Global Expression Analysis of Endometrial Cancer Cells in Response to Progesterone Identifies New Therapeutic Targets

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Declarations

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Abstract

Progesterone prevents development of endometrial cancers through its receptor (PR) although the molecular mechanisms have yet to be fully characterized. In this study, we performed a global analysis of gene regulation by progesterone using human endometrial cancer cells that expressed PR endogenously or exogenously. We found progesterone strongly inhibits multiple components of the platelet derived growth factor receptor (PDGFR), Janus kinase (JAK), signal transducer and activator of transcription (STAT) pathway through PR. The PDGFR/JAK/STAT pathway signals to control numerous downstream targets including AP-1 transcription factors Fos and Jun. Treatment with inhibitors of the PDGFR/JAK/STAT pathway significantly blocked proliferation in multiple novel patient-derived organoid models of endometrial cancer, and activation of this pathway was found to be a poor prognostic signal for the survival of patients with endometrial cancer from The Cancer Genome Atlas. Our study identifies this pathway as central to the growth-limiting effects of progesterone in endometrial cancer and suggests that inhibitors of PDGFR/JAK/STAT should be considered for future therapeutic interventions.

Keywords

1. Introduction

Endometrial cancer is the most common gynecologic malignancy and one of only a few cancers in the United States with an increasing incidence and mortality [1]. Remarkably, from 2008 to the present, endometrial cancer cases have increased over 30%, equating to 65,620 new cases in 2020. Moreover, in 2020, 12,590 women in the US lost their lives to this pervasive and under-studied disease [1]. Patients with early-stage disease have a reasonably good prognosis following hysterectomy; however, for advanced or recurrent cases, effective treatments are severely limited. We have proposed that the natural hormone progesterone serves as a powerful endometrial tumor suppressor [2–4]. Our objective in this study was to uncover the anti-tumor effects of progesterone at the molecular level, with the goal of using these progesterone-controlled pathways as a novel roadmap to design more effective targeted therapies for women with advanced or recurrent endometrial cancer.

Progesterone exerts differentiating effects in the endometrium through progesterone receptors A and B (PRA and PRB), which are DNA-binding transcription factors in the steroid hormone receptor family. PRA and PRB arise from alternative translation initiation sites of a single gene, *PGR*, with PRA being a shorter isoform due to N-terminal truncation of 164 amino acids. The two isoforms form homo- and heterodimers with different functional activities with respect to the control of gene transcription and co-localize in the cell nuclei of normal endometrium. In addition to binding directly to the progesterone response elements (PREs) in DNA, PRs are also believed to interact with other DNA-bound transcription factors such as members of the AP-1 family, including Fos and Jun, as well as other steroid receptors such as the glucocorticoid, androgen, mineralocorticoid and estrogen receptors (GR, AR, MR and ER) to further modulate gene transcription [5–7]. It is believed that both PR isoforms are required for optimal, normal endometrial differentiation [8]. PRs are also present in endometrial carcinomas; however, expression of only one isoform is common [9]. The lack of PR expression may lead to the loss of the differentiating effects of synthetic progesterone (progestins) and render hormonal therapy ineffective for advanced carcinoma [10]. It is therefore critical to assess the independent and combined transcriptional effects of the PR isoforms to fully understand how progesterone controls cell function and inhibits endometrial cancer development and progression.

The expression of PR in endometrial glands is under the control of estrogen and progesterone, where estrogen induces PR gene expression and progesterone down-regulates the expression of its own receptor [11]. Indeed, we have documented the hormone-mediated down-regulation of PR in two independent clinical trials of endometrial cancer [12, 13]. Upon exposure to progesterone, the immediate down-regulation of PR is a sign of its transcriptional activity owing to ligand-dependent destruction in the proteasome that must occur to maintain PR-dependent gene activation. On the other hand, the loss of PR in the long run is an epigenetic mechanism associated with hormone resistance [14, 15]. Thus, the lack of robust PR expression hinders the use of progestin therapy for advanced or recurrent endometrial tumors. An alternative approach would be to target signaling pathways downstream from PR to elicit endometrial differentiation in the absence of functional receptors. To identify these pathways, we examined the effects of progesterone on gene expression in cell models of PR-positive endometrial carcinoma. We demonstrate that

progesterone inhibits the activity of many growth-promoting genes. These results identify new pathways worthy of exploration as targets for future therapeutics in endometrial cancer, even in PR-dysregulated tumors.

2. Materials and Methods

2.1. Ethical Approval

All studies using human tumor specimens were reviewed and approved by the University of Iowa Institutional Review Board (IRB), protocol # 201809807 and performed in accordance with the ethical standards as laid down in the 1964 Helsinki Declaration and its later amendments. Informed consent was obtained from all subjects participating in the study.

2.1. Cell Lines

Ishikawa endometrial cancer cells were purchased from ATCC. Hec50 endometrial cancer cells were provided by Dr Erlio Gurpide (New York University) [16]. An aggressive subclone, Hec50co, has been previously characterized and validated as a model for advanced endometrial cancer; these cells are negative for ER and PR [17]. Cells were cultured in DMEM (Sigma, St Louis, MO) supplemented with 10% FBS (Gemini Bio Products, Inc., Calabasas, CA) when grown prior to experiments and without FBS during hormonal treatment. Antibiotic/antimycotic solution containing 100 units/ml penicillin-G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (GIBCO Life Technologies, Grand Island, NY) was also added to the growth media. Cell line identity is confirmed twice per year using STR testing (BioSynthesis, Inc., Lewisville, TX).

