PROGESTERONE RECEPTOR ISOFORM SIGNALING IN OVARIAN CANCER CELLULAR SENESCENCE

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Abstract

As the deadliest of all gynecologic malignancies, ovarian cancer has a death rate of more than 50% due to late detection and diagnosis of the disease and intrinsic or acquired resistance to current therapeutic regimens. The identification of robust biomarkers for early detection will have a substantial impact on survival rates, while prognostic molecular markers may allow for efficacious targeted therapeutic strategies.

Progesterone plays a pivotal role in the development and progression of hormone-regulated tumors. In the breast, progesterone promotes a proliferative, pro-survival response, but inhibits growth in the uterus and ovary. The opposing biology in ovarian versus breast cancer cells may be largely dependent on cell context. The paradoxical effects of progesterone observed in ovarian relative to breast cancer cells may be attributed to actions of the nuclear progesterone receptor (PR) and its isoforms, PR-B and PR-A and their relative regulation (i.e. by post-translational modifications and co-factor binding partners), differential cross-talk between PR and growth factor-mediated signaling pathways (i.e. protein kinases), and/or altered expression levels in the target cells. In contrast to breast cancer, the detailed mechanisms of progesterone action in ovarian cancer are poorly understood. The goals of our studies were to determine the level of PR isoform expression in primary and cell models of ovarian cancer, to define the biological consequences of PR expression and activity on ovarian cancer cell

biology, and to identify the key cofactor(s) required for mediating PR-dependent signaling actions.

We demonstrated that ligand-activated PR-B induced a non-proliferative cell fate, known as cellular senescence, through a FOXO1-dependent mechanism in ovarian cancer cells. PR-B and FOXO1 were co-recruited to the same PRE-containing region of the upstream promoter of p21 upon progestin (R5020) treatment. Both proteins are required to cooperatively activate p21 expression based on data from PR-null control cells and lentiviral-delivered shRNA FOXO1-knockdown studies. Stable knock-down of FOXO1 inhibited progestin-induced p21 expression in ES-2 cells stably expressing GFP-tagged PR-B and blocked PR-dependent cellular senescence.

Next, we investigated the biological consequences of PR isoform-specific gene regulation in ovarian cancer models, as well as directly compared PR isoform-selective transcriptomes between ovarian and breast cancer models. In ovarian cancer cells, PR-A is relatively insensitive to hormone, and PR-B (but not PR-A) is capable of inducing FOXO1 and p21 expression required for progestin-mediated cellular senescence in ovarian cancer cells. Furthermore, our studies revealed FOXO1 as a critical cofactor and determinant for the regulation of PR hormone sensitivity. Namely, activated FOXO1 confers PR-B-like behavior to PR-A, and in the presence of FOXO1, PR-A trans-activates classical PR-B target genes and induces robust cellular senescence. Finally, we utilized a novel *ex*

vivo explant culture system of human primary ovarian tumors that recapitulated our *in vitro* findings.

Significantly, identifying the mechanisms governing PR-A versus PR-B specific gene regulation driven by FOXO1 may provide a means to promote PR-B driven cellular senescence in ovarian cancer and provide clues to inducing the protective actions of PR-A in other hormone-driven cancers, such as breast and uterine. PR-targeted strategies could provide a safe and useful means to improve treatment outcomes and increase overall ovarian cancer patient survival.

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CHAPTER 1

INTRODUCTION

1.1 Progesterone: sources and functions

Progesterone is a steroid hormone that is produced primarily by the corpus luteum in the ovaries during the second half of the menstrual cycle (i.e. luteal phase). Progesterone is also produced, to a lesser extent, in the adrenal glands and, during pregnancy, the placenta. Thus, cyclical hormone exposure beginning at menarche and ending in menopause occurs monthly and regulates the growth and differentiation of specialized tissues within the reproductive tract and breast tissues.[1, 2] Pregnancy interrupts this process, and is characterized by high progesterone levels, which are required for fetal development, breast development for lactation, maintenance of uterine/placental integrity, and myometrial quiescence.[3]

1.2 Epidemiological role of progesterone in ovarian tumors

A considerable body of epidemiological data suggests that progesterone and progestins play a protective role against ovarian carcinogenesis. Progesterone deficiencies due to increasing age, infertility, or a genetic LOH at the PR gene locus are associated with increased ovarian cancer risk.[4, 5] In

contrast, elevated progesterone levels decrease ovarian cancer risk. The protective effect of pregnancy has been documented in Asian, European, and North American populations[6]; progesterone levels during pregnancy are 10-fold greater than luteal phase levels measured during the menstrual cycle. Similarly, hormonal oral contraceptive use has been consistently associated with a reduced risk. In an analysis of 20 epidemiological studies between 1970-1991, it was estimated that a 35% reduction in risk was associated with ever-use of oral contraceptives.[7] Additionally, the risk of ovarian cancer is correlated with duration of oral contraceptive use: 10-12% decrease in risk with one year of use and 50% decrease after 5 years of use in both nulliparous and parous women.[7] Progesterone exerts a protective effect on ovarian cancer risk by reducing ovulation through elevated progesterone levels from oral contraceptive use or during pregnancy. Furthermore, PR expression, the PR-B isoform specifically[8-10], in ovarian tumors is a favorable prognostic marker associated with longer progression-free survival.[8, 11-18]

BRCA1/2 mutations may alter the production and sensitivity to estrogen and progesterone as carriers have an increased risk for breast and ovarian cancer. Studies in mice carrying a BRCA1 mutation in ovarian granulosa (i.e. hormone producing) cells[19-21] and in humans with either a BRCA1 or BRCA2 mutation[22] demonstrated that BRCA mutations confers higher serum (circulating) levels of both estrogen and progesterone. Moreover, serous tubal intra-epithelial carcinoma in the distal end of the fallopian tube was discovered in 10-15% of

BRCA carriers who had prophylactic salpingo-oophorectomy.[23, 24] Ultimately, little mechanistic information exists related to the impact that hormones have on the prevention and/or pathogenesis of ovarian cancer. The evidence related to the pathophysiology of ovarian cancer suggests a strong connection with estrogen, progesterone, and, more recently, androgen actions in the development and progression of ovarian cancer. Steroid hormone action in ovarian cancer is grossly understudied, and there is an urgent need to focus on the early events related to the contribution of hormones in the context of altered signaling events (loss of p53 or PTEN, elevation of AKT signaling) that predisposes women including those with BRCA mutations to an increased risk of breast and ovarian cancer.

1.3 Progesterone Receptor Isoforms

The actions of progesterone are mediated through the nuclear progesterone receptors (PRs). PR belong to the steroid receptor superfamily of related ligand-activated transcription factors that includes estrogen, androgen, mineralocorticoid, and glucocorticoid receptors.[25] PRs are composed of a modular domain structure that includes an N-terminal domain (NTD), DNA binding domain (DBD), hinge region (H), and hormone binding domain (HBD) (Figure 1). There are three PR isoforms: full length PR-B, N-terminally truncated PR-A (-164 amino acids), and the non-functional PR-C, consisting of only the hinge region and HBD. PR-B and PR-A are typically expressed in equimolar ratios and function as ligand activated transcription factors, whereas PR-C

expression is limited and may serve largely to sequester ligand, as it is incapable of binding DNA.[26]

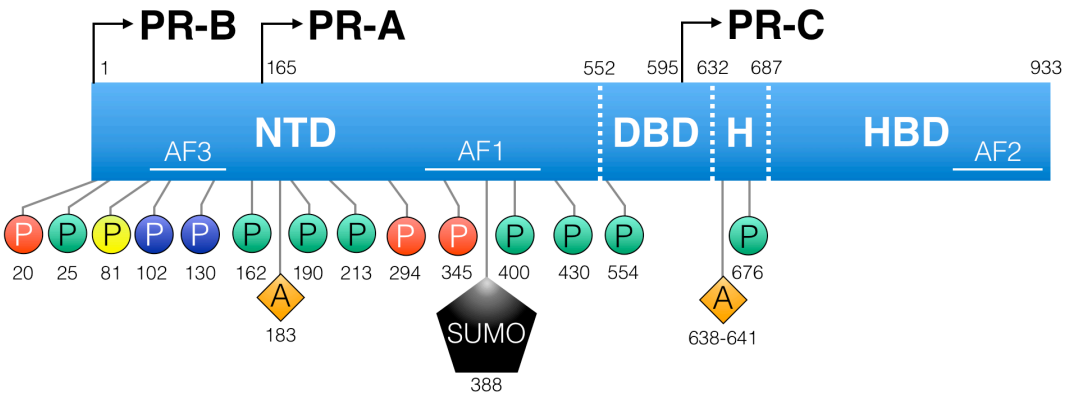

Figure 1-1: The post-translational modifications of progesterone receptors. 17 post-translational modification sites that impact PR-mediated transcriptional action. PR-B, but not PR-A, includes 164 additional amino acids in the NTD (called B upstream segment) where the third activation function domain and multiple phosphorylation sites are located. PR-B and PR-A are transcribed from the same gene and their protein isoforms are identical from amino acids 165-993. The protein tertiary structure results in a folding at the hinge region between the DBD and HBD. Post-translational modifications (phosphorylation, acetylation, and SUMOylation) can occur basally or in response to ligand binding and affect PR transcriptional activity. In particular, activated protein kinase pathways input to PR via phosphorylation and these pathways are heavily altered in breast, ovarian, and uterine carcinomas. Numbering reflects amino acid residue positions. The color of phosphorylation sites is associated with the following: red = MAPK; green = CDK2; yellow = CK2; purple = unknown kinases. PR, progesterone receptor protein isoforms A, B, or C; NTD, N-(amino)-terminal domain; DBD, DNA binding domain; H, hinge region; HBD, hormone binding domain; AF, activation function 1-3; P, phosphorylation; A, acetylation; SUMO, small ubiquitin-like modifier (SUMOylation).

Progesterone diffuses through the lipid membrane and interacts with the HBD of PR-A or PR-B. This interaction alters the conformation of PR favoring nuclear localization, dimerization (A:A, B:B, or A:B dimers are possible), and DNA binding. Classically, PR binds Progesterone Response Elements (PRE) in the DNA and recruits cofactors and transcriptional machinery to initiate gene transcription. PR can also participate in the transcriptional complexes of other

DNA bound transcription factors to alter gene expression.[27-30] Non-classical or extranuclear signaling of PR involves PR direct binding to kinases complexed at the membrane with growth factor receptors (such as EGFR or IGF1R) to initiate rapid activation of downstream signaling cascades.[31, 32] For example, progesterone induces rapid activation of ERK1/2 MAP kinase pathways, which function to activate a variety of transcription factors via phosphorylation events, including PR itself.[28, 31, 32] Notably, PR-B, but not PR-A, is capable of rapid signaling, likely in part owing to its relatively increased occupancy in the cytoplasm.[33]

Although PR isoforms share structural and sequence similarity, PR-A and PR-B are functionally distinct transcriptional regulators exhibiting recruitment to different subsets of PR-target gene promoters[34, 35]. PR-B, but not PR-A, functions outside of the nucleus to rapidly activate protein kinases (MAPK, AKT, c-Src) in part by a ligand-induced interaction between PR and c-Src kinase.[31, 32, 36] Rapid progesterone-induced c-Src/MAPK activation phosphorylates PR-B[37, 38] (Figure 1), and potentiates the actions of nuclear PR to specific target promoter regions. Recently, MAPKs (namely ERK2 and p38 MAPK) have been shown to participate in transcriptional complexes as part of steroid hormone receptor binding partners.[39, 40] Thus, differences between PR isoform activities may be attributed to differential cross-talk between PRs and protein kinases downstream of growth factor-mediated signaling pathways and/or differential recruitment of required co-

factors. It is unknown how PR isoform activities are regulated in ovarian cancer cells.

