Oncomine and Prognostic data analysis

Patient outcome information was downloaded from the Cancer Genome Atlas Data Portal (<https://tcga-data.nci.nih.gov/tcga/>) maintained by the National Cancer Institute (NCI) and the National Human Genome Research Institute. Gene expression was assayed based on RNASeq conducted on the Illumina platform and was downloaded from NCI's Cancer Genomics Hub (<https://cghub.ucsc.edu/>). PR mRNA expression data from normal and tumor specimens were obtained from the Oncomine Database (<https://www.oncomine.org>). The Prognostic Database (PROGgene V2) was used to determine the correlation of PR expression with patient survival [27].

Adenoviral expression of PR

Cells were transfected with adenoviral vectors encoding PRA, PRB, PRA/B or an empty vector control (Adcontrol) using a multiplicity of infection (MOI) of 5 viral particles per cell as previously described [28, 29]. An MOI of 5 viral particles per cell was applied to obtain PR expression levels roughly equivalent to the late proliferative phase of the menstrual cycle.

Flow cytometry

H1299 cells were transduced with an empty virus or PRAB adenovirus followed by treatment with or without progesterone (P4) and incubated for 15 minutes with PE-Annexin V (BD Pharmingen) in the presence of CaCl_2 . Hoechst 33258 (Thermo Fisher) was used to label the nucleus. Flow cytometry was used to read the population of apoptotic cells labeled with PE-Annexin V.

Real-time PCR

Quantitative real-time PCR (qPCR) was performed in triplicates as previously described [25, 26, 28]. Primer sequences are listed in [Table S2](#). Comparisons of normalized expression values (ΔCt) were applied using the conventional $\Delta\Delta\text{Ct}$ fold change method [30].

Western blotting

Expression of PR, FOXO1, p21, p27, cyclin D1 and β -actin were assessed by Western blotting as previously described [25, 26].

Methylation-specific PCR

Genomic DNA was prepared using the DNeasy kit (Qiagen) followed by bisulfite conversion (ZYMO Research). Methylation-specific PCR was amplified with the primers for either methylated (PRB-M) or unmethylated (PRB-U) PRB promoter CpG islands as previously reported [31]. PCR products were resolved by agarose gel electrophoresis and the DNA bands were visualized by ethidium bromide staining.

Luciferase assay

The pQXCIN luciferase vector (a gift from Dr. Michael Henry, the University of Iowa) was transfected into GP2-293 cells for retrovirus production. Cells were transfected with lipofectamine 2000 (Invitrogen), and stably transfected cells were selected with neomycin

(G418) treatment. Luciferase activity was determined in triplicates using the Luciferase Reporter Assay System (Promega) and quantified with Synergy HT (BioTEK).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling). Results are representative of at least three independent experiments.

Normal and malignant ovarian tissue

Tissue from non-malignant ovaries isolated from post-menopausal women (n=5) and ovarian serous adenocarcinomatous tumors (n=14) were obtained from University of Iowa, Department of Obstetrics and Gynecology Tissue Bank under informed consent (IRB#201605-841). Patient age and tumor clinical characteristics are listed in [Table S3](#). Following histologic confirmation, all specimens were snap-frozen and stored at -80°C .

Statistical analysis

Student's *t*-test was used for comparisons between two groups. All pairwise multiple comparisons were performed by one-way ANOVA using the Holm-Sidak method or Bonferroni post-hoc tests with the overall significance level at 0.05 ($P \leq 0.05$).

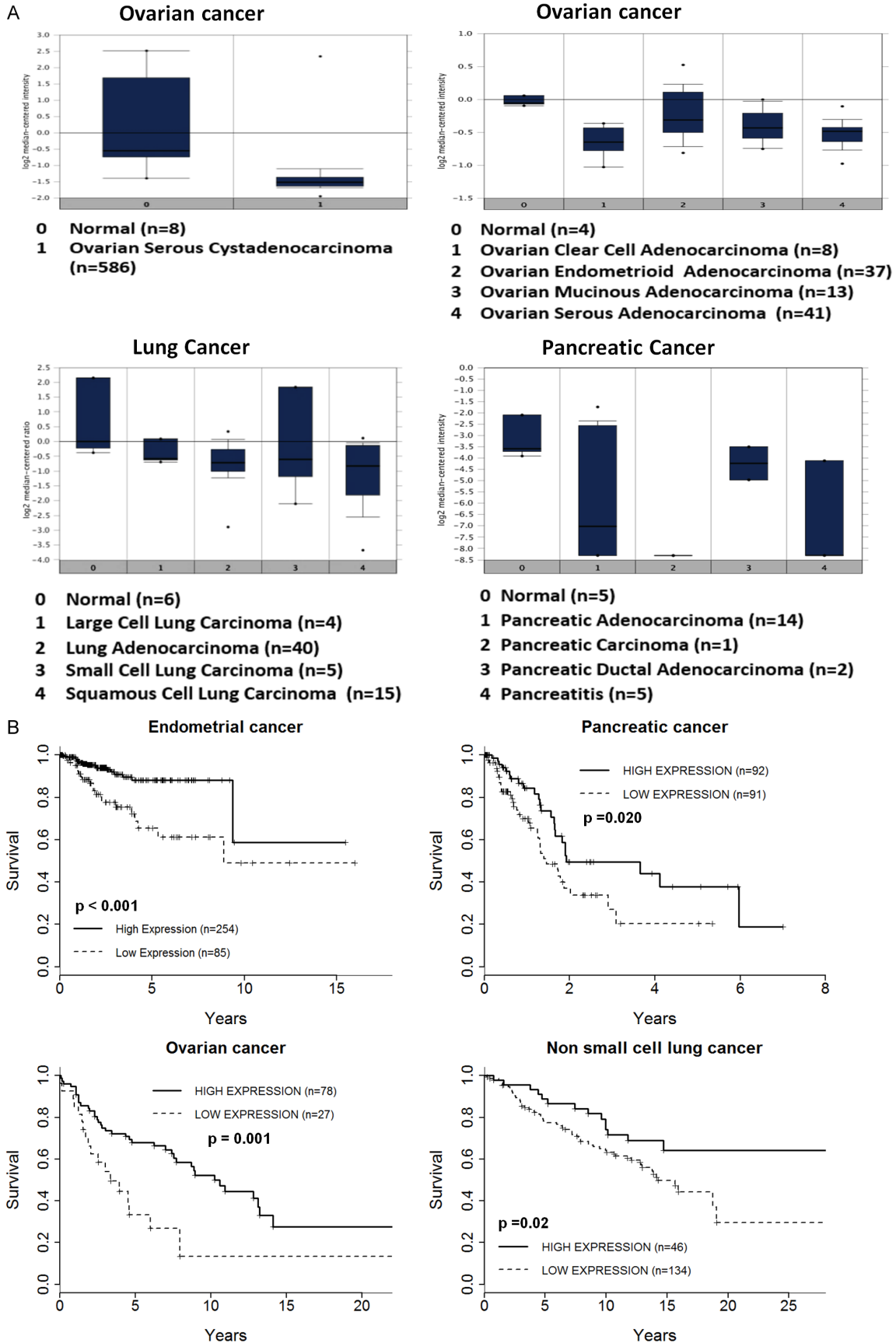
Results

PR expression is lost in multiple solid tumors and predicts unfavorable clinical outcomes

We analyzed PR mRNA expression data from the Cancer Genome Atlas (TCGA) and OncoPrint Database. In 586 ovarian cancers from TCGA, we found that PR expression is significantly lower than normal non-malignant tissue (**Figure 1A**). Analysis of another ovarian cancer database (Hendrix Ovarian [32]) also confirmed that PR expression is low and consistently across multiple forms of ovarian cancer (**Figure 1B**). From the OncoPrint dataset, we found that PR expression is lost in lung and pancreatic cancer specimens compared with normal tissue (**Figure 1A**).