2.2. Patient-derived organoid models

Fresh tumor tissue was collected from the patient's debulking surgery. Samples were washed with 10 ml PBS and cut into small pieces, then collected in a 50 ml tube with 5 ml digest buffer (AdDF+++ media (Advanced DMEM-F12 with 1X Glutamax,10 mM HEPES and pen/strep) supplemented with 2 U/ml Dispase Ⅱ, 1 mg/ml collagenase P and 50 μg/ml DNAse I), and incubated for 0.5–1 hour at 37°C. Dissociated cells were filtered through a 40 μm cell strainer, centrifuged, washed and pelleted. Erythrocytes were removed with red blood cell lysis buffer. Cells were counted and embedded in Matrigel (Corning Life Sciences, USA) on ice and seeded as 50 μl domes in pre-warmed 24-well cell culture plates (Costar, Corning); 500 μl of Organoid Culture Media [18] was added on the top of the Matrigel to each well. Assessment of cell viability was performed as previously described [18] as follows. Organoids were collected with Organoid Harvesting Solution (Cultrex) and digested to single cells with TrypLE Express (Gibco, USA). Single cells were suspended in AdDF+++ medium with 10% Matrigel and seeded at a density of 10,000 cells/well in an ultra-low attachment 96-well U-bottom white plate (S-Bio). After 24 hours, organoids were exposed to progesterone (100 nM), cediranib (1 μM), ruxolitinib (15 μM), tofacitinib (1 μM), napabucasin (0.5 μM) or combinations for 72 hours at 37°C. At the end of the incubation, an equal volume of CellTiter-Glo 3D reagent (Promega, Fitchburg Center, WI, USA) was added to each well and incubated for 25 minutes at room temperature. Luminescence, reflective of cell viability, was measured using the Gen5 Microplate Reader

(BioTek, Vermont, USA). All tests were conducted in triplicate and data normalized to untreated controls (set at 100% viability).

2.3. Adenoviral Infections

Adenoviral vectors of PRA and PRB were constructed as described previously [19]. Hec50co cells were infected the AdPRA + AdPRB for 15 h prior to treatment as previously described [19].

2.4. RNA Sequencing and Analyses

RNA quality was analyzed using the Agilent BioAnalyzer 2100 with RNA integrity number greater than eight used for the library preparation at the University of Iowa Institute of Human Genetics, Genomics Division. QC qualified RNA (500ng) was treated with DNase-1 and used to enrich for poly-A-containing transcripts using oligo-dT primers bound to beads. The enriched RNA pools were then fragmented, converted to cDNA and ligated to sequencing adaptors containing indices using the Illumina TruSeq stranded mRNA sample preparation kit (Illumina, San Diego, CA). Molar concentrations of the indexed libraries were then measured on an Agilent Model 2100 Bioanalyzer and mixed in equimolar amounts in sequencing pools. Pools were sequenced on the Illumina NovaSeq 6000 genome sequencer using 100bp paired end SBS chemistry. RNA-seq data went through rigorous quality control as outlined in our previously established quality control protocols and tools [20]. RNA-seq data alignment was performed by STAR [21] followed by gene quantification into read counts using featureCounts [22]. Differential expression analysis was carried out using DESeq2 [23]. Genes expressed at greater than 1.0 fragments per kilobase per million reads and with greater than two-fold changes in gene expression were compared using Ingenuity Pathway Analysis (Qiagen). Heatmaps of differential gene expression were generated using the average $\log_2$ fold-change relative to time-matched vehicle control in three independent biological replicates (Prism version 9.0.0 (GraphPad)).

2.5. Affymetrix Expression Microarrays

RNA was extracted using RNeasy spin columns (Qiagen Corp, Valencia, CA). The RNA quality was checked using the Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA) and the concentration determined with the Nanodrop (Thermo Scientific, Wilmington, DE). Functional replicate Affymetrix microarrays were performed for two independent experiments using the human HG-U133A plus 2.0 chips (Santa Clara, CA), thereby querying the expression of 54,000 transcripts. All procedures for the chip preparation and cDNA production were performed according to instructions from the manufacturer's manual, version 701025 Rev.5. Briefly, total RNA was used to generate double-stranded cDNA with an oligo dT-primer containing the T7 RNA polymerase promoter site and the One-Cycle Target Labeling Kit. cDNA was purified via a column using the GeneChip Sample Cleanup Module, and biotinylated cRNA was synthesized by *in vitro* transcription using the GeneChip IVT Labeling kit. Biotin labeled cRNA was purified (GeneChip Sample Cleanup Module), and the absorbance measured at 260 nm to determine yield (Nanodrop spectrophotometer). Twenty μg of the labeled cRNA was fragmented, and its quality was assessed for purified cRNA and fragmented cRNA using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano LabChip kit. The labeled fragmented cRNA was hybridized

to Affymetrix GeneChip HGU133 Plus 2.0 arrays for 16 h at 45°C. Array washing and staining was performed on the Affymetrix fluidics (450) station according to the antibody amplification protocol (Fluidics script: EukGE-WS2v5). The GeneChips were scanned using the Affymetrix GeneChip Scanner 3000 (a wide-field, epifluorescent near-confocal microscope with a patented flying objective).

Initial data analysis was performed using Affymetrix Microarray Suite v 5.0 software, setting the scaling of all probe sets to a constant value of 500 for each GeneChip. Silicon Genetics' (now Agilent Technologies) GeneSpring version 7.3 (Redwood City, CA) was used to filter data using several criteria. First, only transcripts with a fluorescent signal above the background level were retained for the subsequent 2-fold change filter. Starting with over 54,000 transcripts, this eliminated all but 5592 transcripts. Unsupervised clustering and heatmaps were constructed using the R package heatmap3 [24]. Pearson's correlation coefficients were used as the distance between two samples. An ANOVA calculation was used to identify transcripts with a p-value less than 0.05. Heatmaps of differential gene expression for select genes were generated by plotting the $\log_2$ fold-change in response to progesterone relative to time-matched vehicle control (Prism version 9.0.0 (GraphPad)).

2.6. Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

2.6.1. Hec50 cells expressing PRA+B: cDNA was synthesized from 1μg of total RNA and PCR reactions were carried out in 50μl reaction mixtures using 50ng of template per well. Amplification of GAPDH was used as an endogenous control to standardize the amount of RNA in each sample. The Assay on Demand ™ protocol was carried out as directed in the ABI manual (Applied Biosystems, Foster City, CA). The raw data were presented as the cycle number associated with initial amplification. The data were then normalized to an endogenous control to allow for variance in RNA template amounts added to the reverse transcription reaction. The data could then be compared to a calibrator and analyzed using the $2^{-\Delta\Delta CT}$ method [25].