1.4 PR as a prognostic marker in ovarian tumors

Recent studies have revealed that 'ovarian cancer' is not a single disease, and a significant portion of ovarian tumors may not originate from ovarian tissue. At present, five major histopathological subtypes of epithelial ovarian cancer have been characterized and are phenotypically and molecularly distinct[41]: high-grade serous, low-grade serous, endometrioid, clear cell, and mucinous. Pathological and genomic studies indicate that cancers of these major subtypes are frequently derived from non-ovarian tissues that have metastasized and homed to the ovary (Figure 2). Clear cell and endometrioid ovarian cancers are derived either from the cervix or endometriosis, which itself is associated with retrograde menstruation from the endometrium.[42, 43] Invasive mucinous ovarian cancers are metastases from the lower intestinal tract (e.g. stomach, colon, appendix) to the ovary.[44] High-grade serous ovarian cancers are derived from the distal fallopian tubes.[23, 45] A recent study demonstrated that ovulation, the release of hormones (e.g. estrogen and progesterone), growth factors, and inflammatory factors among others, promoted the migration of intrauterine-injected malignant cells towards the ovarian stromal compartment to form "ovarian" tumors.[46] Thus, it is plausible that the unique hormonal milieu provided by functional ovaries serves to attract pre-malignant and malignant cells that may remain dormant (i.e. under progesterone concentrations) or fully progress to tumors (i.e.

postmenopausal contexts or upon loss of progesterone or functional PRs). Approximately 90% of ovarian cancers are detected in the ovary, with over 50% ovarian cancers diagnosed in post-menopausal women.[47]

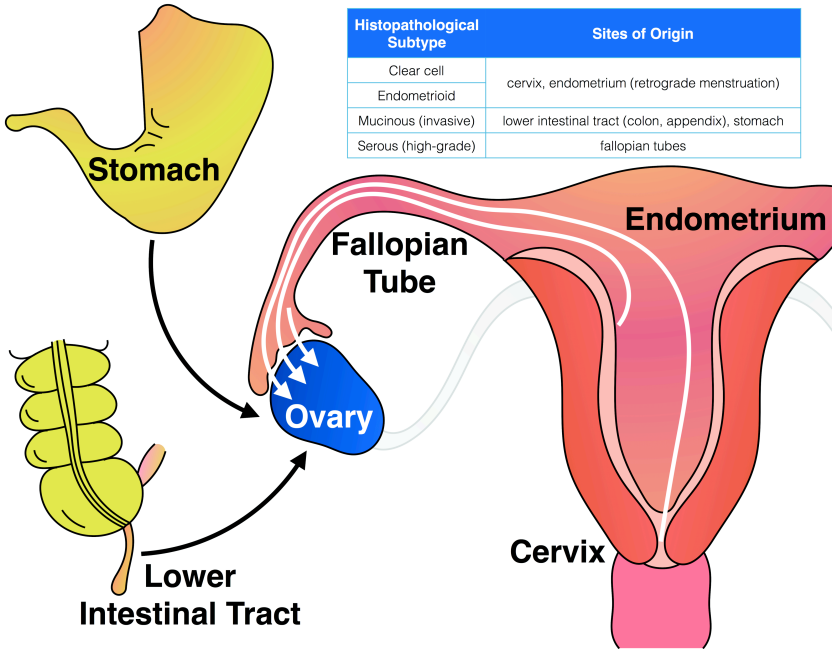

Figure 1-2: The cellular origins of ovarian cancer. Ovarian cancer is a collective term for several distinct invasive diseases that originate in the peritoneal cavity. *Inset*, the known sites of origin associated with the major histopathological subtypes of ovarian cancer. Mucinous ovarian cancers are metastases on the ovary from the gastrointestinal tract, including the stomach, colon, or appendix. Endometrioid and clear cell ovarian cancers are derived either from the cervix or from the uterus via progression of endometriosis, which is linked to retrograde menstruation from the endometrium. High-grade serous ovarian cancers are either derived from metastases from the distal fallopian tube or from the surface of the ovary.

Until recently, little was known about the relative distribution of PR within the subtypes of epithelial ovarian tumors. In a cohort of 504 tumors, we reported that 35% of ovarian tumors are PR-positive, with the highest total PR expression in endometrioid (67%) and serous (35%; low grade serous, 64%) subtypes.[48] In accordance with our study, the international Ovarian Tumor Tissue Analysis

consortium examined the association of ER and PR expression with subtype-specific survival in nearly 3,000 invasive epithelial ovarian tumors reporting positive total PR expression in endometrioid (67%), low-grade serous (57%), and high-grade serous (31%) tumors.[49] Additionally, the study confirmed the prognostic significance of PR expression in ovarian tumors strongly expressing PR (≥50% tumor cell nuclei staining). Strong PR expression in high-grade serous ovarian carcinomas was associated with a significant improvement in survival; positive PR expression (weak or strong) in endometrioid carcinomas was associated with significantly improved disease-specific survival independent of patient age and tumor grade, site, and stage. Of note, ER expression conferred a patient survival advantage in endometrioid ovarian tumors only. ER may contribute to the favorable prognosis in endometrioid ovarian tumors via regulation of PR expression; a functional ER signaling pathway promotes robust PR expression.[49] While total PR levels are routinely measured in breast and endometrial cancers (but rarely in ovarian cancer) for clinical management and disease treatment, very few studies have examined the levels of PR isoforms in ovarian tumors. To our knowledge, only three studies[8-10] have reported differential expression of PR isoforms in ovarian tumors. These studies have reported a dominance of PR-B expression in ovarian tumors across all sub-types, with PR-B frequently expressed in the serous subtype. In contrast, PR-A expression was weakly expressed in mucinous and serous ovarian carcinomas

and comparison of normal to malignant ovarian tissues revealed reduced to absent expression in malignant tumors relative to PR-B.[9]

1.5 Progesterone actions in ovarian cancer

The molecular mechanisms of progesterone's protective role in ovarian cancer are not well understood; both proliferative and inhibitory actions of progesterone have been reported in ovarian cancer cell line models. Several independent *in vitro* studies demonstrated anti-proliferative actions of progesterone at higher concentrations (≥1 μM) in ovarian cancer cells, primarily through the induction of apoptosis[50-53], while fewer studies reported progesterone as proliferative in these cells at lower concentrations.[54, 55] The opposing cellular responses of ovarian cancer cells to progesterone may be attributed to cell context-dependent regulatory inputs to PR, such as progesterone dosing, kinase activation state of the cells, cofactor availability, or PR-A and PR-B expression ratios. Ovarian cancer cells are susceptible to concentration-dependent and biphasic effects within the same cell model systems. Similar to breast and uterus, crosstalk between PR and growth factor-mediated signaling pathways (i.e. protein kinases) presumably directs PR promoter selection and specific cell fates (e.g. apoptosis). The relative abundance of cofactors that associate with PR also varies in a tissue-specific manner.[56, 57] As in other tissues (i.e breast and uterine), shifts in PR isoform ratios (PR-A and PR-B) and cofactor availability may contribute to variations in biological responses to progesterone.

Taken together, these observations and findings serve as a strong rationale for defining progesterone receptor signaling as a strategy to inhibit the malignant transformation of ovarian epithelial cells and suppress ovarian cancer progression. The mechanisms responsible for mediating the tumor suppressive effects of progesterone in the ovaries are grossly understudied and therefore are not understood. Accordingly, the aims of these studies are the following: I) Determine the level of expression of PR isoforms in human ovarian primary tumors and in well-characterized cultured ovarian cancer cell lines, II) define the biological impact of PR expression and activity on ovarian cancer cell biology, and III) identify the post-translational modifications to PR and the cofactors required for mediating PR-dependent signaling actions.

CHAPTER 2

PROGESTERONE RECEPTORS INDUCE FOXO1-DEPENDENT SENESCENCE IN OVARIAN CANCER CELLS

2.1 INTRODUCTION

Although ovarian cancer accounts for approximately 3% of all cancers among women, it is the deadliest among gynecologic cancers. An estimated 15,500 deaths were expected in 2012[58], a death rate of more than 50% due to late detection of the disease and intrinsic or acquired resistance to current therapeutic strategies. The identification of reliable biomarkers for early detection of OC will have a substantial impact on survival rates, while molecular markers that predict outcome may allow for efficacious targeted therapies.

Progesterone receptors (PR) belong to the steroid receptor superfamily of related ligand-activated transcription factors that includes estrogen, androgen, mineralocorticoid, and glucocorticoid receptors.[25] PR is a classical estrogen-regulated ER-target gene. Two PR isoforms (full-length PR-B and N-terminally truncated PR-A) have been identified and characterized as ligand-activated transcription factors with distinct transcriptional activities, while a third (PR-C) modulates the other two in selected tissues.[34, 59-62] Upon ligand binding, PR binds

directly to specific progesterone response elements (PREs) or tethers to other DNA-binding transcription factors to alter gene expression.[25]

PR has become an attractive target in OC. Progesterone deficiencies and a genetic loss of heterozygosity at the PR gene locus (Chr 11q23.3-24.3)[63] are associated with increased OC risk. While elevated progesterone levels appear to play a protective role, multiparity and elevated circulating progesterone levels (10-fold) during pregnancy, as well as the suppression of ovulation, are associated with decreased OC risk.[64] Similarly, the use of progestin-containing oral contraceptives is associated with decreasing lifetime risk of OC.[65] The expression of PR is a favorable prognostic marker in ovarian tumors and associated with longer progression free survival.[8, 11-18]

PR transcriptional activity is commonly linked to the expression of many cell cycle regulators including members of the cyclin, cyclin-dependent kinase (CDK), and p21/p27 families.[66] PR is often associated with survival and cell-cycle progression in breast and prostate cancer cells.[38, 67, 68] Specifically, PR-B isoforms are more potent transcription factors in reporter gene assays and at selected PR target genes relative to PR-A isoforms, including genes that encode cell cycle regulators.[34, 35] PR-B but not PR-A isoforms mediate mammary gland alveologenesis during normal breast development[60] and induce cyclin D1-driven proliferation and pro-survival in breast cancer cells.[69] Interestingly, however, a handful of reports have suggested that progesterone may inhibit these effects in ovarian cancer cells.[50, 54, 55, 70-72] Of particular interest, is the association of PR-B

expression with the induction of cell-cycle arrest first observed in Ras-transformed NIH3T3 cells[73] and later extended to include OC cells.[74] Furthermore, expression of PR-B isoforms in ovarian tumors is associated with longer progression-free patient survival and an indicator of positive prognosis.[8, 10] Herein, the goal of our studies was to further investigate the impact of PR-B expression and activation on OC cell proliferation and to determine the signaling mechanisms responsible for PR-B-mediated cell-cycle control.

2.2 MATERIALS AND METHODS

Human Tissue Microarray and Immunohistochemistry

Tissue microarrays (TMA's) were constructed as previously described[75] from representative tumor areas using a tissue arrayer (Beecher Instruments, Silver Spring, MD and Pathology Devices, Westminister, MD). Arrays were stained for PR (Ventana, #790-2223) and ER (LabVision, #RM9101S0). Immunohistochemistry was scored using a subjective assessment of percent positive cells in a TMA core where scores of '0' = no positive cells, '1' = > 0% but < 15%, '2' = ≥ 15% but < 50%, and '3' = ≥ 50%. Recursive partitioning was used to determine the binarisation cutpoint for optimal separation of the five major sub-types of ovarian carcinoma and resulted in raw scores of '0' being considered negative (coded as '0') and raw scores greater than '0' being considered positive (coded as '1'). Contingency analysis was used to determine if there was differential expression of PR across the five major sub-types of ovarian

carcinoma, and the significance of those differences was determined by the Pearson Chi Square (X^2) statistic.

Cell Culture and stable cell line generation

All cell lines were grown at 37ºC under 5% CO_2 in water-jacketed incubators (Forma Scientific, Asheville, NC). 1816-575 cells were kindly provided by Dr. Patricia Kruk (University of South Florida). HEY and ES-2 cells were kindly provided by Dr. Amy Skubitz (University of Minnesota). OVCAR-3 cells were kindly provided by Dr. Sundaram Ramakrishnan (University of Minnesota). OVCAR-8 cells were purchased from the DCTD Tumor/Cell Line Repository. PEO4 cells were kindly provided by Dr. Scott Kaufmann (Mayo Clinic). TOV-112D were purchased from American Type Culture Collection. The T47D and T47D-YB breast cancer cell lines were maintained as described.[76] The parental ES-2 ovarian cancer cell line was maintained as described.[77] With the exception of the experiments performed on unstarved and untreated growing cell lines in Fig. 1, 24 hours prior to all experiments, cells were washed with PBS and placed in Modified IMEM supplemented with 5% charcoal-stripped FBS (i.e. DCC) (Hyclone, #SH30068.03).

Empty GFP-vector control and GFP-PR stable cell lines were generated using the parental ES-2 cell line as a model system. Stable cell lines were generated by transfecting cells with 2.0 µg of their respective plasmids using FuGene HD® transfection reagent (Roche, #04709691001) according to manufacturer's instructions. After transfection, the cells were selected and

maintained with 0.5 mg/mL of G418 sulfate. The human PR-B gene was previously cloned into the pEGFP-N3 vector (Clontech Laboratories, Inc.), which also served as the Empty Control GFP vector.[59] Fluorescence-activated cell sorting (FACS) with a FACSDiva™ cell sorter (BD Biosciences, San Jose, CA) was used to purify Empty GFP-vector control and GFP-PR-B containing stable cell lines by removing any low and non-GFP-expressing cells.

Stable shRNA expressing cell lines were created by transducing ES-2 GFP-PR cells with pLKO.1 lentiviral vectors containing target gene shRNA sequences from the Open Biosystems Expression Arrest™ TRC Library (Thermo Scientific). shRNA cell colonies were selected in and maintained as described earlier with 1 μg/mL of puromycin. Stable control shRNA GFP-PR cells (sh-control) were created using an empty vector (clone RHS4080) containing an 18 bp non-targeting, non-specific "stuffer sequence". Stably expressing p21 shRNA GFP-PR cells were created using a pLKO.1 lentiviral vector (clone TRCN0000010401), and FOXO1 shRNA GFP-PR cells were created using pooled pLKO.1 lentiviral vectors (clones TRCN0000039578 and TRCN0000039579).