To assess the relationship between PR expression and clinical outcomes, we further queried TCGA and supplemented these data with find-

PR loss in solid tumors



PR loss in solid tumors

Figure 1. PR expression is lost in multiple solid tumor types and predicts unfavorable clinical outcomes. A. *PGR* mRNA expression levels were evaluated from tumors compared to nonmalignant tissues reported in the Oncomine Database. B. Kaplan-Meier survival analysis of PR mRNA levels in endometrial cancer and pancreatic cancer from TCGA dataset. Ovarian cancer (GSE73614) and NSCLC cancer (GSE50081) data were analyzed from the Prognostic Database. Patients were divided into two groups based on relative *PGR* mRNA levels: high expression and low expression. Cut offs for high and low expression were determined based upon the level of endogenous PR expression in each tumor type. For endometrial and ovarian cancer with overall more robust PR expression, the lowest quartile was used to select the low group, with the remainder assigned into the high group. For pancreatic cancer, the high and low demarcation was the median PR expression value. For non-small-cell lung cancer with overall low PR expression, the highest quartile was used to select the high group, with the remainder assigned into the low group. Significance was assigned as $P < 0.05$ by the log-rank test.

ings from the Prognostic Database. A notable finding was that high PR mRNA (*PGR*) expression predicts longer overall patient survival (OS) for endometrial and pancreatic cancer from TCGA; ovarian (GSE73614) and NSCLC (GSE50081) patients also achieved longer OS as determined from the Prognostic Database (**Figure 1B**). Whether PR is a marker for a more differentiated cancer cell subtype, and thus is more highly expressed in cancers with better prognosis, or alternatively, the improved outcomes are a direct result of PR expression, is not yet known. However, the studies below indicate that progesterone/PR limit cell growth and enhance differentiation in the representative cell models studied.

Figure S1: Screening PR mRNA expression in multiple cancer cell lines

PR mRNA (*PGR*) expression was tested by qPCR in four pancreatic, four NSCLC and six ovarian cancer cells lines. Comparisons are provided with two well-characterized endometrial cancer cell lines, Ishikawa (PR positive) and Hec50 (PR negative). Overall, PR is expressed, albeit at low levels, in the majority of cell lines studied (**Figure S1**).

Expression of exogenous PR activates PR downstream genes

As a transcription factor, progesterone-activated PR directly regulates the expression of its target genes through progesterone response elements (PREs). Promoters with PREs include FOXO1, Myc and Cyclin D1, among others. Secondly, other important genes are regulated by progestins through alternative promoters such as Sp1. These indirectly regulated genes include p21 and p27 [33]. To determine progesterone's direct effects in NSCLC, pancreatic and ovarian cancer cells, we expressed PRA and PRB protein through adenovirus transduction. Protein expression was confirmed by

Western blotting from cells transfected with expression viruses encoding PRA only, PRB only or PRAB (**Figure 2A-F**). Addition of progesterone (+P4) activated PRA, PRB or PRAB in all six selected cell lines. As a positive control, PR was also expressed by adenovirus in Hec50 (PR null) serous endometrial cancer cells. As a result of progesterone/PR activity in these endometrial cancer cells, pro-growth factors Myc and Cyclin D1 were mostly downregulated. On the other hand, growth inhibiting factors such as p21 and FOXO1 were upregulated in some cell lines. These findings are consistent with the known effects of progesterone/PR on these genes [28, 33, 34] (**Figure 2A**).

Cells from non-reproductive tissue origin responded similarly. In H1299 NSCLC cells, expression of PRA protein decreased Myc protein (**Figure 2B**), while addition of P4 ligand further diminished the expression of this important oncogene. Addition of P4 to PRA-expressing H1299 cells resulted in the upregulation of p27, p21 and FOXO1, but decreased Cyclin D1 expression (**Figure 2B**). Co-expression of PRA and PRB had the strongest effect on protein expression of p27. There was repression of pro-growth factors such as Myc and Cyclin D1 expression, while at the same time, anti-growth factors such as p27, p21 and FOXO1 were significantly upregulated (**Figure 2B**). Similar results were obtained when another NSCLC line was studied, H358 (**Figure 2C**). Compared with NSCLC H1299 cells, the pancreatic cancer cell line Panc-1 is not as sensitive to the growth regulatory effects of progesterone/PR, although modest degrees of Myc down regulation and p21 and FOXO1 upregulation were observed, particularly in the presence of PRB (**Figure 2D**).

We also confirmed the effects of progesterone through PR in two ovarian cancer cell lines, ES-2 and Ovacar-8. In ES-2 cells, addition of P4

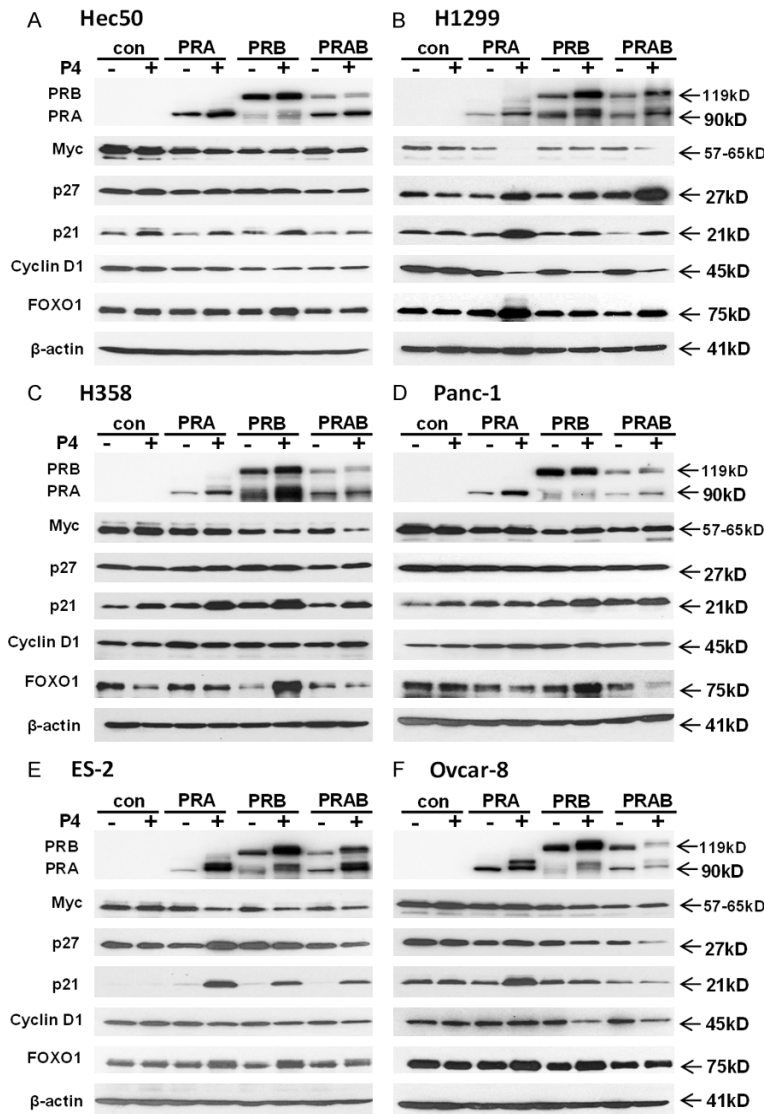


Figure 2. Expression of exogenous PR activates PR downstream genes. The indicated cell lines grown in media supplemented with charcoal-stripped serum were transduced with control adenovirus (Adcontrol) or adenovirus containing PRA, PRB, or PRA+PRB for 24 hr, followed by 100 nM P4 for an additional 24 hr. Expression of PR, Myc, p27, p21, Cyclin D1, FOXO1 was evaluated by Western blotting. β -actin served as the loading control.

to PRA expressing cells decreased Myc expression and increased p27, p21 and FOXO1 expression (**Figure 2E**). In Ovarcar-8 cells, progesterone increased p21 and FOXO1 expression in PRA expressing cells, while expression of PRB and PRAB decreased Cyclin D1 expression (**Figure 2F**).