2.6.2. Ishikawa cells and patient-derived organoid models.—Ishikawa cells were treated with progesterone (100 nM) −/+ estrogen (10 nM) or vehicle control (ethanol) in serum-free media. Cells were harvested at the end of treatment and subjected to RNA purification. PDO models were treated with 100 nM progesterone or vehicle control in organoid culture media (see Supplemental Methods in ref. [18]). At the end of treatment, Matrigel was dissolved using Organoid Harvesting Solution (3700–100-01, Cultrex, R&D Systems) per the manufacturer's instructions, and cell pellets obtained for RNA isolation. Total RNA was purified from Ishikawa cells or organoid models using the RNeasy Plus RNA purification kit (QIAGEN). Yield and purity were assessed on a Trinean DropSense 16 spectrophotometer and an Agilent Model 2100 Bioanalyzer in the Genomics Division of the Iowa Institute of Human Genetics. QC qualified total RNA (500 ng) from each vehicle only or P4 treated sample was reverse transcribed using oligo-dT primers in the SuperScript III kit following manufacturer's recommendations (Invitrogen). The resulting cDNAs were then amplified in the presence of SYBR Green (Thermo Fisher) on an Applied Biosystems Model 7900HT platform in the Genomics Division of the Iowa Institute of Human Genetics. Locus-specific primers are shown in Table 1.

qPCR assays were designed using PrimerQuest software available online at Integrated DNA Technologies (www.idtdna.com). Primers were synthesized at Integrated DNA Technologies (Coralville, IA). All experiments were performed with at least three biological and three technical replicates. Raw expression values (Ct) were normalized (ΔCt) against the endogenous control *18S rRNA* and then used to calculate relative expression (ΔΔCt) versus untreated cells. Fold changes in expression resulting from treatment were calculated as $2^{-\Delta\Delta Ct}$ following the standard method [25, 26]. Heatmaps of fold change relative to time-matched controls were generated using Prism version 9.0.0 (GraphPad).

2.7. Immunohistochemistry

Patient tumor specimens from which organoid models were derived were subjected to immunohistochemistry for expression of PR and ER. All staining was performed by the University of Iowa Histology Research Laboratory. Formalin-fixed paraffin-embedded (FFPE) tumor blocks were sectioned to 4 μm thickness. PR (1:200 dilution, #M3568, Dako) and ER (1:50 dilution, #M7048, Dako) staining was performed on each sample as previously described [13] using a polymer-based detection system on the Biocare Medical IntelliPATH FXL system. IHC staining evaluated in a semiquantitative manner by two reviewers blinded to sample identity following the American Society of Clinical Oncology/ College of American Pathologists guidelines for scoring PR and ER [27]. Per the guidelines, at least 1% of the cells in the specimen must be immunoreactive to consider the tissue positive. The intensity of tumor staining (0 to 3+) was scored using a positive control tissue as 3+, and all samples were also assessed for presence of internal positive control staining (stroma, myometrium) to ensure tissue integrity. Reviewers also scored each slide with a percent of cells staining positive (0–100), which was multiplied by the intensity score (0, 1+, 2+, or 3+) to calculate a modified H-score. The values from two independent reviewers were averaged for each specimen and biomarker. Representative images were acquired at 20*X* magnification.

2.8. TCGA Analysis

RNA-seq data of uterine corpus endometrial carcinoma (UCEC) (N=560) from the cancer genome atlas (TCGA) were downloaded from Genomic Data Commons. Genes of interest identified using our data were extracted from the TCGA dataset for further prognostic evaluation. Each gene of interest's disease specific prognostic power was measured by Cox proportional hazard model in R treating gene expression as a continuous variable. Furthermore, the overall prognostic relevance of the genes of interest was evaluated using two methods. First, the overall prognostic power was measured by a meta analysis. Then, we combined genes of interest using a previously established method AESA [28] to compose a gene expression composite score (GECS) and used it for prognostic analysis.

3. Results

3.1. Gene regulation by progesterone through PR

To understand the basis of progesterone's distinct actions in endometrial cancer cells and to determine which genes this hormone regulates, we first performed studies Hec50co endometrial cancer cells engineered to transiently co-express PRA and PRB

[19]. As determined by Affymetrix microarray, PR-transfected Hec50co cells responded to progesterone (P4) by down-regulating multiple *STAT* (signal transducer and activator of transcription) genes and the downstream AP-1 transcription factors (Fos and Jun), as well as *PDGFR* (platelet derived growth factor receptor) as compared to vehicle control (Figure 1A).

We next asked if this pathway is also significantly altered in endometrial cancer cell lines with endogenous PR expression. Ishikawa cells express moderate levels of PR isoforms as compared to other endometrial cancer cell lines [14–16]. We performed RNA-sequencing (RNA-seq) of Ishikawa cells after treatment with progesterone (P4) for 12, 24 or 48 h and compared results to time-matched vehicle controls. Global gene expression analysis indicates a clear demarcation between control vs. progesterone-exposed cells (Figure 1B and Supplemental Figure S1). Ingenuity Pathway Analysis identified several pathways that were differentially regulated by progesterone, with the overwhelming majority being downregulated (Figure 1C). In line with the microarray results, the PDGFR and JAK/STAT signaling pathways were downregulated by P4 treatment, though progesterone required a longer time frame (48 h) in order to effect the down-regulation in cells with endogenous vs. exogenous PRs (Figure 1D). A similar pattern of effects was observed by qRT-PCR in Ishikawa cells (Figure 1E). While the magnitude and specific timecourse of the effects on genes in the PDGFR/JAK/STAT/Fos/Jun pathway differed between cells with exogenous vs. endogenous PR expression and by the method used for expression analysis, these results nevertheless indicate that the downstream transcriptional effectors of this pathway were strongly inhibited by P4. A schematic of genes in this pathway is provided in Figure 1F.

Studies were extended to a more translational model of endometrial cancer, patient-derived organoids (PDOs). To date, we have established a biorepository of 29 endometrial cancer PDO models and characterized expression of ER and PR in the primary tissue specimens from which these models were derived (Table 2).

We selected PDO models of stage 1/grade 1 endometrioid endometrial cancer to determine the effects of progesterone on expression of genes in the PDGFR/JAK/STAT pathway. Expression of PR and ER in the tissue from which these models were derived is shown in Figure 2. In the majority of cases we tested, hormone receptor staining was most intense in the glandular structures and was primarily nuclear (e.g,. see ONC-8743). Whereas PR expression was variable across specimens studied as PDO models, all cases harbored relatively high ER levels (Figure 2; note the clinical standard for ER is an H-score of 75; one case, ONC-8734, is borderline high).