Primary Human Ovarian Cancer Cell Isolation

Cancerous human tissue samples were harvested at the time of surgery and immediately placed in 37°C PBS. Tissue samples were dissected into small pieces and placed in 6-well plates containing 0.25% trypsin-EDTA (1mM) (Invitrogen, #25200-056) for 3 hours at 37°C. Trypsinization was neutralized 1:1

with serum supplemented cell culture media and cells were allowed to plate overnight. The following day, partially digested tissue was removed and adherent cells were extensively washed with PBS before cell culture media was replaced. All tissue samples were typed and graded by in-house surgical pathologists.

Reagents

Cells were treated with the following reagents (when applicable): R5020 (Perkin Elmer, #NLP004005MG), RU486 (Sigma, #M8046), β-estradiol (Sigma, #E8875), and AS1842856 (EMD Millipore, # 344355). For experiments with AS1842856, cells were pretreated for one hour prior to the addition of R5020 in combination treatment studies.

Immunoblotting

Western blots were performed as previously described.[77] Western blots were probed using the following primary antibodies: PR-A/B (Ab-8, Fisher Scientific Lab Vision #MS298P) and PR (H-190) antibody (Santa Cruz Biotechnology, #sc-7208); full-length and cleaved-PARP (#9546), p21 (#2946), and FOXO1 (#2880) were purchased from Cell Signaling Technologies; and Actin (Sigma-Aldrich, #A4700). Actin (Sigma, #A4700) and ERK (Cell Signaling Technologies, #9102) were used as loading controls where indicated. HRP-conjugated goat anti-rabbit and goat anti-mouse (Bio-Rad, #170-6515 and #170-6516) secondary antibodies were used to detect their respective primary antibodies, and immunoreactive proteins were visualized on Kodak X-OMAT LS

film (Carestream Health, #864-6770) following ECL detection with Super Signal® West Pico Maximum Sensitivity Substrate (Pierce, #34087).

Real-Time Quantitative-PCR (RT-qPCR)

Total RNA was extracted from cell samples using TriPure Isolation Reagent (Roche, #11667165001) and isopropanol precipitation. RNA (1.0 μg) was reverse transcribed to cDNA according to manufacturer's instructions using the qScript cDNA SuperMix (Quanta Biosciences, #95048-100). qPCR was performed using Light Cycler® FastStart DNA Master SYBR Green I (Roche, #12239264001) on a Light Cycler® 480 II Real-Time PCR System (Roche). Human primer sequences are listed in Supplementary Table 1. qPCR cycling conditions were as follows: initial denaturation at 95°C for 10 min; denature at 95°C for 10 sec, anneal at 60ºC for 10 sec, and extension at 72º C for 5 sec for 45 cycles.

Luciferase Assays

Cells were co-transfected overnight using FuGene HD® transfection reagent (Roche) according to manufacturer's instructions with 0.9 μg of either a PRE-containing[34] or a p21 promoter-containing[60] firefly luciferase reporter construct and 0.1 μg of a constitutively active pRL-TK-*Renilla* luciferase construct (Promega, #E2241). Luciferase assays were performed as previously described[37] using the dual luciferase reporter assay (Promega, #E1910).

Histology and Microscopy

All brightfield and fluorescent cell images described herein were acquired with an Axioplan 2 upright microscope (Zeiss, Thorwood, NY) and captured using a SPOT camera (Diagnostic Instruments, Inc., Sterline Heights, MI) with the ProgRes Capture Pro software (Version 2.8.8) (Jenoptik Optical Systems Inc., Easthampton, MA). Cellular membranes were stained for 15 min at RT with Texas Red®-X-conjugated wheat germ agglutinin (1 µg/mL) (Invitrogen, # W21405), and all cell nuclei were stained with DAPI containing ProLong® Gold antifade reagent (Invitrogen, #P-36931). Fixed and live cell soft-agar images were acquired with a Leica DM IL inverted microscope (Leica Microsystems, Inc.) and captured using a MagnaFire® camera and MagnaFire® imaging software (Olympus, Melville, NY).

Cell proliferation and survival assays

Cell proliferation was measured by using MTT assays as described previously.[78]

Anchorage-independent growth and survival were measured by using soft agar assays as previously described[79] with the exception of seeding 2 X 10^4 cells. After 4 weeks of growth at 37º C, cell colonies were stained with 0.1% crystal violet for 1 hr at RT and washed with PBS. 1,000 randomly chosen cell colonies per well were separated according to size (total pixels/colony area) and quantified using Photoshop® version 7.0 (Adobe Systems, Inc.).

Senescence Associated-β-galactosidase (SAβGal) Activity Assays

Cells were continuously treated for four days in 5% DCC. Cells were washed, fixed, and stained for SAβGal activity according to manufacturer's instructions using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, #9860).

Flow Cytometry

Cells were treated in 5% DCC for 96 hrs. Cells were collected, fixed and stained as described previously.[76] Propidium iodide staining was detected using a FACSCalibur (BD Biosciences). Cells were gated for cell cycle phases using FlowJo software (Tree Star Inc.). Cells were stained with 2 μg/mL of Hoechst 33342 DNA-specific dye (Invitrogen, #H3570) and 0.5 μg/mL of Pyronin Y RNA-specific dye (Polysciences, #18614-5). FACS analysis performed on a BDTM LSR II Flow Cytometer System (BD Biosciences) was used for cell-cycle analysis and separation of the G0 (Hoescht-low and Pyronin Y-low) and G1 (Hoescht-low and Pyronin Y-high) phases of the cell cycle as previously described.[80]

Chromatin Immunoprecipitation (ChIP)

Cells were treated for 1 hr with either R5020 or ethanol vehicle in 5% DCC and cell samples were fixed, harvested, and lysed according to optimized manufacturer's instructions using the ChIP-IT™ Express Magnetic Chromatin Immunoprecipitation Kit (Active Motif, #53008). Samples were homogenized using a Bioruptor sonicator (Diagenode, Inc.). ChIP reactions were incubated overnight on an end-to-end rotator using 95 μL of isolated chromatin and either 2

μg of PR-A/B antibody (Ab-8), FKHR (H-128) antibody (Santa Cruz Biotechnology, Inc., # sc-11350) or 0.4 μg of normal mouse or rabbit IgG (Santa Cruz Biotechnology, Inc., #sc-2025 # sc-2027). Samples were washed, eluted, reverse cross-linked, and treated with Proteinase K according to manufacturer's instructions (Active Motif). DNA was analyzed by RT-qPCR as described above.

Statistical Analysis

All reported values represent data representative of the indicated replicates of each experiment (mean ± the standard deviation (SD)). Statistical analyses were performed using a Student's two-tailed *t*-test where significance was determined with 95% confidence (*$p \leq 0.05$, **$p \leq 0.01$).

2.3 RESULTS

PR expression in OC tumors and cell lines.

Studies of limited sample size report decreased or absent PR expression in human OC tissue samples and little information exists on the relative distribution of PR within OC subtypes.[63] We evaluated the percentage of PR-positive tumors from each major histological sub-type of ovarian surface epithelial (OSE) derived OC in a cohort of 504 tissue samples (Figure 1A). While percentages varied between sub-types, each group contained PR-positive tumors. PR expression was highest in endometrioid (67%) and serous (35%; low-grade, 64%) tumors. Overall, 35% of ovarian tumors were PR-positive; a value consistent with larger mixed cohort studies.[10, 16] The distribution of estrogen

receptor (ER)-positive tumors in the same cohort was similar to that of PR with endometrioid (77%) and serous (>70%) tumor sub-types displaying the highest portion of ER-positivity; the overall percentage of ER-positive tumors was 55%.

We next examined expression levels of PR in a panel of six established human OC cell lines of epithelial origin and one immortalized normal OSE cell line (1816-575). PR protein expression was low to negligible in all cell lines (data not shown), regardless of histologic sub-type (clear cell, ES-2; serous, HEY, OVCAR-3, OVCAR-8, PEO4; endometrioid, TOV-112D). Additionally, RT-qPCR analysis detected minimal levels of PR mRNA in each of these cell lines when compared to T47D human breast cancer cells (Figure 1B). Cell line models of human tumors frequently lose steroid hormone receptor expression when grown in tissue culture. Alternatively, estrogen responsiveness of the PR promoter may be diminished.[81]

To determine if ER-positive ovarian cancer cells are capable of inducing endogenous PR expression, PEO4 cells were treated with β-estradiol (E2, 1 nM) and PR mRNA and protein expression was evaluated (Figure 1C). E2 treatment of PEO4 cells significantly induced PR mRNA expression (4.3-fold) compared to vehicle treated controls. Untreated PEO4 cells contained low levels of PR-B protein. The expression of both PR isoforms (PR-A and PR-B) was significantly induced upon estrogen (E2) treatment relative to vehicle-treated and R5020-only, while R5020 treatment caused a slight up-shift in gel mobility of both PR-A and PR-B proteins in E2-treated cells (Figure 1D). The transcriptional activity of

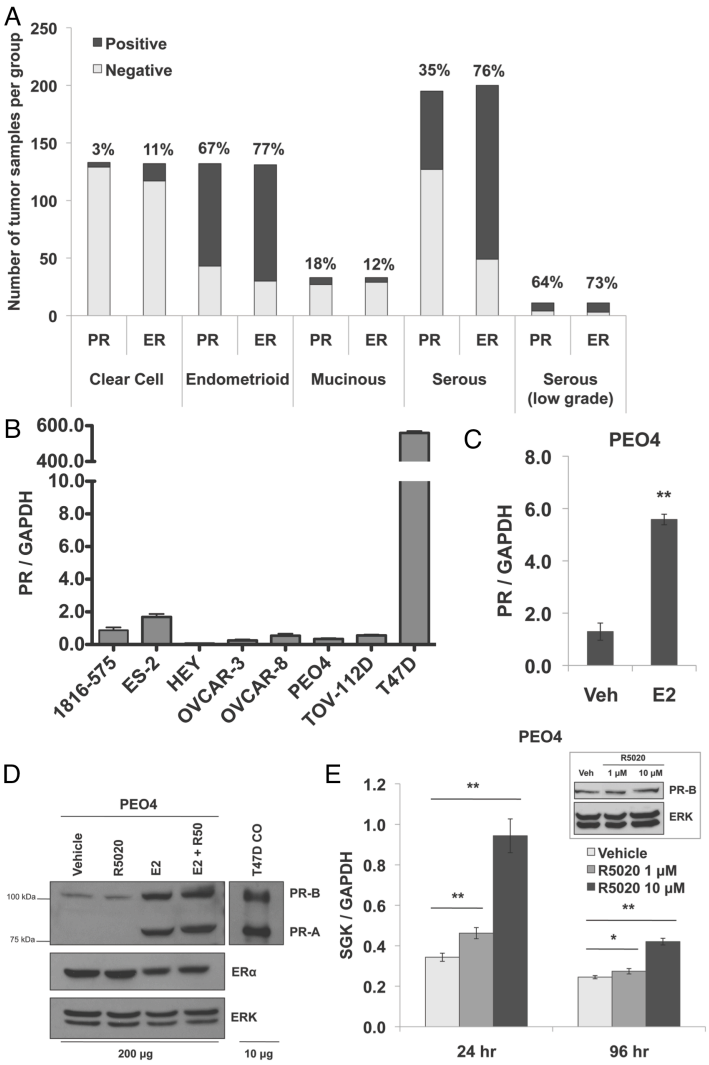

Figure 2-1: PR is expressed in human ovarian cancer tissues and cancer cell lines. A) Immunohistochemical staining for PR in human ovarian cancer tissues representing the five major sub-types of ovarian surface epithelial (OSE) origin (n=504). B) RT-qPCR analysis of PR mRNA expression in a panel of six ovarian cancer cell lines and one immortalized, non-transformed cell line relative to PR+ T47D breast cancer cells. All values were normalized to GAPDH levels. C) RT-qPCR analysis of PR mRNA expression of PEO4 cells treated with vehicle (ethanol) or β-estradiol (E2, 1 nM) for 24 hr (n=3, **p≤0.01). D) Western blot analysis of PR and ERα protein expression in PEO4 cells treated with vehicle, R5020 (10 nM, 1 hr), E2 (1 nM, 48 hr), and E2 (1 nM, 48 hr) followed by R5020 (10 nM, 1 hr). T47D CO total cell lysate was loaded on the same gel as a positive control for PR expression. Total ERK was used as a loading control. E) Inset, Western blot analysis of PR expression in PEO4 cells treated with vehicle and R5020 (1 and 10 µM) for 48 hr. RT-qPCR analysis of SGK mRNA expression after 24 hr and 96 hr R5020 treatment (1 and 10 µM) in PEO4 cells (n=3, *p≤0.05, **p≤0.01). All values were normalized to GAPDH levels.

endogenous PR in PEO4 cells was evaluated in response to increasing doses of the PR-specific agonist, R5020, a synthetic progestin, at 24 and 96 hr. In the absence of estrogen, expression of the classic PR-target gene, serum/glucocorticoid regulated kinase (SGK)[82, 83] was significantly induced by R5020 (10 mM) treatment relative to vehicle controls (Figure 1E), consistent with the modest up-shift in gel mobility of PR-B protein (Figure 1D inset). Pretreatment with estrogen did not further sensitize these cells to progestin (data not shown). These data suggest that OC cell lines express low abundance functional PR-B, as measured by the ability of progestins to induce PR-target gene (SGK) expression.