Expression of exogenous PR induces cell death

We next asked if expression of PR and treatment with progesterone induces cell death con-

sistent with previous findings in endometrial cancer cells [29, 35]. Using NSCLC H1299 cells as a model (since these cells were shown to be the most sensitive cells to progesterone treatment), addition of progesterone to H1299 cells in the presence of PRAB induced apoptosis as shown by accumulation of annexin V positive cells (**Figure 3A**). Cell death resulting from progesterone/PR treatment resulted in the appearance of fragmented debris (**Figure 3B**). The impact of therapy on cell viability, as assessed by the number of attached cells in culture dishes, was also determined. The attached (living) cells were stained with crystal violet after transduction with viruses encoding PRA and PRB with or without P4 treatment. Cell numbers decreased dramatically after P4 treatment specifically in PRA or PRAB expressing cells (**Figure 3C**). Western blotting confirmed the PARP and caspase-3 cleavage after PRAB+ P4 treatment. Cell viability was quantified with luciferase activity since only living cells have intact luciferase activity. Since luciferase activity is an easy and quantitative method to monitor cell viability, we evaluated luciferase activity in Hec50, H358, A549 and Ovarcar-8 cells to validate PR

effects on cell proliferation. Progesterone was added and found to promote death in PRA, PRB and PRAB-expressing cells.

Epigenetic modulators restore functional PR expression

We next explored the use of epigenetic modulators to restore functional PR expression. NSCLC cells H358 and A549, pancreatic cancer cells Panc-1 and Miapaca-2 and ovarian cancer cells TOV-112D, Ovarcar-3, ES-2 and Ovarcar-8 cells were treated with clinically rele-

PR loss in solid tumors

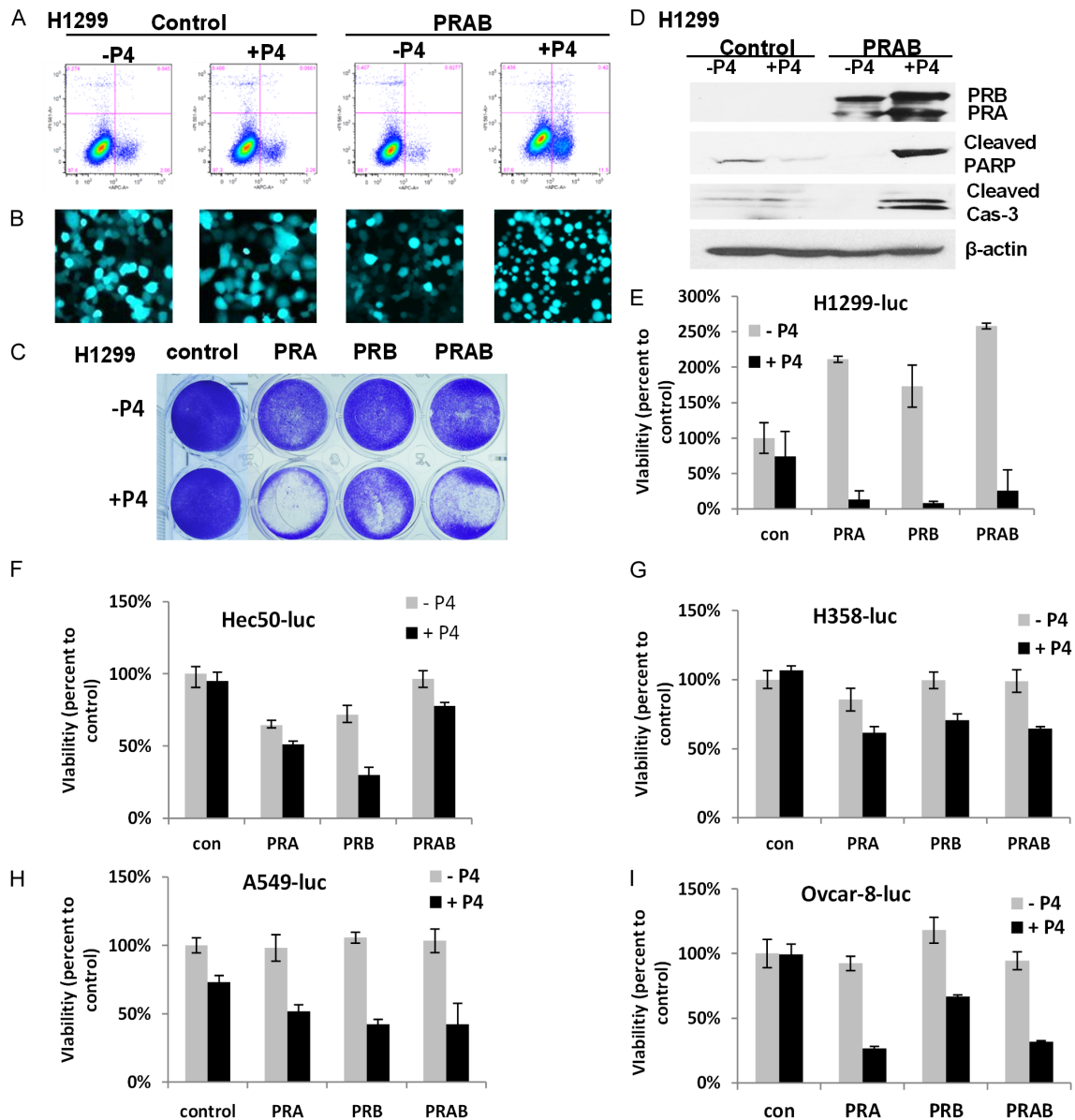
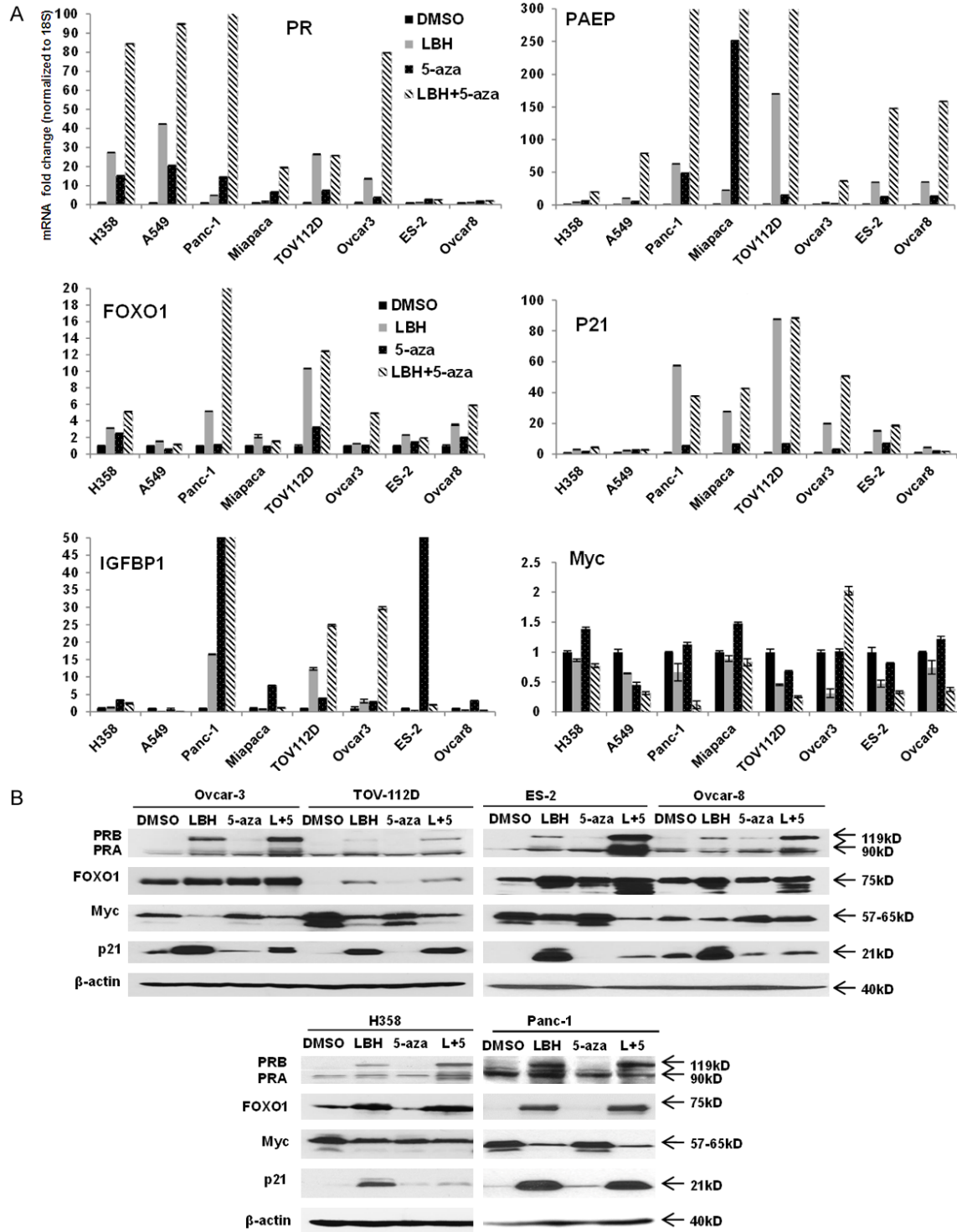