Gene expression changes were assessed in PDO models with varying levels of PR expression. Consistent with results in cell lines, we observed an overall downregulation of the genes the PDGFR/JAK/STAT/Fos/Jun pathway in response to progesterone treatment (Figure 3). The models with higher PR expression had greater downregulation in *PDGFR, STAT, FOS* and *JUN*. We also observed the anticipated increase in PR expression 24 h after P4 treatment and downregulation at 48 h in the models with PR.

3.2. Relevance of PDGFR/JAK/STAT pathway activation on outcomes in patients with endometrial cancer

Studies were extended to understand the prognostic significance of the PDGFR/JAK/STAT/Fos/Jun pathway in endometrial cancer. The Cancer Genome Atlas (TCGA) dataset for uterine corpus endometrial cancer (UCEC) was queried for the expression of genes within the PDGFR/JAK/STAT pathway. Based on gene expression changes by microarray and RNA-seq, we selected the following ten genes for analysis: *PDGFRA, STAT1, STAT2, STAT3, STAT4, STAT6, FOS, JUN, JUNB* and *JUND*. Of these ten genes, four had significant prognostic results: *PDGFRA, STAT1* and *JUN* were inversely associated with survival (higher expression = worse survival), whereas *STAT6* was positively associated with survival (higher expression = better survival). A meta analysis of the ten genes together revealed an inverse association with survival (hazard ratio = 1.002, 95% confidence interval: 0.0006 – 1.0035). We also determined the impact of high (highest quartile) versus low (lowest quartile) expression on survival using disease-specific survival probability Kaplan-Meier curve assessment (Figure 4). Using the AESA [28] method, high expression of genes in the PDGFR/JAK/STAT/Fos/Jun pathway portended a significantly decreased survival (p = 0.006).

3.3 Effect of PDGFR/JAK/STAT/Fos/Jun pathway targeted inhibitors on the viability of endometrial cancer platelet derived organoids

To link progesterone activity with gene expression changes, we exposed PDOs to targeted agents that block the PDGFR/JAK/STAT/Fos/Jun pathway. The agents studied included cediranib (an anti-angiogenic inhibitor that targets multiple receptor tyrosine kinases, including PDGFR), ruxolitinib (an inhibitor of JAK1 and JAK2), tofacitinib (an inhibitor of JAK3) and napabucasin (an inhibitor of STAT3). These agents have been evaluated to a very limited degree in clinical trials of endometrial cancer (Supplemental Table S1). In general, all models displayed some sensitivity to inhibitors of the PDGFR/JAK/STAT as single agents (Figure 5). We also combined the anti-angiogenic inhibitor cediranib with the JAK or STAT targeted agents in order to evaluate the impact of dual therapy on cell viability. With the exception of one model (ONC-7677), the combinatorial regimens produced more marked reductions in cell survival as compared to single agent alone. There was only one combination for which the combination of cediranib with a JAK/STAT inhibitor was synergistic: ONC-8004, cediranib+ruxolitinib (Supplemental Figure S2). All other combinations were additive (e.g., ONC-7756, cediranib+tofacitinib) or driven predominantly by a single agent (e.g., ONC-8004, cediranib+napabucasin was similar to napabucasin alone). These data indicate that, regardless of PR expression, inhibition of the PDGFR/JAK/STAT pathway represents a potential therapeutic strategy for endometrial cancer.

4. Discussion

Progesterone acts to control cell function and proliferation through PR. Gene expression profiling in response to steroid hormones through their cognate receptors has provided information on large numbers of pathways controlled by these factors. Dai *et al.* [5] found that progesterone inhibits endometrial cancer cell growth and invasiveness by

down-regulation of cellular adhesion molecules through PR. We have also shown that progesterone inhibits inflammation by inducing anti-inflammatory cytokines and inhibiting pro-inflammatory cytokines and their receptors [29]. Indeed, given the multiple mechanisms of action of progesterone in the endometrium, it can be considered the ultimate endometrial tumor suppressor [30]. It follows that elucidating the repertoire of progesterone/PR targets will potentially shed light on other pathways with therapeutic potential in endometrial cancer and relevant even for tumors that do not express PR.

In addition to *VEGF* and pro-angiogenic factors, as previously reported [31], the most relevant pathway summarizing our data as assessed by Ingenuity Pathway Analysis is the PDGFR/JAK/STAT/Fos/Jun pathway, which is often constitutively up-regulated in endometrial cancer [32], and inhibited by progesterone through PR. This pathway enhances many proliferative signals in cells and is linked to tumor angiogenesis. Our analysis of TCGA dataset suggests that elevated expression of genes in the PDGFR/JAK/STAT/Fos/Jun pathway is associated with poor outcome in patients with endometrial cancer.

Like VEGF, PDGF is a key regulator of angiogenesis as well as proliferation. High intratumor microvessel density in endometrial cancer is associated with advanced clinical stage and an increased risk of recurrent disease [33–38]. A study by Slomovitz *et al.* showed that 33 (91%) of primary endometroid endometrial cancer tumors express PDGFR, and 8 of 11 (73%) primary uterine papillary serous tumors express PDGFR [32]. Importantly, in this study, all recurrent tumors expressed PDGFR [32]. The Gynecologic Oncology Group has also confirmed the relevance of PDGFR as a target through GOG Study 229-J wherein the tyrosine kinase inhibitor cediranib, which targets PDGFR, FGFR, and VEGFR, showed independent activity against advanced endometrial cancer [39]. Here, we have shown that progesterone inhibits *PDGFR* transcription as well. In breast cancer cells, PDGF ligand has been identified as a PR target gene, and exposure of smooth muscle cells, which express high levels of PDGFRs, to conditioned media from progesterone-treated PR-positive breast cancer cells results in activation of PDGFR [40]. In addition, PDGFR inhibition with imatinib blunts proliferation of PR-positive breast cancer cells [41].

The Signal Transducers and Activators of Transcription (STATs) have been implicated in cross talk between steroid hormones and growth factors. There are seven STAT genes. The activation of STATs is controlled by JAKs, which are kinases linked to the cytoplasmic tails of cytokine receptors. Once activated through phosphorylation by JAKs, STATs have dual functions as signaling molecules in the cytoplasm and as transcription factors following nuclear translocation [42]. The STAT proteins mediate diverse biological processes including cell growth, differentiation, apoptosis, transformation and survival. In endometrial cancer cells, our data consistently demonstrated that progesterone through PR inhibits the transcription of multiple pro-growth *STAT* genes. In contrast, ER/PR/STAT signaling is linked to estradiol and progesterone's proliferative activity in the normal breast and possibly also in breast cancer [43]. While our data confirm down-regulation of multiple *STAT* transcripts by progesterone in endometrial cancer cells, treatment of T47D human breast cancer cells with the synthetic progestin R5020 results in the up-regulation of STAT3 and STAT5 proteins [44, 45]. This interesting dichotomy may relate to the potential

proliferative effects of progesterone in breast cancer compared to its almost universal anti-proliferative effects in endometrial cancer.