Progestin induces non-proliferative cell survival in OC cells.

In order to investigate the impact of PR expression and signaling on OC cell biology without the confounding effects of exogenously added estrogen, we created a PR-expressing OC cell line. We chose ES-2 cells due to their inherently aggressive nature, rapid growth rate, ability to form tumors in xenograft[84, 85], and low endogenous PR mRNA levels (Figure 1B). Notably, these cells are resistant to estrogen-induction of endogenous PRs (data not shown). ES-2 cells were transfected with GFP-tagged PR-B or GFP-only vectors, and PR+ clones were selected for PR protein expression levels that were comparable to similarly created T47D-YB breast cancer cells[86] (Figure 2A inset). PR transcriptional activity in GFP-PR and control ES-2 cells was evaluated by PRE-luciferase reporter assays. R5020 stimulated luciferase activity in GFP-PR cells,

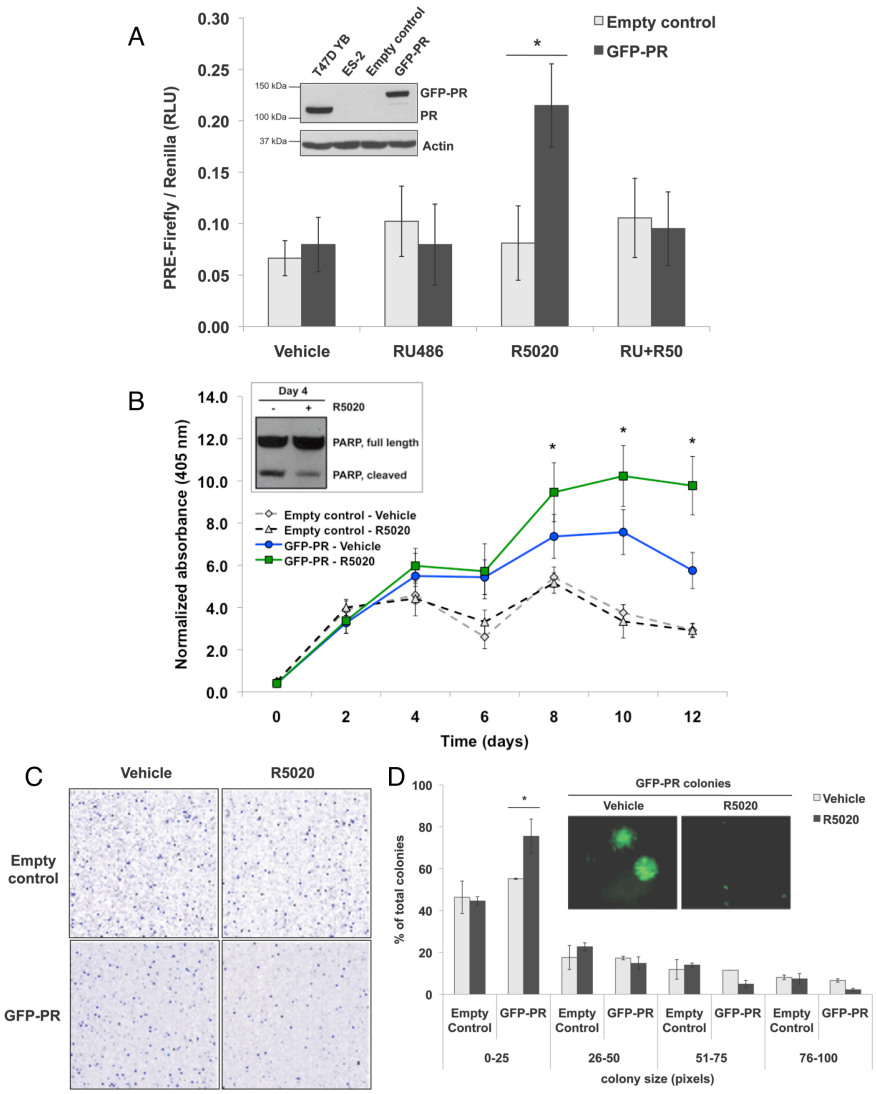

Figure 2-2: Stable expression of PR in ES-2 cells increases cell survival and inhibits cell colony formation. A) *Inset*, Western blot analysis showing stable expression of GFP-tagged PR-B in ES-2 cells (GFP-PR) as compared to parental ES-2 cells stably expressing GFP-tagged empty vector construct (Empty control), and PR-B expressing T47D breast cancer cells (T47D-YB). ES-2 GFP-PR cells transiently transfected with a progesterone response element (PRE) containing luciferase reporter construct were treated for 48 hours with R5020 (10 nM) or RU486 (1 uM). Relative luciferase units (RLU) were normalized to the mean result ± standard deviation (SD) for Renilla luciferase expression (n=4, *p≤0.05). B) Inset, Western blot analysis of total and cleaved PARP in GFP-PR-containing ES-2 cells treated with R5020 for 4 days. Viable GFP-PR cells continuously treated with R5020 (10 nM) as measured by MTT assay (all values normalized to Day 0 readings, mean ± SD, n=3, *p≤0.05). C) Empty control and GFP-PR expressing cells grown in soft-agar and stimulated with R5020 (10 nM) for 4 weeks. Colonies were stained with crystal violet. D) Quantification of equal numbers of

colonies grown in soft-agar for 4 weeks (mean ± SD, n=3 fields/sample, 10^2 colonies/field, *p≤0.05). *Inset*, Representative live-colony image taken at 100X magnification demonstrating the presence of viable, single- and two-cell colonies in 4-week R5020 (10 nM) treated GFP-PR samples.

but not in GFP-vector control cells; PR transcriptional activity was blocked by co-treatment of the cells with the competitive PR antagonist RU486 (Figure 2A). Endogenous PR target genes (p21 and KLF4)[28, 78] were similarly regulated in breast and ovarian cancer cells stably expressing PR-B (data not shown). Fluorescence microscopy indicated that GFP-PR-B accumulated in the nucleus upon R5020 stimulation (Figure 3).[87] Interestingly, we observed an increase in the overall size of nuclei present within GFP-PR-B expressing cells exposed to R5020 for as little as 24 hours (Figure 3).

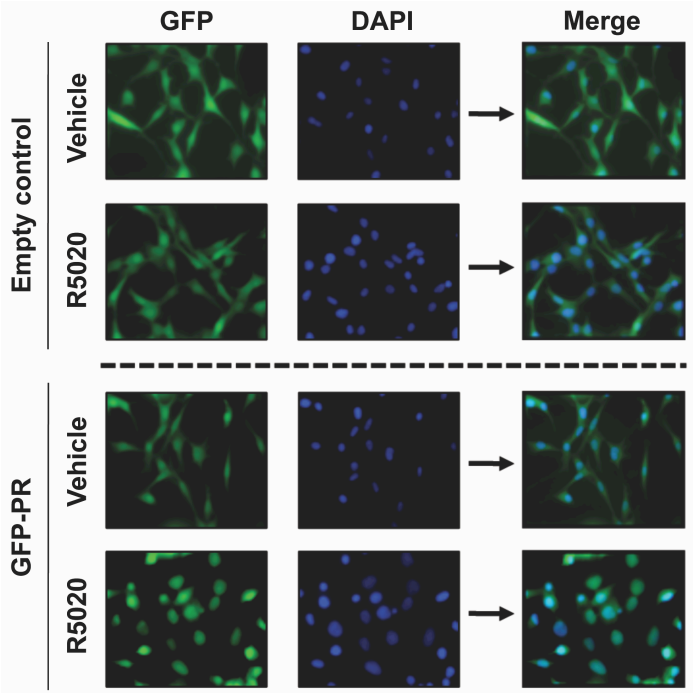

Figure 2-3: Functional activity of stably expressed GFP-tagged PR in ES-2 ovarian cancer cells. Immunofluorescence microscopy of Empty control and GFP-PR-containing cells stimulated with vehicle (ethanol) control or R5020 (10 nM) for 24 hr. All images were acquired at 200X magnfication.

Prior studies have shown that progesterone exhibits both proliferative[54] and anti-proliferative effects on the growth of OC cells[51, 52, 88] with inhibitory effects observed at particularly high concentrations (≥ 10^{-6} M) of ligand.[53-55] We investigated the effects of progestin on growth characteristics of GFP-PR ES-2 cells. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assays were initially utilized to study long-term effects of progestin treatment. GFP-PR and vector control cells were plated in equal numbers and stimulated with R5020 for 12 days. Measurements taken at 2-day intervals revealed an increase in growth, as measured by the number of viable cells expressing GFP-PR (treated or untreated with R5020) relative to vector-matched controls beginning at Day 2, with significantly increased growth at Days 4, 6, and 8. By Day 8, cells containing GFP-PR treated with progestin significantly outnumbered vehicle-treated control cells expressing GFP-PR (Figure 2B). Interestingly, proliferation of ligand-stimulated cells expressing GFP-PR ceased by Day 8 and the number of viable cells present through day 12 remained unchanged. Cell numbers in all groups began to diminish at late time points, likely due to nutrient (i.e. in media) starvation. However, cells in the R5020-treated cohort failed to die off in a predictable manner over a long period of time without media replenishment (Figure 2B). These findings suggest that PR may positively influence ovarian cancer cell number by promoting increased cell survival. We utilized poly (ADP)-ribose polymerase (PARP) cleavage as an indicator of apoptotic cell death. Beginning as early as Day 4, the amount of

cleaved-PARP was greater (2.7-fold) in vehicle-treated samples relative to R5020-treated samples, suggesting that PR activity inhibits apoptosis of GFP-PR cells (Figure 2B; inset).

To further examine the effects of liganded PR on OC cell survival and specifically, anchorage-independent growth, we performed soft agar colony formation assays where the constraints of 2-D growth and serum starvation are non-limiting over a four-week time course. When GFP-PR and vector-matched controls were either cultured with vehicle or R5020 for four weeks, R5020 stimulation dramatically inhibited the formation of GFP-PR-containing cell colonies compared to GFP control cells (Figure 2C). Additionally, when an equal number of colonies were objectively sorted based on size by computer analysis, there were significantly more small colonies (0-25 pixels) and fewer large colonies (51-75 and 76-100 pixels) present in the ligand-stimulated group of GFP-PR-containing cells compared to vehicle controls (Figure 2D). After 4 weeks of soft-agar growth, single- and two-celled GFP-PR "colonies" or cell clusters remained viable but appeared dormant in the R5020-treated condition (Figure 2D, inset). In sharp contrast, progestins induce pro-survival but are clearly mitogenic in PR-B+ breast cancer cells (MCF-7, T47D) grown in similar conditions.[37, 79]

PR mediates OC cellular senescence.

Contrary to the pro-survival and pro-proliferative impact of progestins in breast cancer models, our data suggest that in the presence of progestin, PR-B

promotes a pro-survival but anti-proliferative phenotype in ES-2 OC cells. This paradoxical scenario where cells exist in a viable and metabolically active but non-proliferative state may be explained by the phenomenon of cellular senescence. The exit of proliferating cells from the cell-cycle (i.e. into G0) can be separated into a quiescent arm, where appropriately stimulated cells are capable of re-entering the cell-cycle, and a senescent arm that is classically defined as a state of permanent cell-cycle arrest.[89] Therefore, we analyzed whether GFP-PR cells undergo a senescent transition following prolonged progestin exposure based on three criteria commonly used to identify cellular senescence: the expression of senescence-associated β-galactosidase (SAβGal), altered cell morphology, and cell cycle profiling.