Figure 3. Expression of exogenous PR induces cell death. H1299 NSCLC cells were transduced with control adenovirus or adenovirus containing GFP-PRAB for 24 hr, followed by 100 nM P4 for an additional 48 hr. A. Cells were stained with APC-annexin V and PI and detected by flow cytometry. B. GFP expressing whole cells and cell fragments (small dots) were imaged with a fluorescent microscope. C. Attached cells were stained with crystal violet. D. Cleaved PARP and caspase-3 were evaluated by Western blotting. E-I. Cell viability was determined by luciferase activity.

vant doses of the histone deacetylase inhibitor (HDACi) LBH589, the hypomethylating agent 5-aza-decibatin (5-aza-dC, a DNMTi) or the combination in an attempt to increase *PGR* mRNA expression. Treated cells demonstrated the upregulation of numerous targets of HDAC inhibitors as well as DNMT inhibitors including PR (Figure 4A). The effects were cell specific: *PGR* mRNA was enhanced by 10 to 40-fold in

H358, A549, Panc-1 and Ovar-3 cells, and 3 to 10-fold in Miapaca-2, TOV-112D, ES-2 and Ovar-8 cells. Treatment with 5-aza-dC also increased *PGR* mRNA expression as well, albeit the effects were more modest compared to the HDACi. The combination of LBH589 and 5-aza-dC increased *PGR* mRNA significantly more than either drug alone. Western blotting supported that the enhanced mRNA was translat-

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ed into PR protein (**Figure 4B**). In addition, genes known to be regulated by both PR and

epigenetic modulators were induced, including progesterone associate endometrial protein

(Glycodelin, PAEP), FOXO1, p21 and insulin-like growth factor binding protein 1 (IGFBP1), while Myc mRNA and protein expression was reduced (**Figure 4A** and **4B**).

Figure S2: Epigenetic modulators reduce cell viability in multiple cancer cells lines.

The impact of epigenetic modulators on cell viability, as assessed by the number of attached cells in culture dishes, was also determined. Attached (living) cells were stained with crystal violet after treat with different dose of LBH589 or LBH589 in combination with hypomethylating agent 5-aza for 72 hours. Cell numbers decreased dramatically after the epigenetic modulators' treatment (**Figure S2**).

Epigenetic modulators sensitize cells to progesterone treatment

Next, we explored whether the PR induced by epigenetic modulators further sensitizes cells to progesterone treatment. **Figure 5A** demonstrates that the combination of LBH589 with 5-aza increased PR and its target genes in ES-2, Ovar-3, H358 and A549 cells. These cell models were chosen because of the dramatic increase in PR protein in response to therapy. The addition of progesterone further promoted the expression of PR and resulted in upregulation of PR target genes including *AREG*, *PAEP*, *FOXO1*, *p21* and *IGFBP1* and downregulation of oncogene *Myc*. We tested whether the upregulated PR expression resulted in the expected inhibition of cell growth in response to progesterone treatment. LBH589+5-aza treatment decreased cell viability in ES-2 cells, and the addition of progesterone further decreased cell growth compared to cells that were treated with vehicle alone (**Figure 5B**). Similar growth inhibition was observed in A549 and MiaPaca-2 cells in response to progesterone treatment (**Figure 5B**).

Potential mechanisms of PR downregulation

The ability of epigenetic modulators to restore PR expression indicates that PR is repressed at the epigenetic level. Our group and others have reported that the poly-comb repressing complex 2 (PRC2) binds to the *PGR* promoter and inhibits transcription [25, 36]. Therefore, chromatin immunoprecipitation assays (CHIP) were performed in ES-2 and H358 cells to assess occupancy on the promoter. Histone modification and RNA polymerase II were used as mark-

ers of *PGR* gene transcriptional status based upon the fact that H3 lysine 4 methylation (H3K4Me3) indicates active gene transcription, while H3K27 methylation (H3K27Me3) and H3K9 methylation (H3K9Me3) are consistent with transcriptional repression. We hypothesized that the mechanism of LBH589+5-aza induced PR expression is the dissociation of the PRC2 component SUZ12 from the *PGR* promoter, increasing binding of H3K4Me3 and enhancing RNA polymerase II binding. **Figure 6A** indicates that LBH589+5-aza treatment increased the binding of activation marker H3K4Me3 and decreased the repression markers H3K27Me3 and H3K9Me3 to the *PGR* promoter in ES-2 cells. Dual therapy also enhanced the binding of RNA polymerase II to the *PGR* promoter in this cell model. In H358 cells, a similar pattern was noted with the exception of the repression complex SUZ12, which did not dissociate from the *PGR* promoter in these experiments. Nevertheless, RNA polymerase II was recruited to the *PGR* promoter, and H3K9Me3, one of the markers of transcriptional repression, was reduced. We propose that this finding indicates that other mechanisms of repression in addition to modulation of the PRC2 and SUZ12 contribute to PR silencing, and that these effects are somewhat cell and tumor type specific.