Interestingly, signaling downstream of activated PDGFR in turn activates STAT3 [46]. STAT3 induces progression through the cell cycle and prevents apoptosis. The candidate target genes regulated by the STAT pathways, such as cyclin D1, appear to contribute to oncogenesis by inducing cell proliferation and survival [47]. It is likely that the inhibition of STAT3 contributes to the mechanisms by which progesterone through PR inhibits the cell cycle.

The most robust changes in gene expression were detected in Hec50 cells expressing artificially high levels of PR. These findings provided a clue into the signaling repertoire of progesterone and the motivation to validate studies in other models. However, a limitation of this study is the lack of endometrial cancer cell lines with endogenously high expression of PR. In Ishikawa cells, the best characterized PR-expressing cell line, we detected only modest changes in gene expression in response to progesterone. For this reason, we extended studies to the more clinically relevant model system, patient-derived organoid, and to patient datasets. Indeed, our analysis of TCGA data underscores the relevance of the PDGFR/JAK/STAT pathway as a prognostic indicator of patient outcome.

Now that signaling through the PDGFR/JAK/STAT/Fos/Jun pathway has been identified as a key proliferative pathway that likely promotes tumor progression and is inhibited by progesterone, how can this pathway be blocked therapeutically even in tumors lacking PR? Inhibitors of JAK/STAT including ruxolitinib, targeted against JAK1 and 2, and the pan-JAK inhibitor tofacitinib are now in clinical use, although they have yet to be evaluated in endometrial cancer. In addition to JAK inhibitors, a series of direct inhibitors of STAT3 are in development, including BBI-608 (napabucasin) and TTI-101, both of which have been studied in early phase clinical trials in cancer. In addition, AZD9150 is an antisense oligonucleotide against STAT3 that is in development [48]. The data provided through these experiments demonstrate that JAK/STAT inhibitors may be highly effective in blocking proliferation of early stage/grade endometrial cancer and deserve further consideration for testing.

A limitation is that the majority of studies were performed in ER-positive endometrial cancers, and future experiments in ER-negative (e.g., serous or high-grade endometrioid histologies) are necessary to understand the generalizability of our findings. Of particular interest to our group is the application of combinatorial strategies that can reactivate PR expression in advanced disease, which is commonly devoid of ER and PR expression. We have extensively studied mechanisms of PR downregulation in endometrial cancer and identified both chromatin modification through the polycomb repressor complex (PRC2) as well as DNA methylation as mechanisms of suppressed PR gene expression [14, 15, 49, 50]. Importantly, PR suppression can be overcome *in vitro* and *in vivo* with histone deacetylase inhibitors. Based on these results, we performed a non-therapeutic surgical window trial comparing medroxyprogesterone acetate (MPA) alone to MPA + a histone deacetylase inhibitor, entinostat [13]. Unfortunately, we have been unable to garner support from NCI to test the combination of MPA + a histone deacetylase inhibitor as a treatment strategy for

endometrial cancer; this is due in large part to 1) the shifted emphasis on immunotherapy in endometrial cancer and competition for the same patient cohort for concurrent clinical trials; and 2) reticence from pharmaceutical companies to further pursue epigenetic modulators in solid tumors, which have shown limited efficacy as single agents. As the field makes more progress in understanding which patients are ideal candidates for immunotherapy and those that are less likely to respond, it is our hope that we will be able to renew interest in combinatorial treatment regimens using targeted agents as proposed in this study.

5. Conclusions

In summary, we have identified progesterone-controlled genes through PR to shed light on the global effects of this important tumor suppressor. We have further highlighted a proliferative pathway, PDGFR/JAK/STAT/Fos/Jun, which is inhibited by progesterone through PR. We hypothesize that JAK/STAT inhibitors may improve outcomes for patients with advanced endometrial cancer, even those who do not express PR, and hope that these preclinical data will provide the initial support for early phase clinical trials testing this concept. Use of specific inhibitors of the pathways normally down regulated by progesterone has the potential to compensate for the loss of PR in advanced endometrial cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

Data availability:

The RNA-seq and Affymetrix array datasets generated during the current study will be deposited in GEO prior to publication. All other data generated or analyzed during this study are included in the submitted article and its supplementary information files.

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Highlights

- Progesterone inhibits PDGFR/JAK/STAT pathway in endometrial cancer preclinical models

- PDGFR/JAK/STAT pathway activation predicts for worse outcomes in endometrial cancer