The most common marker of cellular senescence is the accumulation of endogenous lysosomal beta-galactosidase, as measured by assay of senescence-associated beta-galactosidase (SAβGal) activity at pH 6.[90] GFP-PR cells exposed to R5020 for four days significantly induced SAβGal (40%) relative to minimal SAβGal (10%) in empty vector and vehicle-treated cells, indicating senescence is induced in a ligand-dependent fashion specific to PR-positive cells (Figure 4A-B). We examined GFP-PR cells treated with R5020 (12 days) under high magnification (400X) for morphologic changes consistent with senescence (Figure 4C). SAβGal positive cells (white arrows) possessed enlarged nuclei and exhibited a wide, flattened appearance relative to SAβGal-negative cells in the same culture (asterisk).[91] Finally, cell cycle analysis of GFP-PR cells by

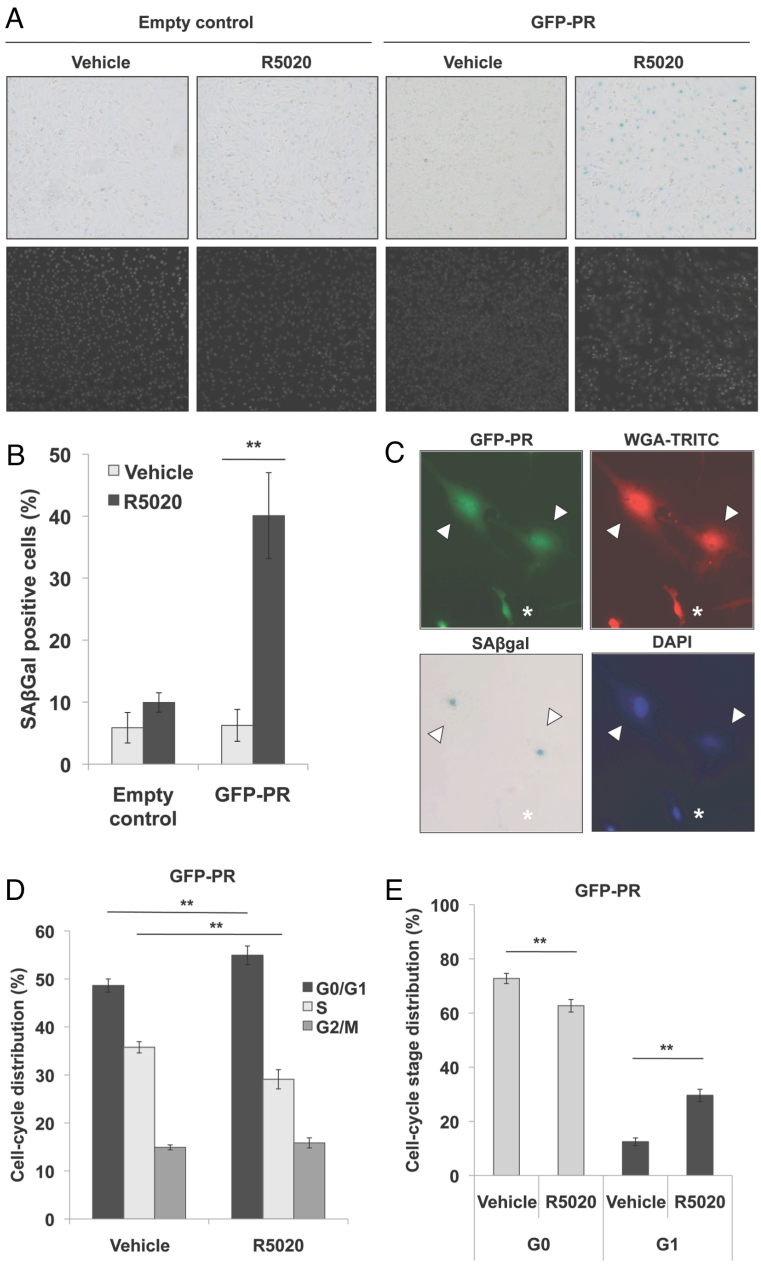

Figure 2-4: PR expression and activity induces cellular senescence.
A) Representative staining for SAβGal activity of Empty control and GFP-PR cells treated with R5020 (10 nM) for 96 hrs. (Magnification = 100X). Cell samples were mounted onto glass slides using ProLong® Gold Antifade Reagent with DAPI (Invitrogen) for brightfield microscopy. B) Percentage of positive SAβGal cells was determined from quantitating three fields at 100X magnification. Values were normalized to total nuclei present in each field from DAPI staining (n=3, **p≤0.01). C) Exposure of ES-2 cells expressing GFP-PR to R5020 (10 nM) for 12 days induced cellular senescence as indicated by cells (arrowheads) with increased SAβGal activity, while non-senescent cells (asterisk) remain SAβGal negative. Senescent GFP-PR-containing

cells also develop a characteristically larger, more flattened morphology as revealed by TRITC-labeled wheat germ agglutinin (WGA-TRITC) cell membrane staining. Nuclei were identified by DAPI counterstaining. All images were acquired at 400X magnification. D) Cell cycle analysis by propidium iodide staining of GFP-PR-containing cells treated with R5020 (10 nM) for 96 hr (n=3, **p≤0.01). E) Flow cytometric analysis of DNA and RNA by Hoescht 33342 and Pyronin Y staining, respectively, of GFP-PR-containing cells treated with R5020 (10 nM) (**p≤0.01).

propidium iodide staining for DNA content demonstrated a significant increase in the percentage of cells in the G0/G1 phase accompanied with a decrease in the percentage of cells in the S phase of the cell cycle following four days of ligand exposure relative to vehicle controls (Fig. 4D). Quiescent cells, arrested in G0 phase, have lower RNA content levels compared to transcriptionally active senescent cells in G1 phase.[92] To further discriminate between G0 and G1 populations from the combined G0/G1 compartment, we conducted differential staining of DNA (Hoescht 33342) and RNA (Pyronin-Y) prior to flow cytometry.[80, 93] We observed a significant decrease in the percentage of GFP-PR cells in G0 phase relative to vehicle-treated cells, while the percentage of cells accumulating in the G1 phase was significantly increased (Fig. 4E). Taken together, these results suggest that OC cellular senescence occurs by a progestin and PR-B-dependent pathway.

Progestin-induced p21 expression mediates rapid OC cell senescence.

Cell cycle mediators are critical drivers of senescence.[94] p21 is a well-known cell-cycle inhibitor best characterized for its ability to prevent the transition from the G1 phase to the S phase by blocking cyclin E-CDK2 activity and to a

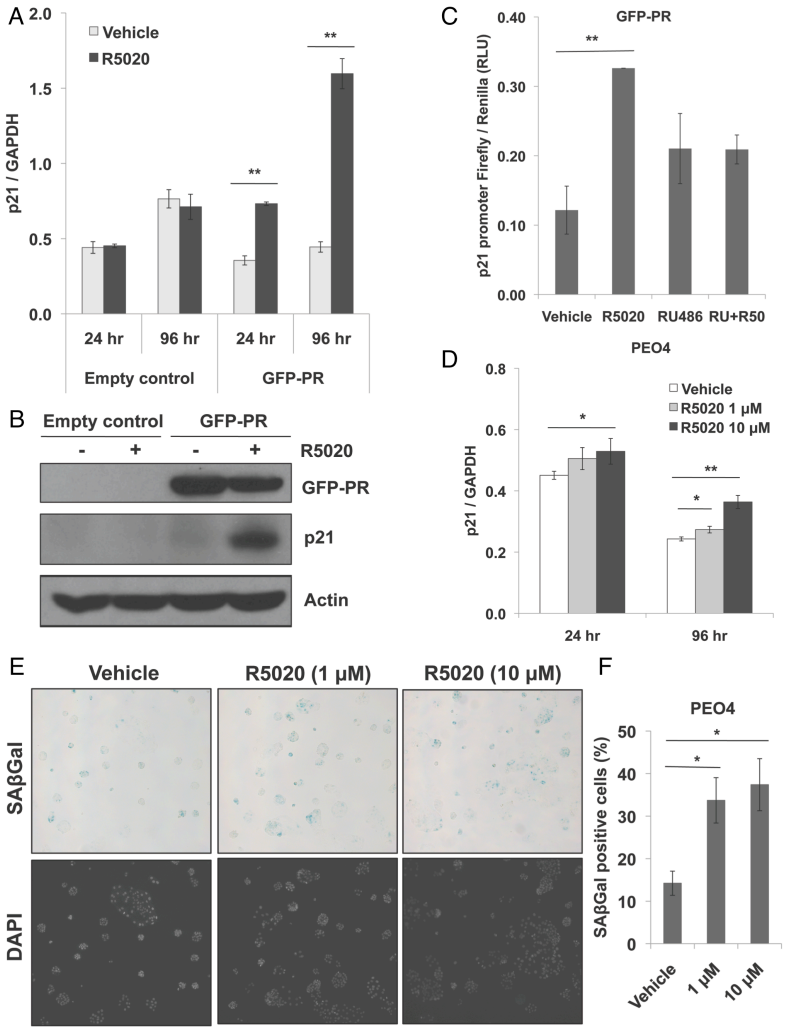

Figure 2-5: Progestins upregulate p21 expression to mediate cellular senescence. A) RT-qPCR analysis of p21 mRNA levels after 24 hr and 96 hr R5020 (10 nM) treatment in Empty control and GFP-PR-containing cells (n=3, **p≤0.01). B) Western blot analysis of p21 protein expression in Empty control and GFP-PR-containing cells after 8 days of R5020 (10 nM) treatment. C) ES-2 GFP-PR cells transiently transfected with a p21 promoter-containing luciferase reporter construct were treated for 24 hours with R5020 (10 nM) or RU486 (1 uM). Relative luciferase units (RLU) were normalized to the mean result ± standard deviation (SD) for Renilla luciferase expression (n=4, **p≤0.01). D) RT-qPCR analysis of p21 mRNA expression after 24 hr and 96 hr R5020 (1 and 10 μM) treatment in PR+ PEO4 cells (n=3, *p≤0.05, **p≤0.01). E) Representative staining for SAβGal activity in PEO4 cells treated with R5020 (1 and 10 μM) for 96 hr. (Magnification = 100X). Cell samples were mounted onto glass slides using ProLong® Gold Antifade Reagent with DAPI (Invitrogen) for brightfield microscopy. F) Percentage of positive SAβGal cells was determine from quantitating three fields at 100X magnification. Values were normalized to total nuclei present per field from DAPI staining (n=3, *p≤0.05).

lesser extent cyclin A-CDK1/2 and cyclin B1-CDK1 activities.[95] For this reason, increased p21 expression and activity have been directly linked to the induction of cellular senescence.[96-98] Known PR target genes (p21, p15, p16, p27)[34, 35, 78] were examined in progestin-treated GFP-PR and control cells. Notably, in the presence of progestin, p21 exhibited significantly increased mRNA and protein expression (Figure 5A-B). Similar although somewhat blunted p21 induction was observed in R5020-treated PEO4 cells (Figure 5D). In addition, we observed increased p21 levels following up to eight days progestin treatment of primary isolates of PR+ OC cells (Figure 6B). p21 upregulation occurred at the level of transcription, as R5020 (24 hr) induced p21 promoter activity, as measured by luciferase reporter-gene assays; p21 transcriptional activity was blocked by co-treatment of the cells with RU486 (Figure 5C).

Consistent with our results in ES-2 cells expressing GFP-PR, PR+ PEO4 cells exhibited a significant increase in SAβGal following progestin exposure (Figure 5E-F). In addition, primary isolates of human OC cells expressing PR (Figure 6A) exhibited increased SAβGal activity following 10 days progestin-treatment (Figure 6C). These data confirm that endogenously expressed PR drives senescence upon progestin stimulation of unmodified OC cells.

We next sought to determine whether progestin-induced senescence was dependent upon p21-induced cell cycle inhibition. We generated GFP-PR cells stably expressing p21-targeted shRNA (sh-p21) or a non-targeting vector control

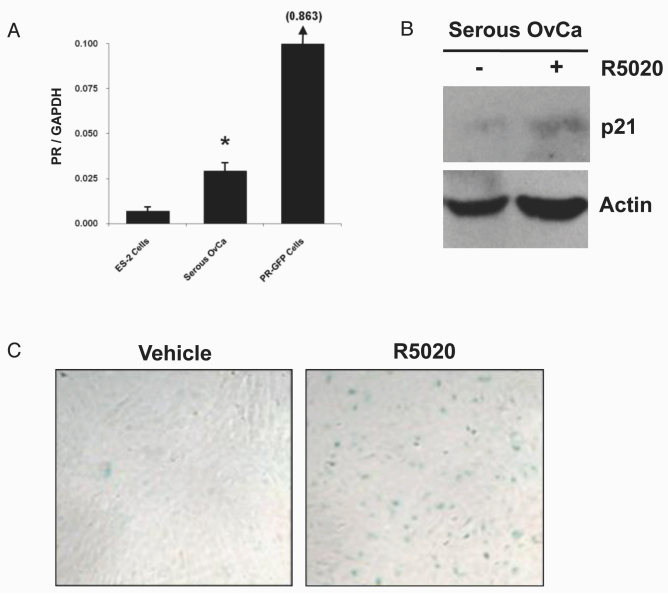

Figure 2-6: Functional activity of stably expressed GFP-tagged PR in ES-2 ovarian cancer cells. Immunofluorescence microscopy of Empty control and GFP-PR-containing cells stimulated with vehicle (ethanol) control or R5020 (10 nM) for 24 hr. All images were acquired at 200X magnfication.

(sh-control); p21 knockdown was confirmed by Western blotting (Figure 7A). We anticipated that p21 loss would block R5020-induced senescence. Surprisingly, we observed a significant increase in SAβGal activity and the development of a senescent phenotype after only four days of R5020 stimulation compared to sh-control cells (Figure 7B-C). To account for the increase in SAβgal activity, we evaluated other senescence-associated cell cycle regulators, p15, p16, and p27 in cells expressing sh-p21 (Figure 7D). Interestingly, upon R5020 (96 hr) treatment, p15 (23-fold), p16 (2-fold) and p27 (2-fold) mRNA levels were significantly induced in cells expressing sh-p21 relative to vehicle-treated cells or sh-controls. p21 expression was induced upon R5020 treatment in cells expressing either sh-control or sh-p21, although total expression was blunted following knock-down. These data suggest a mechanism for progestin-regulated

molecular compensation upon knock-down of p21 in cells expressing GFP-PR, and further implicate PR as a driver of cell cycle inhibition and, ultimately, cellular senescence in OC cells.