To address alternative mechanisms to explain epigenetic silencing of PR, *PGR* promoter methylation was assessed, as previously reported in endometrial cancer [25, 31]. We hypothesized that PR silencing may also occur at this level and may be important in pancreatic, NSCLC and ovarian cancer cells. Therefore, we performed methylation-specific PCR on the *PGR* promoter. Our studies indicate that the PRB promoter is highly methylated in MiaPaca-2, H358, Ovar-8 and ES-2 cells. The hypomethylating agent 5-aza-dC partially reversed the methylation of PRB in H358 and Ovar-8 cells compared to control samples and compared to ES-2 cells, where the methylation signal is unchanged (**Figure 6B**). These data are consistent with the alternative modality of PR silencing in ES-2 cells through the poly-comb repressor complex.

Mechanisms of PR downregulation in ovarian tumors

To prove that the above-mentioned PR downregulation mechanisms occur in patient tissue,

PR loss in solid tumors

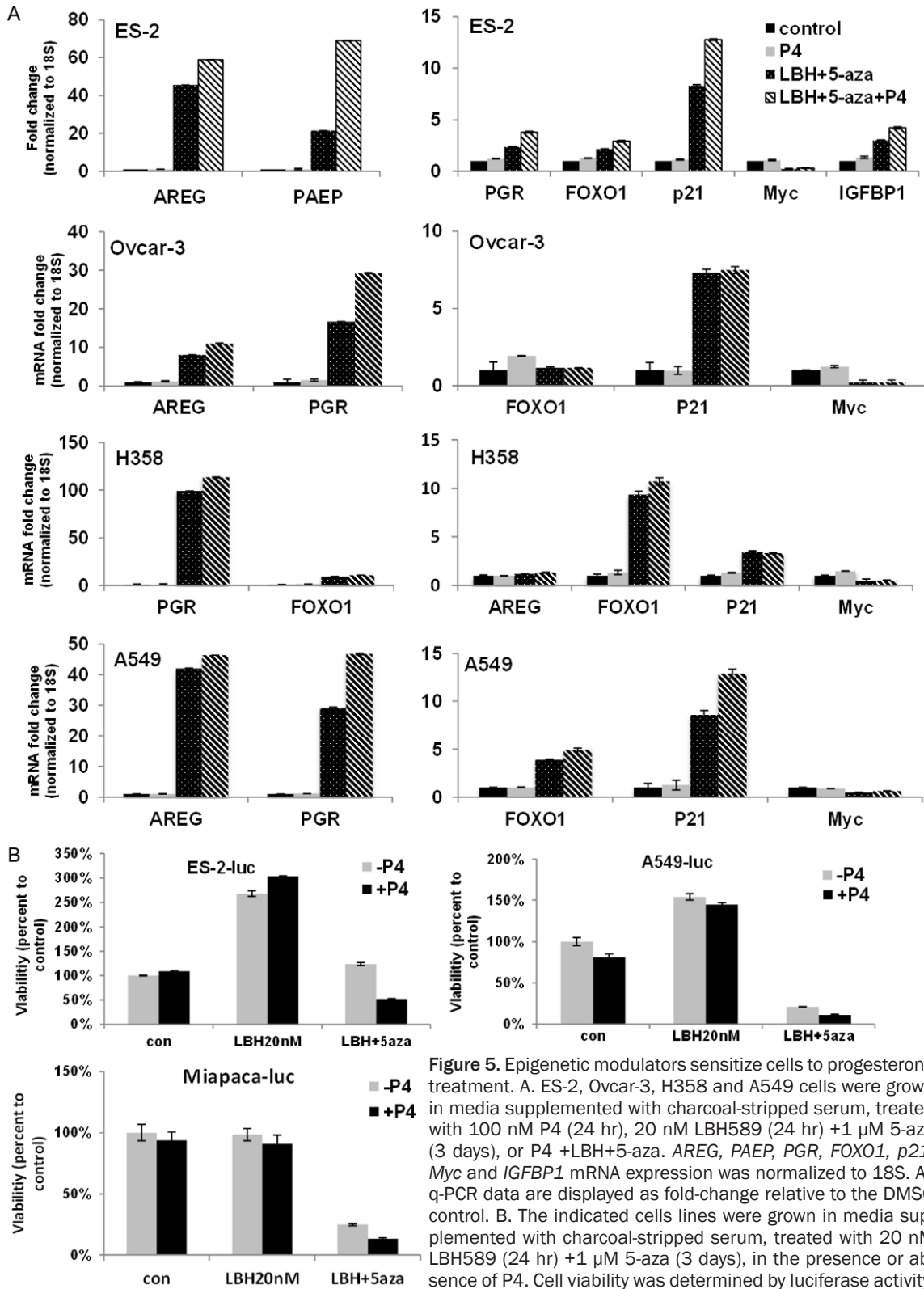


Figure 5. Epigenetic modulators sensitize cells to progesterone treatment. A. ES-2, Ovar-3, H358 and A549 cells were grown in media supplemented with charcoal-stripped serum, treated with 100 nM P4 (24 hr), 20 nM LBH589 (24 hr) +1 μ M 5-aza (3 days), or P4 +LBH+5-aza. *AREG*, *PAEP*, *PGR*, *FOXO1*, *p21*, *Myc* and *IGFBP1* mRNA expression was normalized to 18S. All q-PCR data are displayed as fold-change relative to the DMSO control. B. The indicated cells lines were grown in media supplemented with charcoal-stripped serum, treated with 20 nM LBH589 (24 hr) +1 μ M 5-aza (3 days), in the presence or absence of P4. Cell viability was determined by luciferase activity.

we performed tissue ChIP and DNA methylation studies from malignant and nonmalignant tissues collected from the University of Iowa

Tissue Bank with IRB approval. Cell extract from 14 ovarian tumors was compared to 5 non-malignant ovarian samples. Patient age

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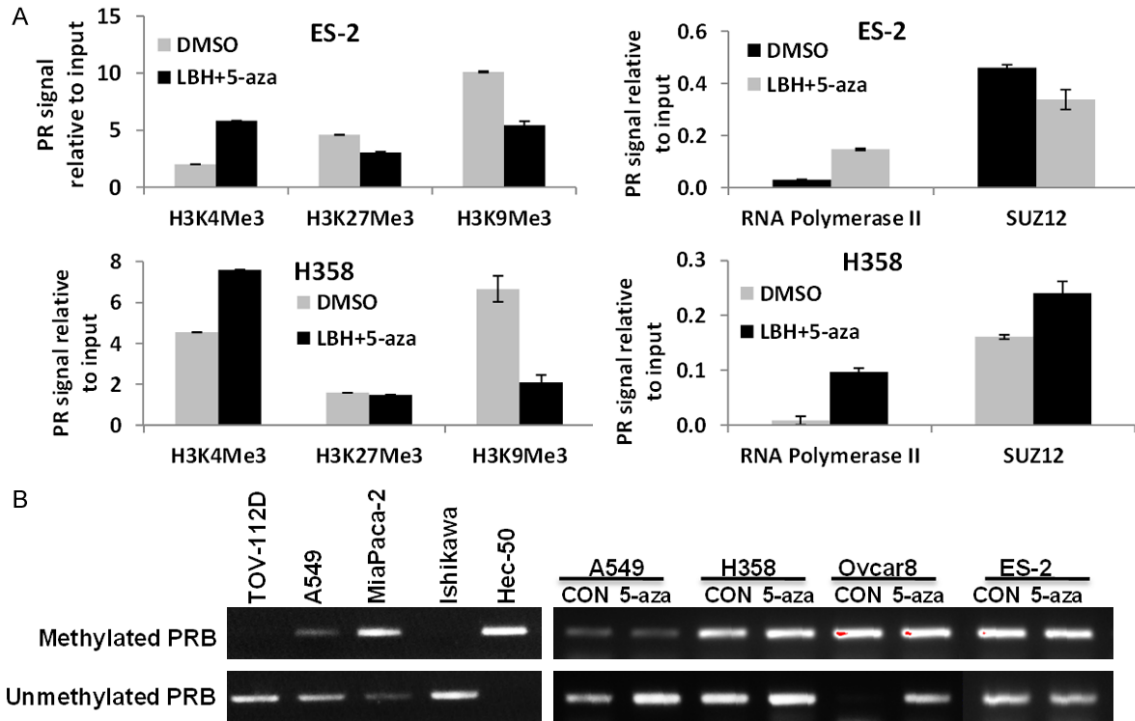