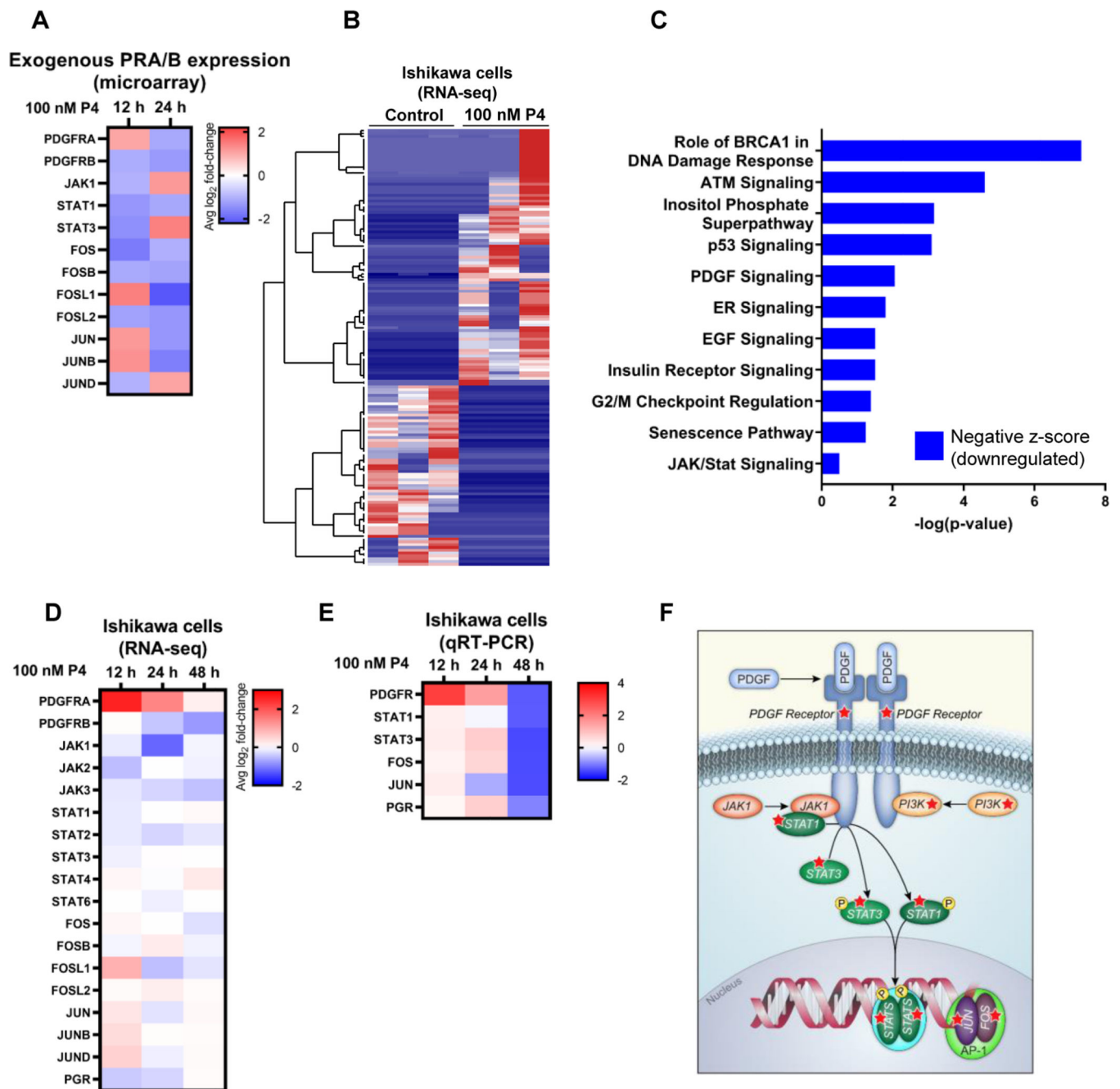- PDGFR/JAK/STAT inhibition is cytotoxic in organoid models of endometrial cancer

Figure 1. Significantly altered pathways in endometrial cancer cells response to progesterone.
(A) Heatmap of representative Affymetrix microarray data from Hec50co cells expressing
exogenous PRA and PRB. Gene expression changes in response to 100 nM progesterone
(P4) were compared to vehicle control. (B) Heatmap of unsupervised clustering analysis
of RNA-seq data. Ishikawa cells were treated with 100 nM progesterone or vehicle control
for 12 h, followed by analysis of gene expression by RNA-seq. Each column represents
a biological replicate (N=3 per treatment) and each row represents a gene. (C) Ingenuity
Pathway Analysis (IPA) of significantly altered pathways based on RNA-seq data from
Ishikawa cells treated with P4 or vehicle control for 24 h. (D) Heatmap of representative

differential gene expression from RNA-seq data from Ishikawa cells treated with either progesterone or vehicle control. (E) Heatmap of qRT-PCR of Ishikawa cells treated with P4 or vehicle control. (F) Schematic representation of the PDGFR/JAK/STAT/Fos/Jun pathway. Red stars indicate proteins in the pathway encoded by genes inhibited by progesterone. Heatmaps reflect the $\log_2$ fold-change in gene expression relative to time-matched vehicle control. Values less than 1 indicate a decrease in transcript levels in response to progesterone, whereas values greater than 1 indicate an increase. Results are the average of N=3 biological replicates.
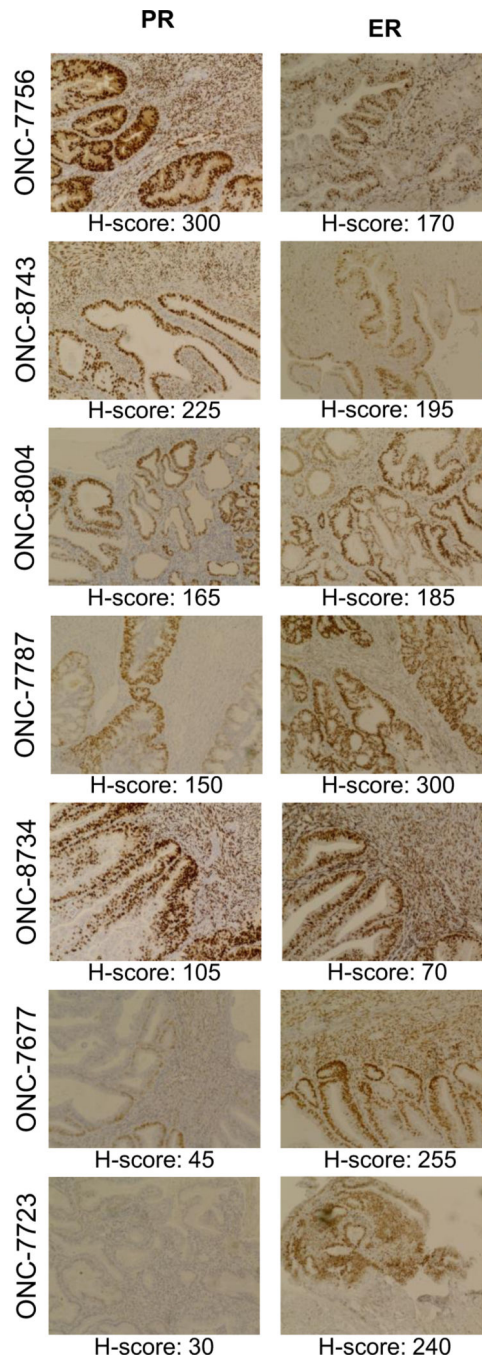
Figure 2: Hormone receptor expression in endometrial patient tumor specimens used to create organoid models.