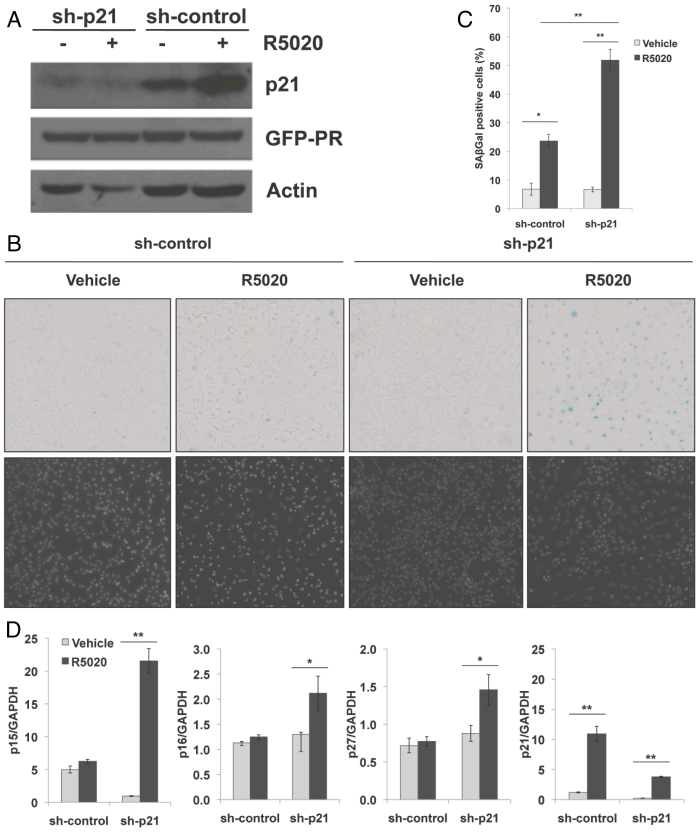

Figure 2-7: High p21 expression is dispensable for PR-induced cellular senescence. A) Western blot analysis of p21 expression after lentiviral infection of shRNA oligonucleotides containing a scramble, non-targeting sequence (sh-control) or p21-targeting sequence (sh-p21). Cells were treated with R5020 (10 nM) for 8 days after stable infection of shRNA oligonucleotides. B) Representative staining for SAβGal activity in cells expressing either sh-control or sh-p21 and treated with R5020 (10 nM) for 96 hrs (magnification= 100X). Cell samples were mounted onto glass slides using ProLong® Gold Antifade Reagent with DAPI (Invitrogen) for brightfield microscopy. C) Percentage of positive SaβGal expressing cells was determined from quantitating three fields at 100X magnification. Values were normalized to total nuclei present from DAPI staining (n=3, *p≤0.05, **p≤0.01). D) RT-qPCR analysis of p15, p16, p21, and p27 mRNA expression in the sh-p21 knockdown cells treated with R5020 (10 nM) for 96 hrs (n=3, *p<0.05, **p<0.01).

PR and FOXO1 cooperate to induce p21.

Previous studies identified FOXO1 as a PR target gene and master regulator of cell cycle mediators (p15, p16, p21, p27). Notably, FOXO1 and PR interact within transcription complexes in human endometrial stromal and cancer cells.[99, 100] Like PR, FOXO1 expression is down-regulated in ovarian cancers.[101] FOXO1 mRNA and protein expression was significantly upregulated following 24 hrs R5020 treatment (Figure 8A-B) and remained sustained for 96 hrs. Similar results were observed in progestin-treated PR+ PEO4 cells (data not shown). Chromatin immunoprecipitation (ChIP) assays revealed that upon R5020 treatment (1 hr) of GFP-PR cells, PR was significantly recruited (18-fold) to a PRE-containing region downstream of the p21 transcriptional start site identified by PR ChIP-Seq studies conducted in breast cancer models[102]; PR recruitment in GFP-control cells was minimal and similar to background (IgG control) levels (Figure 8C). In contrast to the dramatic ligand-dependent recruitment of PR-B, FOXO1 was basally present within the same PRE-containing region in both control and GFP-PR cells; FOXO1 recruitment to this site was not significantly modulated in response to R5020 treatment. Cell fractionation experiments indicated that FOXO1 protein was nuclear in both the absence and presence of progestin (data not shown).

Since progestin treatment induces FOXO1 expression in PR-expressing cells, we next evaluated if FOXO1 was required for the induction of p15, p16, p27, and p21 in cells expressing sh-p21 shRNAs. To block the activity of FOXO1,

the selective small-molecular inhibitor, AS1842856 (AS), was utilized.[103] Upon R5020 (96 hr) treatment, p15 (17-fold), p16 (2-fold), and p21 (18-fold), and p27 (2-fold) mRNA levels were again significantly induced in cells expressing sh-p21

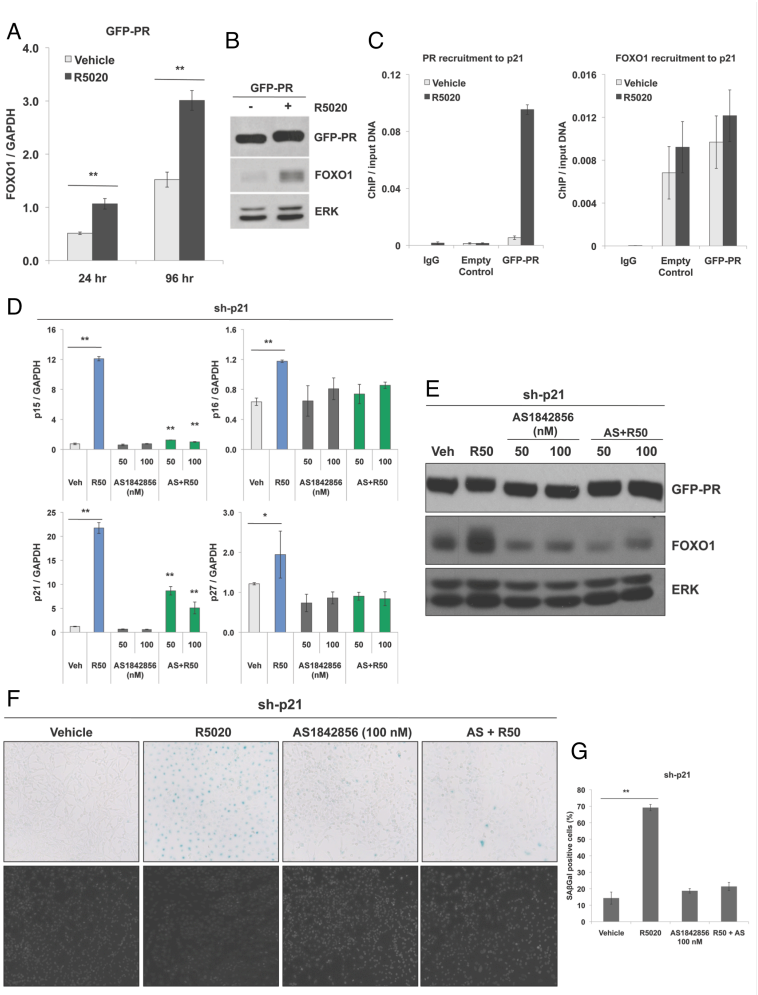

Figure 2-8: Progestin treatment of GFP-PR-containing cells stimulates FOXO1 expression and promotes PR recruitment to the p21 promoter. A) RT-qPCR analysis of FOXO1 mRNA expression after 24 hr and 96 hr R5020 (10 nM) treatment of GFP-PR-containing cells (n=3, **p≤0.01). B) Western blot analysis of FOXO1 expression in response to 96 hr of R5020 (10 nM) treatment in GFP-PR-containing cells. C) RT-qPCR analysis of PR and FOXO1 recruitment to p21. Empty control and GFP-PR expressing cells were stimulated with vehicle or R5020 (10 nM) for 1 hr. Fixed lysates were chromatin immunoprecipated with antibodies to PR or FOXO1 and qRT-PCR was performed on isolated DNA. D) RT-qPCR analysis of p15, p16, p21, and p27 mRNA expression in sh-p21 knockdown cells treated with R5020 (10 nM), FOXO1 inhibitor, AS1842856 (AS) (50 and 100 nM), or the combination of AS1842856 and R5020 for 96

hrs (n=2, *p<0.05, **p<0.01). E) Western blot analysis of FOXO1 expression in cells expressing sh-p21 after treatment with vehicle, R5020 (10 nM), AS1842856 (50 and 100 nM), or the combination of AS1842856 and R5020 for 96 hrs. F) Representative staining for SAβGal activity of sh-p21 knock-down cells treated with R5020 (10 nM), AS1842856 (AS, 100 nM), or the combination of AS1842856 and R5020 for 96 hrs. (Magnification = 100X). Cell samples were mounted onto glass slides using ProLong® Gold Antifade Reagent with DAPI (Invitrogen) for brightfield microscopy. G) Percentage of positive SAβGal cells was determined from quantitating three fields at 100X magnification. Values were normalized to total nuclei present in each field from DAPI staining (n=2, **p≤0.01).

relative to vehicle-treated cells (Figure 8D). Treatment with AS (50 or 100 nM) alone did not affect basal (vehicle) mRNA levels. However, the addition of AS to R5020-treated cells significantly blunted induction of p15, p16, p21, and p27, indicating that FOXO1 activity is required for progestin-induced expression of these cell cycle regulators. Furthermore, AS blocked basal and progestin-induced FOXO1 protein expression in cells expressing sh-p21 (Figure 8E). Importantly, AS treatment had no effect on GFP-PR-B expression (Figure 8E). Moreover, progestin-induced senescence, as measured by SAβGal activity, was significantly reduced upon inhibition of FOXO1 (i.e. in the presence of AS) relative to R5020-treated controls (Figure 8F-G). Together, these data implicate FOXO1 as a key mediator of cell cycle gene expression associated with progestin-induced ovarian cancer cell senescence.

FOXO1 expression is required for PR-induced senescence in OC cells.

Our data suggest that in the presence of progestin, liganded PR may primarily tether to pre-existing FOXO1 (i.e. located at nearby or distant sites) in order to facilitate hormone-regulated expression of p21 and senescence induction. A similar paradigm has been defined for ER tethering to FOXA1

pioneer factors in breast cancer models.[104] To test the FOXO1-dependence of PR-induced senescence, we generated GFP-PR cells stably expressing FOXO1-

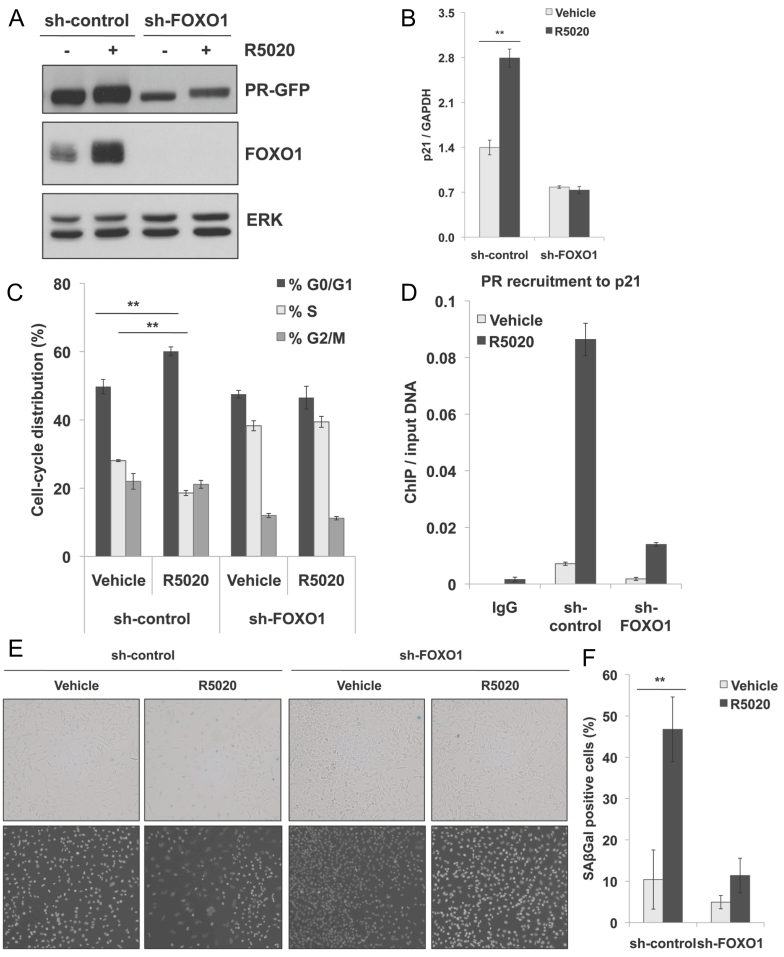

Figure 2-9: PR-induced cellular senescence is dependent on FOXO1 expression. A) Western blot showing FOXO1 expression in cells expressing either sh-control or sh-FOXO1 after treatment of R5020 (10 nM) for 96 hr. B) RT-qPCR analysis of p21 mRNA expression in sh-control and sh-FOXO1-containing cells after 96 hr of R5020 (10 nM) treatment (n=3, **p≤0.01). C) Cell cycle analysis by propidium iodide staining of cells expressing either sh-control or sh-FOXO1 and stimulated with R5020 for 96 hrs. (n=3, **p≤0.01). D) RT-qPCR analysis of PR recruitement to p21. Sh-control and sh-FOXO1-containing cells were stimulated with vehicle or R5020 (10 nM) for 1 hr. Fixed lysates were chromatin immunoprecipated with an antibody to PR and qRT-PCR was performed on isolated DNA. E) Representative staining of SAβGal activity of sh-control and sh-FOXO1-containing cells after treatment of R5020 for 96 hrs. Cell samples were mounted onto glass slides using ProLong® Gold Antifade Reagent with DAPI (Invitrogen) for brightfield microscopy. F) Percentage of positive SAβGal cells was determine from quantitating three fields at 100X magnification. Values were normalized to total nuclei present from DAPI staining. (n=3, **p≤0.01).

targeted shRNAs (sh-FOXO1); FOXO1 knockdown was confirmed by Western blotting (Figure 9A). Interestingly, PR expression also diminished slightly in cells stably expressing sh-FOXO1; some loss of PR occurred in multiple clones and using distinct FOXO1-targeted shRNAs (not shown), suggesting a mechanism for co-expression of these “coupled” factors. Early passage clones expressing sh-FOXO1 that retained significant PR expression were used for further study. As expected, p21 mRNA levels were greatly diminished in cells expressing sh-FOXO1 relative to sh-controls in both vehicle and R5020-treated conditions (Figure 9B). Similarly, R5020 treatment did not induce p15, p16, or p27 mRNA expression in cells expressing sh-FOXO1 (Figure 10).