Figure 6. PR downregulation mechanisms in cell lines. A. ES-2 ovarian cancer cells and H358 NSCLC cells were treated with DMSO or 20 nM LBH589 (24 hr) +1 μ M 5-aza (3 days). ChIP followed by q-PCR for H3K4Me3, H3K27Me3, H3K9Me3, RNA polymerase II (RNA PII), and SUZ12 was used to determine recruitment of these factors to the *PGR* promoter. B. The indicated cells were treated with DMSO or 1 μ M 5-aza for 3 days. DNA methylation-specific PCR was performed using specific PRB methylated primers or PRB unmethylated primers.

and tumor clinical characteristics are listed in [Table S3](#). Real-time PCR was then carried out to screen for the mRNA expression of PR and its downstream genes *FOXO1* and *p21*, as shown in [Figure S3](#). We confirmed that *PGR*, *FOXO1* and *p21* mRNA expression was significantly lower in malignant versus nonmalignant tissue ([Figure 7A](#)). Little *PGR* DNA methylation was detected in non-malignant tissue (2 of 5, 40% weak *PGR* DNA methylation band), while 13 of the 14 (93% strong *PGR* DNA methylation band) tumor tissues showed marked *PGR* DNA methylation ([Figure 7B](#) and [7C](#)). Patient samples Ov2 (non-malignant), Ovc7, 33, 34 (malignant) were chosen for further study to determine *PGR* transcriptional status. Compared with normal tissue Ov2, in three tumors, a decrease in the activation marker H3K4Me3 and an increase in the repression marker H3K9Me3 binding on the *PGR* promoter was found ([Figure 7D](#)). Consistent with the repression of PR transcription in the three tumor samples, substantially less RNA polymerase II was detected binding to the *PGR* promoter ([Figure 7D](#)). Enhanced SUZ12 binding, as a reflection

of the presence of the PRC2 complex as a mechanism of repression, was not consistently found in the tumors compared with the nonmalignant Ov2 extract ([Figure 7D](#)), indicating that in these select specimens, the major repression mechanism is DNA *PGR* promoter methylation.

Discussion

It is striking that gynecologic cancers occur predominantly in post-menopausal women. Why is this true? First, of course, is the effect of age itself on carcinogenesis, where longer life exposure to carcinogenetic stimuli clearly is an independent risk factor. However, the loss of progesterone production from the ovary at menopause may indeed be an additional permissive event associated with carcinogenesis. Progesterone is a differentiating hormone for most epithelial cells [37-39]. When binding to PR, progesterone functions through genomic and non-genomic regulatory mechanisms to induce differentiation, apoptosis and cell cycle arrest. Progesterone also inhibits inflammation

PR loss in solid tumors

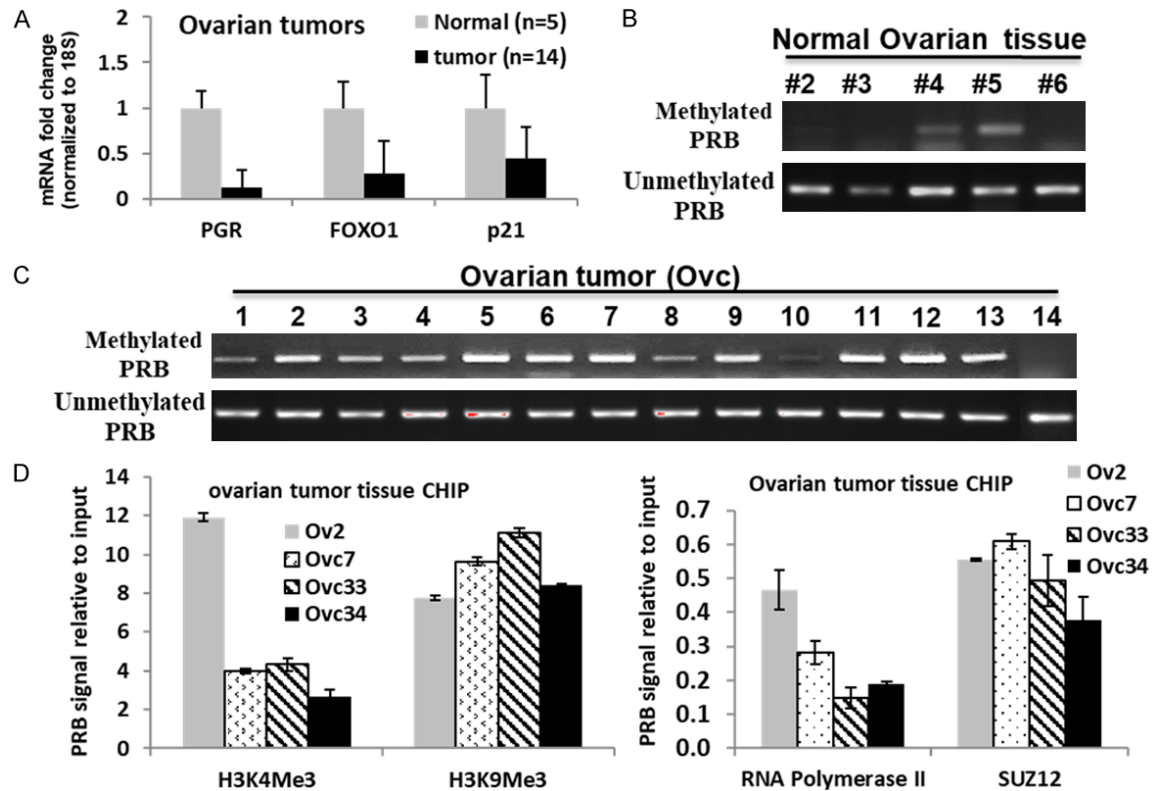


Figure 7. PR downregulation mechanisms in ovarian patient tumors. (A) Five nonmalignant ovarian tissues (normal) and 14 ovarian tumor tissues were collected. *PR*, *FOXO1* and *p21* mRNA expression was normalized to 18S, and all q-PCR data are displayed as fold-change relative to normal tissues. (B, C) DNA methylation-specific PCR was performed using specific PRB methylated primers or PRB unmethylated primers in normal tissues (B) or ovarian tumor tissues (C). (D) Ovarian tissue-ChIP followed by q-PCR for H3K4Me3, H3K9Me3, RNA polymerase II (RNA PII) and SUZ12 was used in nonmalignant ovarian tissue (Ov2) compared to three ovarian tumor tissues (Ovc7, Ovc33 and Ovc34) to determine recruitment of these factors to the *PGR* promoter.

and invasion [33, 40]. However, PR expression is reduced or lost in many solid tumors compared to nonmalignant tissues from those organs, and the lack of endogenous progesterone production in non-cycling women also limits the potentially positive impact this hormone may have to control tumor development and growth. Does progesterone act as a protective factor against the development of cancers even outside the reproductive tract? Could one take advantage of the growth limiting effects of progesterone on the epithelium in many malignancies if PR was expressed? These provocative questions spurred our interest in the studies reported herein. Therefore, the aims of this study were to 1) understand mechanisms of PR loss in multiple solid tumor types, 2) manipulate and restore functional PR expression and 3) determine strategies to re-sensitize cells to progestin therapy.