Expression of progesterone receptor (PR) and estrogen receptor (ER) was determined by immunohistochemistry in patient tumor specimens from which organoid models were derived. Images are annotated with the corresponding de-identified patient ID and the average modified H-score value. Cases are ordered based on expression of PR (highest = ONC-7756; lowest = ONC-7723). All images were acquired at 20X magnification with an EVOS microscope. When possible, serial sections were stained with ER and PR. ONC-7756,

7787, 7767 and 7723 were used for gene expression studies (Fig. 3) and ONC-7756, 7767, 7723, 8004, 8734 and 8743 were used in drug response studies (Fig. 5).
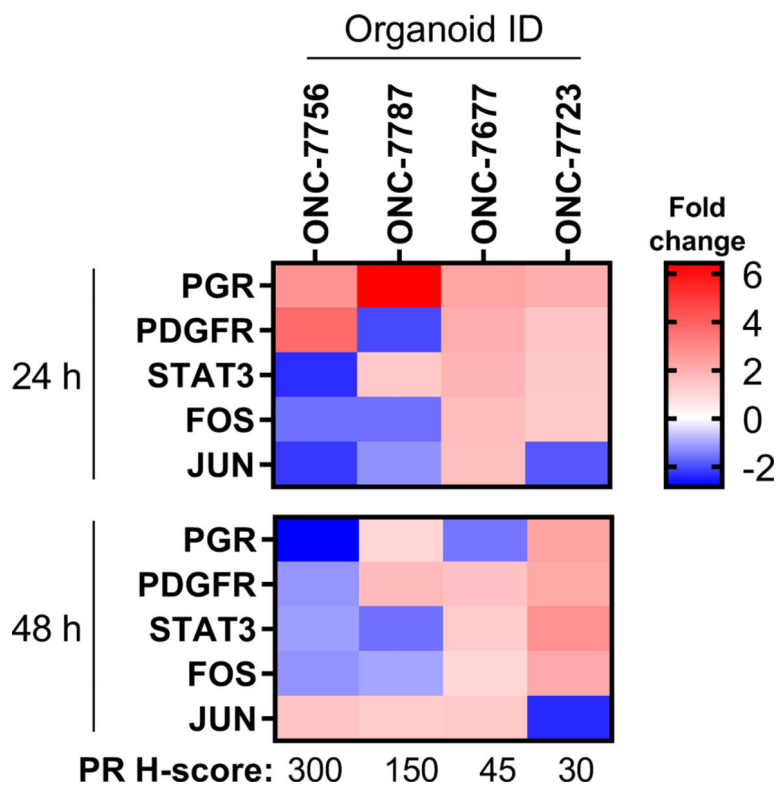
Figure 3: Gene expression changes in PDO models following progesterone treatment.
Expression of the indicated genes was assessed in PDO models exposed to progesterone
(P4) for 24 h or 48 h. Log$_2$ fold-changes were calculated vs. vehicle control at the same time
point and visualized as a heatmap. Data are the average of N=3 biological replicates.
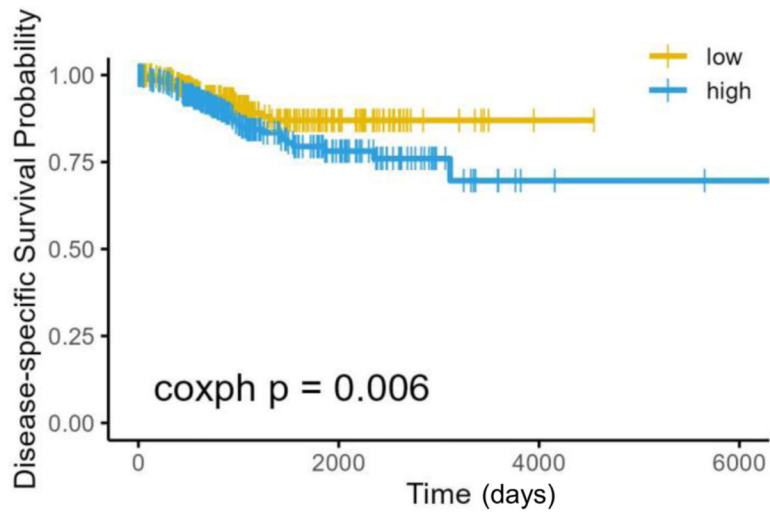
Figure 4. Activation of the PDGFR/JAK/STAT/Fos/Jun pathway in endometrial cancer predicts for worse survival.