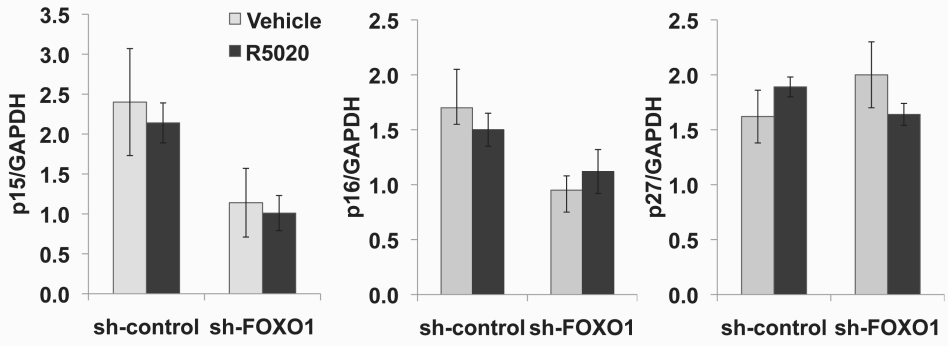

Figure 2-10: RT-qPCR analysis of p15, p16, and p27 mRNA expression in PR+ cells expressing sh-control or sh-FOXO1 after treatment of R5020 (10 nM) for 96 hrs (n=3).

In addition, PR recruitment to the PRE-containing region located downstream of the p21 transcriptional start site was also similarly reduced in cells expressing sh-FOXO1 relative to controls, as measured by ChIP assays (7.7-fold vs. 12.1 fold) (Figure 9D). To confirm that diminished transcriptional regulation of p21 was not entirely due to reduced levels of PR protein observed

in cells expressing sh-FOXO1 (Figure 9A), we performed qRT-PCR analysis of additional PR-target genes; R5020 treatment (24 hrs) of ES-2 GFP-PR cells stably expressing sh-FOXO1 resulted in significant upregulation of a number of PR-target genes, including HSD11B2, KLF4, and NET1 (Figure 11).

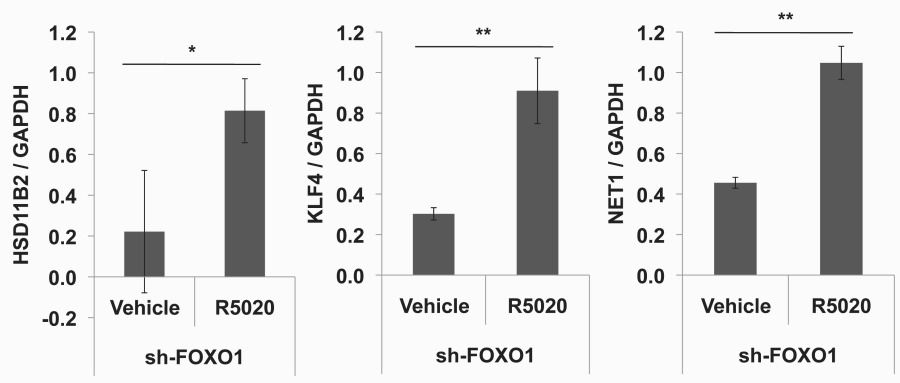

Figure 2-11: RT-qPCR analysis of HSD11B2, KLF4, and NET1 mRNA expression in the PR+ cell expressing sh-FOXO1 after treatment of R5020 (10 nM) for 24 hrs (n=3, *p≤0.05, **p≤0.01).

Consistent with loss of progestin-induced p21 expression, R5020 treatment failed to induce G0/G1 cell cycle arrest up to 96 hrs in cells expressing sh-FOXO1 cells relative to sh-controls (Figure 9C). Interestingly, the percentage of cells in S phase was enhanced upon knock-down of FOXO1 in both vehicle and R5020-treated PR-B+ cells when compared to sh-controls. Consistent with these results, progestin-induced SAβGal activity was also significantly reduced when FOXO1 was stably down-regulated relative to sh-controls (Figure 9E-F).

Overall, these data are consistent with a model in which PR-expressing OC cells induce FOXO1-dependent p21 expression in the presence of

progestins. FOXO1 is a required factor for PR-induced p21 expression and cellular senescence (Figure 12).

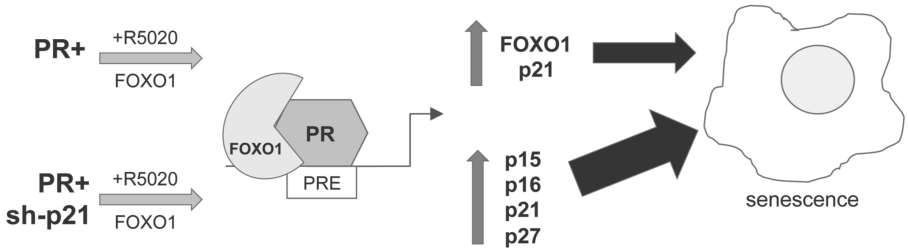

Figure 2-12: Proposed model of PR-induced cellular senescence in ovarian cancer cells. Progestin treatment of PR-expressing OC cells induces expression of p21 and FOXO1. PR and FOXO1 cooperatively upregulate p21 expression to promote cellular senescence. In the absence of p21, PR induces numerous FOXO1 target genes that compensate as mediators of senescence.

2.4 DISCUSSION

Progesterone is a potent breast mitogen, but functions to inhibit proliferation in the uterus. Indeed, PR action is highly context-dependent and heavily influenced by post-translational modifications[105] as well as cofactor availability.[106] The detailed molecular mechanisms of progesterone's protective role in ovarian cancer are not well understood; both proliferative and inhibitory actions of progesterone have been reported[50-53], including concentration-dependent biphasic effects within the same model system.[54] This is perhaps in part due to the complexity of progesterone action. Numerous progesterone receptors exist (PR-A, PR-B, PR-C, mPRa, mPRb, mPRg, and PGRMC1) and their activities and potential interactions are still poorly defined.[77] In this study, we demonstrated that nuclear PR-B receptors exhibit ligand-dependent anti-proliferative effects by inducing cellular senescence in OC models.

Several independent *in vitro* studies have demonstrated the inhibitory action of progesterone on OC cell growth, primarily through the induction of apoptosis[51-55, 88], while other studies report progesterone's proliferative actions.[54] The opposing cellular responses of progesterone's effects in normal and malignant ovarian surface epithelial cells may be attributed to dosage effects: mitogenic effects were observed at low progesterone concentrations ($<10^{-8}$ M) while growth inhibition and apoptosis were associated with high progesterone concentrations ($\geq 10^{-6}$ M).[63] Alternatively, opposing effects of progesterone may be attributed to the presence of two isoforms of the nuclear receptor (PR-A and PR-B).[106] Several *in vitro* studies have demonstrated PR-A to be inhibitory of the transcriptional activity of PR-B, as well as other nuclear receptors, including glucocorticoid, mineralcorticoid, androgen, and estrogen receptors.[106] PR-B is a more potent transcription factor relative to PR-A in gene array studies, but these receptors also regulate overlapping, but distinct gene subsets[34] that are also tissue-specific. Our studies identify ligand-activated PR-B as a mediator of OC senescence. Notably, progesterone can bind to members of the membrane progesterone receptor family, mPRα (PAQR7), mPRβ (PAQR8), mPRγ (PAQR5), and progesterone receptor membrane component-1 (PGRMC1) with varying responses that are likely also tissue-specific and context-dependent.[107] Namely, PAQR have been shown to weakly activate the JNK1/2 MAPK pathway and induce JNK-dependent BAX mRNA expression in ovarian cancer cells[77]. These receptors also rapidly activate p42/p44 MAPKs in breast cancer cells.[108]

However, the primary signaling mechanisms and associated physiological functions of these receptors remain incompletely understood. PAQR bind progesterone with relatively high affinity and do not recognize the synthetic progestin, R5020, or the nuclear PR antagonist, RU486.[109] In addition to PR-null cell controls, our studies used R5020 (10 nM) in order to avoid activating unrelated but ubiquitous membrane progesterone receptors (i.e. PAQR family members) present in OC cells.[77] Interestingly, PR+ PEO4 cells required at least 10 mM progestin for appreciable activation of endogenous PR-B (Figure 1E inset), as measured by gel mobility up-shift of PR-B and regulation of well-characterized PR target genes (SGK). Subtle up-shift in PR gel mobility is indicative of direct global PR phosphorylation, modifications that augment PR nuclear localization and enhance transcriptional activity at selected promoters.[105, 110] The basis of these concentration effects is unknown; the concentrations used in the PEO4 cell model are similar to physiological levels within the microenvironment of the ovary (≥ 1 μM).[53, 111] However, ovarian cancer cells may lack the so-called non-genomic or “rapid” signaling events that are integrated with ER-alpha and PR-B transcriptional activities in response to hormones and are well-characterized in breast cancer models.[112, 113]

Indeed, protein kinases are vital regulatory inputs to steroid hormone receptor action.[114] Takahashi and colleagues characterized cAMP-induced senescence in SKOV ovarian cancer cells overexpressing PR-B and observed induction of both p21 and p27 expression.[74] Although these authors did not

pursue the mechanisms of these effects, cAMP, via the actions of cAMP-dependent protein kinases and/or activation of Ca++ channels[115], may induce changes in PR-B phosphorylation that alter ligand-binding and/or co-factor interactions.[116] Interestingly, cAMP has been demonstrated to regulate the cell cycle in cancer cells[117] as well as induce and/or activate a variety of transcription factors such as STAT5, C/EBPβ, and FOXO1, all proteins that directly interact with PR.[118-120] As such, cAMP may lower the requirement for PR ligand-binding and/or tethering to FOXO1 at PR target genes. We detected little to no change in cAMP accumulation following a time course of progesterone or R5020-treatment of OC models.[77]

Our study demonstrates a detailed molecular mechanism whereby progestins activate nuclear PR-B to upregulate FOXO1 expression leading to robust induction of cell cycle mediators of senescence. In this case, p21 expression required both liganded PR-B and expression of FOXO1. Notably, progestin treatment of OC cells expressing sh-p21 promoted a significantly increased senescence response relative to sh-controls. Interestingly, in the context of diminished p21, hormone-bound PR significantly induced other well-characterized pro-senescence effectors that are also FOXO1 target genes, such as p15, p16, and p27. These PR signaling alternatives reveal FOXO1-dependent tumor suppressive or “fail-safe” mechanisms, which may ensure that senescence occurs in the face of impaired p21. Deregulation of FOXO1 is associated with tumorigenesis and cancer progression. FOXO1 is downregulated in several

carcinomas, including ovarian[101], through alterations in upstream regulators (i.e. inactivating mutations to PTEN, active PI3K-AKT signaling), post-translational deregulation, or by genetic mutations.[121] The targeted re-expression and activation of FOXO1 using chemical and/or biological therapeutic strategies may overcome resistance or sensitize cancer cells to current therapeutics. Ultimately, our studies showed that OC senescence is dependent and specific to both PR-B and FOXO1; the ablation of FOXO1 (a PR target gene) significantly diminished the emergence of progestin-induced p21 expression and cellular senescence. Linkage of PR and FOXO1 signaling has been reported in the uterus[122]. Notably, these molecules trend towards co-occurrence in 570 samples analyzed from The Cancer Genome Atlas (TCGA) ovarian serous cystadenocarcinoma provisional data set (p= 0.209, Fisher's exact test, odds ratio (OR) = 2.14, 95% confidence interval: 0.59-7.73)[123], suggestive that numerous PR-target genes may be sensitive to FOXO1 expression.