Our data indicated that epigenetic transcriptional repression associated with histone modification

and DNA methylation contributes to PR loss in cell models from multiple solid tumor types. We demonstrated significant binding of the histone repressive marker H3K9Me3 on the PR promoter region in ES-2 ovarian cancer cells as well as in NSCLC H358 cells. As a result, less binding of the histone activation marker H3K4Me3 and RNA polymerase II to the PR promoter occurs in both cell lines, indicating transcriptional repression. However, the repression can be reversed using targeted therapeutic agents. Epigenetic modulators such as the histone deacetylase inhibitor LBH589, particularly when combined with the hypomethylating agent 5-aza-decitibine (for reasons discussed below), can reverse this repression. As a result, increased H3K4Me3 and RNA polymerase II coupled with decreased H3K9Me3 binding to the PR promoter is observed. We also demonstrate that less SUZ12 and H3K27Me3 occupied the PR promoter as a consequence of epigenetic modulation in ovarian cancer ES-2 cells. This observation is consistent with SUZ12

as an important component of the poly-comb repressor complex 2 (PRC2), functioning as an H3K27-specific methyltransferase.

The enhancing effect of hypomethylating agents on PR expression strongly suggests that DNA methylation is another mechanism that contributes to decreased PR gene expression. As we have previously reported, significant methylation of the PR gene occurs in serous endometrial cancers [25]. Using two NSCLC cell lines (A549 and H358) and two ovarian cancer cell lines (Ovcar-8 and ES-2) as models, we studied PR DNA methylation by methylation-specific PCR. We observed strong PRB methylation in NSCLC H358 cells, Ovcar-8 and ES-2 cells, but not in A549 cells. Treatment with 5-aza-decitabine increased the de-methylated PGR DNA signal particularly in A549, H358 and Ovcar-8 cells. Not surprisingly, we observed less effect in response to hypomethylating agents in ES-2 cells, where repression by the PRC2 dominates. Regardless of the mechanism of PR repression (PRC2 vs. DNA methylation), we showed that epigenetic modulators reduce cell viability in all cells studied and further sensitize cells to progesterone treatment in most cases.

Our study took advantage of multiple authenticated cell models to determine the mechanism of PR silencing in tumors. Our findings were further validated by studies from human tumor tissues and from analyses of large clinical datasets where we found that PR repression was a consistent finding in cancer. We propose that the findings from these mechanistic experiments may in part explain the results of a recent, important clinical study where a progestin eluting IUD decreased the risk of endometrial, ovarian, lung and pancreatic cancer with long term use in Finnish women [4].

It is well known that the HDACi LBH589 and the hypomethylating agent 5-aza-dC affect many mechanisms in cells, including epigenetic transcription regulation, and nonepigenetic cellular signaling cascades [41-43]. In this study, our data (**Figures 4, 6 and 7**) clearly demonstrate that loss of PR expression occurs through epigenetic mechanisms, but it is not the only mechanism of downregulation. One example is in the ES-2 cell line. LBH+5-aza treatment only increased PGR mRNA expression by 2-fold (**Figure 4A**); however, PR protein expression

increased about 10-fold (**Figure 4B**). This indicated that enhanced transcription may not be the only mechanism that leads to an increase in functional PR protein. Further studies are clearly required to understand the full effects of epigenetic modulators on hormone receptor levels.

In the Finnish study, even though the progestin IUD was reported to be protective against these tumors, it increased the breast cancer risk by 20% [4]. However, the authors pointed out that this result differs from two previous population studies (patients =17,360 and patients =5113), which found no elevation in breast cancer risk when progestin IUDs were used for contraception [44, 45]. The impact of progestins on breast carcinogenesis and progression has elicited widespread controversy among experts. In a recent Nature review paper, Carroll et al attempt to clarify the confusion and point out that the effects of progesterone, the natural hormone, may differ from progestins that have androgenic effects. Synthetic progestins such as medroxyprogesterone acetate (MPA), levonorgestrel and norethindrone acetate appear to be more consistently associated with higher breast cancer risk compared to progesterone [46]. Clinically available forms of progesterone such as oral micronized progesterone (OMP) and dydrogesterone are associated with either no change or a decreased risk of breast cancer [46]. These data suggest that the use of progesterone over the long term may not be a risk factor for breast cancer, but further studies are clearly indicated. If confirmed, the traditional worry of progesterone exposure as a stimulator of breast carcinogenesis may be lessened, particularly considering the protective effect of progesterone on carcinogenesis in other organs.

In this report, we show that epigenetic silencing occurs at multiple levels and by varying mechanisms in different cell types, but the end result is the same: PR expression is lost in malignant cells compared to non-malignant tissue. We confirmed the loss of PR in cancer tissue specimens and in clinical datasets including TCGA, suggesting that lack of PR activity is permissive for cancer development and progression. Our findings go on to support the possibility that reversal of PR silencing is now possible using clinically available epigenetic modulators. Indeed, epigenetic modulators can covert PR

negative to PR positive cells in most models we studied. These targeted agents, when coupled with progesterone, define a new regimen we term “molecularly enhanced hormonal therapy”. We propose that this strategy could be a novel way to limit cell proliferation even in tumors from the ovary, pancreas and lung which are not typically considered hormonally responsive.

Acknowledgements

The abstract of this paper was previously presented in Proceedings: AACR Annual meeting 2017. The authors gratefully acknowledge the assistance of Zhengyi Wan, Sarah Ingwersen and Diane Wu in the performance of some of the experiments. This research is supported by the Holden comprehensive cancer center (HCCC) American Cancer society Seed grant (77-004-34, SY), HCCC-PACT grant (SY), NIH R37CA238274 (SY), NIH R01CA184101 (XM, KKL), NIH R01CA99908 (KKL), and the Department of Obstetrics and Gynecology Research Development Fund (KKL) and the Department of Pathology start up fund (SY). Yiyang Li was supported by Jilin Provincial Department of Finance JLSCZD2019-057 (YL).

Ovarian tissues were obtained from University of Iowa, Department of Obstetrics and Gynecology Tissue Bank under informed consent (IRB#201605841).

Disclosure of conflict of interest

None.

Abbreviations

PR, Progesterone receptor; HDACi, Histone deacetylase inhibitor; PRC, Poly-comb repressor complex; NSCLC, Non-small-cell-lung cancer.

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Supple Table 1. List of cell lines and growth media of the cell lines in this paper. The identity of each cell line was authenticated, as described in the method section

	Pancreatic cancer cell lines	Basic growth media
1	MiaPaca-2	DMEM
2	Panc-1	DMEM
3	AsPC-1	RPMI 1640
4	BxPC-3	RPMI 1640
Non-small-cell-lung cancer cell lines		
5	H1299	RPMI1640
6	A549	RPMI1640
7	H292	RPMI1640
8	H358	RPMI1640
Endometrial cancer cell lines		
9	Ishikawa	DMEM
10	ECC-1	RPMI1640
11	Hec50	DMEM
Ovarian cancer cell lines		
12	OV-90	1:1 mixture of MCDB105 and Medium 199
13	TOV-112D	1:1 mixture of MCDB105 and Medium 199
14	HEY	DMEM +2 mM L-Glutamine
15	Ovcar-8	RPMI1640
16	ES-2	McCoy's 5A
17	Ovcar-3	RPMI1640

All basic growth media supplemented with either 10% fetal bovine serum or 5% charcoal-stripped serum and penicillin-streptomycin (Gibco).