Cases were separated into quartiles based on expression of *PDGFRA, STAT1, STAT2, STAT3, STAT4, STAT6, FOS, JUN, JUNB* and *JUND*. Kaplan-Meier Curve for the survival analysis was conducted on the highest and lowest quartiles using the AESA method.
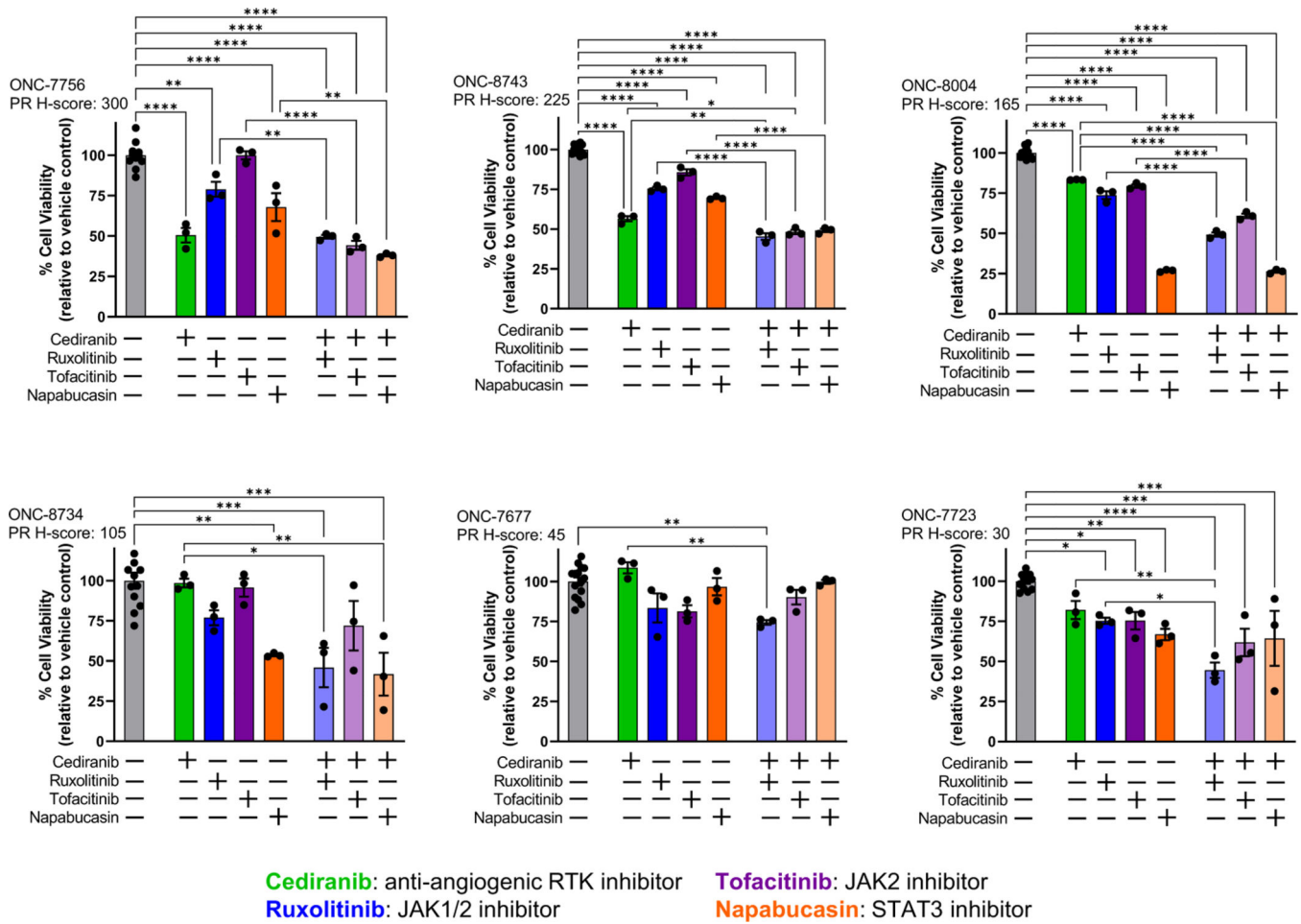
Figure 5: Inhibition of the PDGFR/JAK/STAT signaling pathway is cytotoxic in patient-derived organoid models of early stage/grade endometrial cancer.

PDO models were exposed to the indicated agents for 72 hrs, followed by assessment of cell viability. Drugs concentrations are as follows: 1 μM cediranib; 20 μM ruxolitinib; 1 μM tofacitinib; 0.5 μM napabucasin. Data were normalized to control, which was set at 100% viability. Cases are ordered by PR expression (H-score). * $p < 0.05$; ** $p < 0.01$; *** $p < 0001$; **** $p < 0.00001$ by one-way ANOVA with a Tukey's multiple comparisons test.

Table 1.

Primers used for quantitative PCR (qPCR).

Locus	Primer Sequences	Amplicon	Tm(°C)[*]
PGR	For: ATCCTACAAACACGTCAGTGGGCA Rev: ACTGGGTTTGACTTCGTAGCCCTT	139bp	60.5 60.3
PDGFR	For: GACTTTCGCCAAAGTGGAGGAG Rev: AGCCACCGTGAGTTCAGAACGC	121bp	58.0 62.0
FOS	For: GCCTCTCTTACTACCACTCACC Rev: AGATGGCAGTGACCGTGGGAAT	123bp	56.5 60.9
JUN	For: CGATCTGCACAAGATGAACCACG Rev: CTGCTGAGGTTGGTGTAAACGG	106bp	58.3 58.2
STAT3	For: CTTTGAGACCGAGGTGTATCACC Rev: GGTCAGCATGTTGTACCACAGG	133bp	57.1 58.1
18S rRNA	For: AACTTTCGATGGTAGTCGCCG Rev: CCTTGGATGTGGTAGCCGTTT	104bp	57.3 57.6

[*] Tm is estimated in the presence of 1.5mM $MgCl_2$ using OligoAnalyzer software available on-line at Integrated DNA Technologies (www.idtdna.com).

Table 2.

Expression of steroid hormone receptors PR and ER by immunohistochemistry in tumor tissue from which patient-derived organoid models were created.

Cases are organized by histologic subtype and stage/grade. Cases that were utilized for analysis of progesterone-mediated gene expression changes are underlined, whereas cases that were included in drug testing studies are *italicized.* N.D.: not determined; no tumor: no tumor cells were present for analysis in the stained slide.

Patient ID	Histologic subtype	Stage	Grade	Modified H-Score PR	Modified H-Score ER
ONC-6885	Endometrioid	IA	1	300	190
ONC-7756	Endometrioid	IA	1	300	170
ONC-6790	Endometrioid	IA	1	300	300
ONC-6818	Endometrioid	IA	1	300	300
ONC-6692	Endometrioid	IA	1	285	285
ONC-8743	Endometrioid	IA	1	225	195
ONC-8004	Endometrioid	IA	1	165	185
ONC-7076	Endometrioid	IA	1	160	285
ONC-6096	Endometrioid	IA	1	140	185
ONC-8734	Endometrioid	IA	1	105	70
ONC-6844	Endometrioid	IA	1	70	277.5
ONC-7723	Endometrioid	IA	1	30	240
ONC-6932	Endometrioid	IA	1	0	175
ONC-7026	Endometrioid	IA	2	90	277.5
ONC-6498	Endometrioid	IA	2	70	285
ONC-6173	Endometrioid	IA	2	7.5	180
ONC-6051	Endometrioid	IA	2	0	0
ONC-6925	Endometrioid	IA	3	0	0
ONC-6191	Endometrioid	IB	1	155	300
ONC-7787	Endometrioid	IB	1	150	300
ONC-7677	Endometrioid	IB	1	45	255
ONC-5768	Endometrioid	IIIC2	1	110	292.5
ONC-7623	Serous	IB		45	70
ONC-6720	Serous	IIIC1		0	0
ONC-7991	Serous	IV		45	217.5
ONC-6099	Serous	IVB		0	0
ONC-7757	Clear cell	IV		70	30
ONC-7367	Mixed clear cell & endometrioid	IB	3	225	285
ONC-6742	Mixed serous & endometrioid	IA		285	300
ONC-7003	Mixed serous & endometrioid	IA		85	300