Supple Table 2. Primers for real-time PCR and PGR DNA methylation analysis

Primer name	Forward primer (F)	Reverse primer (R)
PGR	ATGTGGCAGATCCCACAGGAGTTT	ACTGGGTTTGACTTCGTAGCCCTT
FOXO1	TCGTCATAATCTGTCCCTACACA	CGGCTTCGGCTCTTAGCAAA
p21	TGTCCGTCAGAACCCATGC	AAAGTCGAAGTTCCATCGCTC
Myc	GGCTCCTGGCAAAAGGTCA	CTGCGTAGTTGTGCTGATGT
IGFBP1	TTGGGACGCCATCAGTACCTA	TTGGCTAAACTCTCTACGACTCT
AREG	GTGGTGCTGTCGCTCTTGATA	CCCCAGAAAATGGTTCACGCT
PAEP	GAGATCGTTCTGCACAGATGG	CGTTCGCCACCGTATAGTTGAT
18S	AACCTTCGATGGTAGTCGCCG	CCTTGGATGTGGTAGCCGTTT
PRB-U	TGATTGTTGTTTGTAGTATG	CAACAATTTAATAACACACA
PRB-M	TGATTGTCGTTTCGTAGTACG	CGACAATTTAATAACACGCG

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Supple Table 3. Ovarian cancer patient age and tumor clinical characteristics

Patient #	Patient ID	Age	Stage	Grade
1	OVC04	66	IIIC	3
2	OVC05	81	IIIC	3
3	OVC06	44	IV	2
4	OVC07	57	IIIC	3
5	OVC08	57	R	ng
6	OVC10	55	R	ng
7	OVC11	72	IIIC	3
8	OVC13	72	IIIC	3
9	OVC17	49	IIIC	2
10	OVC19	67	IIIC	3
11	OVC21	65	IIIC	3
12	OVC33	53	IV	3
13	OVC34	45	IIIA	3
14	OVC35	27	IIIB	3

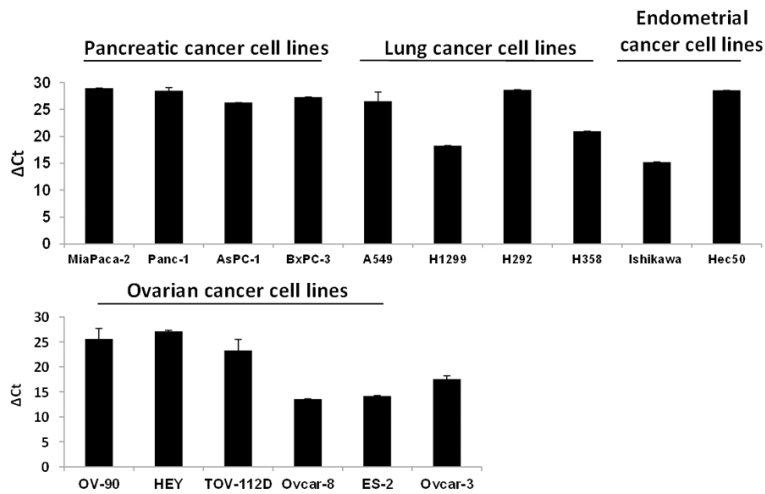


Figure S1. Screening for PR expression in multiple cancer cell lines. *PGR* mRNA expression was measured in four pancreatic, four NSCLC, two endometrial and six ovarian cancer cell lines. The mRNA expression was normalized to 18S and displayed as the ΔC_t value.

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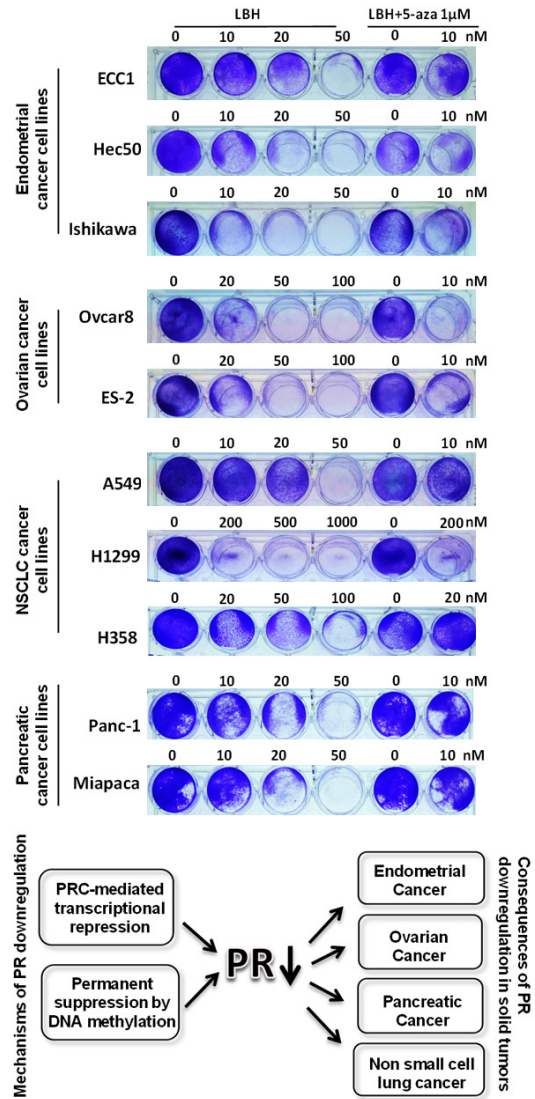


Figure S2. Epigenetic modulators inhibit cell proliferation. The indicated cells were treated with DMSO as a control vehicle or different dose of LBH589 (LBH), 1 μM 5-aza or the combination of both treatments for 3 days, attached cells were stained with crystal violet.

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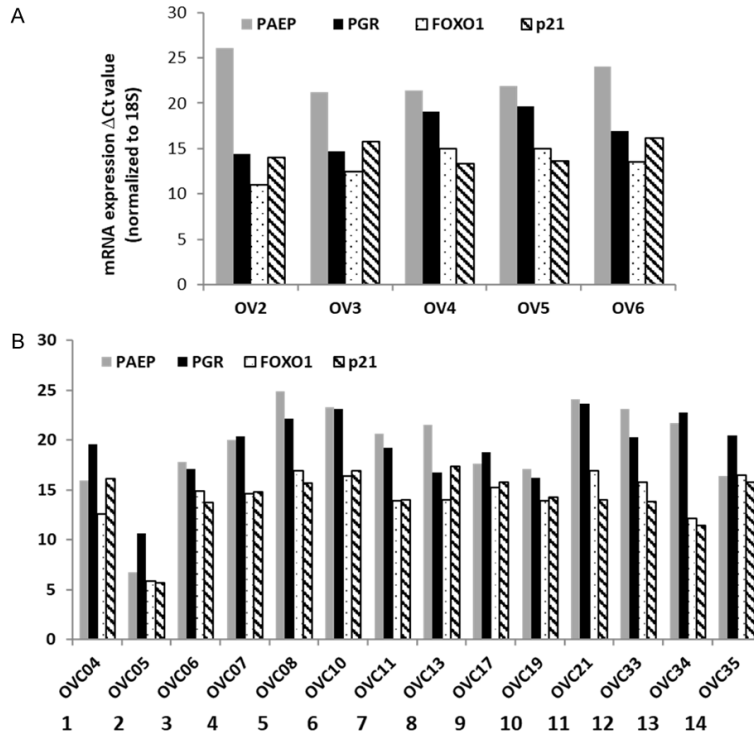


Figure S3. mRNA expression of PR and its target genes in each individual patient samples. A. Nonmalignant ovarian tissues. B. Ovarian tumor tissues.