In contrast to our finding that PR-B is a mediator of OC senescence, the same receptor, when expressed in breast cancer cells, is clearly mitogenic.[25] The opposing biology in ovarian versus breast cancer cells may be largely dependent on cell context. Studies in T47D breast cancer cells growing in 2D culture conditions demonstrated biphasic effects of progestin on cell cycle progression.[124-126] Early effects of progestin treatment are proliferative as T47D-YB cells accelerate through one or more mitotic cycles; cells are growth inhibited at late time points coincident with induction of p21 and p27. A secondary

progestin treatment failed to restore cell cycle progression although PR levels remained high. Interestingly, epidermal growth factor (EGF) restored progestin-stimulated proliferation and prevented cell cycle arrest. In contrast to studies conducted in 2D culture systems, progestin is clearly mitogenic and a mediator of pro-survival and proliferation in breast cancer cells (T47D, MCF-7) cultured in anchorage independent (soft agar) conditions. The paradoxical effects of progestins observed in breast relative to ovarian cancer cells may be attributed to differential cross-talk between PR and growth factor-mediated signaling pathways, differential regulation of PR itself via post-translational modifications, and/or differential recruitment of required co-factors, such as FOXO1. Related to these studies, forkhead family transcription factors such as FOXO1 and FOXA1 are negatively regulated by phosphorylation events. AKT-dependent phosphorylation prevents their nuclear accumulation and thus impairs target gene regulation.[121] As mutations of PI3Ks or PTEN are common events (particularly in breast[127] and ovarian cancers[41, 128]), activated AKT may prevent PR-induced senescence signaling by nuclear exclusion of FOXO1 partners or pioneer factors.

To date, the use of progestins, megestrol acetate and medroxy-progesterone acetate, as OC therapies have been evaluated in a total of 14 relatively small phase II clinical trials with variable inclusion criteria and modest response rates.[129] Data from these trials support the concept that endometrioid and serous ovarian cancers are frequently sensitive to hormones and thus more

likely to respond to endocrine therapy.[129] To optimize response rates, the identification of PR-B as a biomarker within ovarian tumors may be advantageous prior to therapy. Genetic loss of heterozygosity of the PR gene (ch. 11q23.3-24.3) occurs in approximately 75% of ovarian tumors.[130, 131] Alternatively, when the PR gene locus is intact, it may be possible to restore PR expression in PR-low or null OC. For example, robust isoform-specific re-expression of PR has been demonstrated using activating duplex RNAs that target promoter regions in DNA; these reagents are currently in development for therapeutic use.[132]

In sum, our studies suggest that activation of nuclear PR-B may provide a means to force ovarian cancer cells out of the cell cycle and into a form of irreversible “stasis” by inducing cellular senescence. A clear understanding of the mechanisms and mediators of cellular senescence is highly relevant to modern cancer therapy, as the specific targeting of the senescence pathway in tumor cells is predicted to impede tumor progression to advanced and metastatic disease.[133] Senescent cells cannot further divide, but depend upon selected signal transduction pathways for prolonged survival, and thus may be more vulnerable to subsequent therapies that target these survival pathways (i.e. senescence creates a form of synthetic lethality). Thus, the induction of PR-mediated cellular senescence via progestin therapy may provide a safe and useful strategy to limit uncontrolled proliferation of ovarian cancer cells. In particular, translation of these findings may open the way for combination

therapies that couple PR-dependent senescence induction with targeted therapies aimed at blocking cell survival pathways, including AKT signaling. Such “rational targeting” strategies can minimize treatment-related morbidity and may be necessary in order to achieve significant improvements in overall OC patient survival.

2.5 ACKNOWLEDGEMENTS

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2.6 CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

CHAPTER 3

FOXO1 IS A KEY DETERMINANT OF PROGESTERONE RECEPTOR ISOFORM-SPECIFIC SENESCENCE PROGRAMMING IN OVARIAN CANCER CELLS

3.1 INTRODUCTION

Approximately 1.3% of women will be diagnosed with ovarian cancer in their lifetime.[134] Increased ovarian cancer risk is associated with progesterone deficiencies during infertility or with increasing age, and may occur as the result of genetic loss of heterozygosity at the progesterone receptor (PR) gene locus.[4, 5] Conversely, elevated progesterone levels experienced during pregnancy transiently and reversibly increase breast cancer risk[135], but significantly reduce ovarian cancer risk in Asian, European, and North American populations.[6] Consistent with these findings, use of hormonal contraceptives (that include a progestin) is associated with increased risk of breast cancer[136-139] but reduced risk of ovarian cancer.[7, 139, 140] During ovulation the release of a mature follicle into the fallopian tube space requires shedding of ovarian epithelial cell layers followed by local proliferation that is analogous to wound repair. High levels of progesterone experienced during pregnancy or during the luteal phase of the

normal menstrual cycle prevent follicle development. Thus, progesterone is believed to exert its protective effect on ovarian cancer in part by reducing the number of times ovulation occurs (i.e. via fewer cycles of ovulation-associated "damage and repair").

Similar to PR expression in luminal (estrogen receptor (ER)-positive) breast tumors, PR expression in ovarian tumors is a favorable prognostic marker associated with longer progression-free survival.[8, 11-18] Roughly 35% of ovarian tumors express PR; PR expression is highest in the endometrioid (67%) and serous (35%) sub-types.[48, 49] Full-length PR-B and N-terminally truncated PR-A (-164 amino acids) isoforms are encoded by the same gene and mRNAs. PR isoforms are ligand-activated transcription factors with distinct transcriptional activities. Following ligand binding, PRs dimerize (A:A, B:B, and A:B) and are retained in the nucleus where they repress or activate transcription of PR-target genes, either directly through binding to progesterone response elements (PREs) on chromatin, or indirectly via tethering interactions with other transcription factors (e.g. AP1, SP1, STATs, FOXO).[27, 29, 30, 141]

Studies of isoform-specific knockout mice determined that PR-B is required for mammary gland development, while PR-A is required for uterine development and reproductive actions.[1, 60, 142, 143] While PR-A and PR-B share structural and sequence identity downstream of the BUS (B-upstream segment), they are unique transcriptional regulators of distinct gene sets.[34] Little is known about how PR isoform-specific transcription is regulated in PR-expressing tissues

and tumors; total PR rather than PR isoform expression is measured clinically. Progesterone and progestins, acting through PR-B, are proliferative in the breast.[69] However, in the endometrium of the uterus, paracrine signals secreted from PR-A-containing stromal cells antagonize estrogen-induced epithelial hyperplasia.[60]

Our previous study[48] demonstrated that ligand-activated PR-B induces cellular senescence via induction of known cellular senescence mediators, including p21 and p15 via a FOXO1-dependent mechanism in ovarian cancer cells. FOXO1 interacts with steroid hormone receptors (SRs), including the androgen receptor (AR)[144, 145], ER alpha (ERα)[146], and both PR isoforms, PR-A and PR-B.[120, 147] PR-B and FOXO1 were co-recruited to the same PRE-containing region of the p21 upstream promoter. Both proteins were required to activate p21 expression; stable knockdown of FOXO1 or inhibition of FOXO1 activity using the small molecule inhibitor AS1842856 blunted progestin-induced p21 expression in PR-B-expressing cells and blocked PR-dependent cellular senescence.

PR isoform-specific actions have not been studied in ovarian cancer models. *In vitro* studies primarily performed in breast or uterine cancer models have demonstrated PR-A trans-repression of PR-B, as well as other SRs, including AR and ERα.[106] Notably, PR-A expression is markedly reduced relative to PR-B in ovarian tumors.[8-10] To study PR isoform-specific gene regulation and biological consequences, we engineered ovarian cancer (ES-2) cells to stably

express either empty vector (control), PR-A-only, or PR-B-only. Our studies indicate that PR-B is the dominant driver of cellular senescence in ovarian cancer cells and reveal a novel mechanism of regulation of hormone sensitivity via PR isoform-specific target gene expression; the presence or absence of activated FOXO1 confers potent PR-B-like transcriptional activity to PR-A. A clear understanding of PR isoform-specific actions and their co-regulators may reveal novel ways to pharmacologically select for PR-driven inhibitory over proliferative actions in hormone driven cancers.

3.2 MATERIALS AND METHODS

Cell Culture and stable cell line generation

The human PR-B gene was previously cloned into the pEGFP-N3 vector (Clontech Laboratories, Inc.), which also served as the Empty Vector (EV) control vector.[48, 87] GFP-tagged EV control, PR-A, and PR-B (with the isoform A start site mutated to Ala) stable clonal cell lines were generated using the parental ES-2 cell line as a model system. Stable cell lines were generated by transfecting cells with 2 µg of their respective plasmids using FuGene HD® transfection reagent (Roche, #04709691001) according to manufacturer's instructions. Twenty-four hours post-transfection, cells were selected and maintained with McCoy's 5A Modified medium supplemented with 10% charcoal-stripped fetal bovine serum (i.e. DCC) (Hyclone, #SH30068.03), 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.5 mg/mL of G418 sulfate (Corning, #61-234-RG). Fluorescence-activated cell sorting (FACS) with a FACSAria II cell sorter (BD

Biosciences) was used to purify GFP+ EV, PR-A-, and PR-B-containing cells by removing any low and non-GFP-expressing cells. Clones were then established and cultured from the FACS-purified population.

Stable FOXO1 expression cells were generated by infecting ES-2 PR-A-expressing cells (clone #1, #5) with the retroviral pBabe puro L vector (which also served as the EV control) containing the constitutively active FOXO1 (FOXO1-AAA).[148] The pBabe puroL HA FKHR AAA plasmid was a gift from William Sellers (Addgene #9025). Cells were selected in and maintained as described earlier with 1 μg/mL of puromycin.[48]

Ex vivo culture of human ovarian tumors

Ovarian cancer tissues were provided by the University of Minnesota Biological Materials Procurement Network (BioNet). All de-identified tissue samples received in this study were obtained with written informed consent in accordance with the University of Minnesota Institutional Review Board under exemption status.

Dissection, plating, and treatments of ovarian cancer tissue were performed as described previously,[149-151] with a few exceptions. After surgical excision and pathological examination, fresh ovarian cancerous tissue was placed in 10 mL of McCoy's 5A medium supplemented with 5% DCC for transport back to the laboratory. Tissue was dissected into 1-mm^3 pieces and cultured in duplicate wells containing presoaked gelatin sponges (Ethicon, Inc., #1969) in 12-well plates containing 1.5 mL explant media (McCoy's 5A medium

supplemented with 10% DCC, hydrocortisone (0.01 mg/mL), and insulin (0.01 mg/mL)) within one hour of receiving tissue from BioNet. Tissue cultures were placed in a 37°C incubator with 5% CO_2 for 24 hr. Afterwards, media was gently aspirated from each well. 1.5 mL explant media containing 10 nM R5020 or equal volume vehicle (ethanol) was added to corresponding treatment wells and placed in a 37°C incubator with 5% CO_2. To equilibrate the sponges with hormone treatments, media was gently aspirated every 1 hr and replenished with fresh explant media containing 10 nM R5020 or equal volume vehicle (ethanol) for a total three times. Plates were returned to a 37°C incubator with 5% CO_2 for 48 hr. Afterwards, tissues were gently removed from the sponges with sterile forceps and processed for RNA or protein isolation as described below.

Reagents

Cells were treated with the following reagents (when applicable): R5020 (Perkin Elmer, #NLP004005MG) and AS1842856 (EMD Millipore, #344355). For experiments with AS1842856, cells were pretreated for one hour prior to the addition of R5020 in combination treatment studies.

Luciferase assays

Cells were cotransfected overnight using FuGene HD® transfection reagent (Roche) according to manufacturer's instructions with either 0.5 µg of a PRE-containing[34] (2X-PRE) firefly luciferase reporter construct, 1 µg of a p21 promoter-containing firefly luciferase reporter construct[48], or 4 µg (for p21 promoter-luciferase assay) or 0.5 µg (for RT-qPCR analysis of endogenous

genes in PR-B cells) of GFP-tagged N3-PRA vector. The constitutively active pRL-TK-*Renilla* luciferase construct (Promega, #E2241) (10 ng) was cotransfected as a transfection control. Luciferase assays were performed as previously described[48] using the dual luciferase reporter assay (Promega, #E1910).

In experiments using HeLa cells, 10 ng of GFP-tagged EV or PR-A vector, 10 ng pcDNA3 empty vector (pc-EV) or Flag FKHR[152] (pc-FOXO1 WT), 0.5 µg of a PRE-containing[34] (2X-PRE) firefly luciferase reporter construct, and 10 ng of a constitutively active pRL-TK-*Renilla* luciferase construct (Promega, #E2241) were transiently cotransfected. pcDNA3 Flag FKHR was a gift from Kunliang Guan (Addgene plasmid # 13507).

Gene expression profiling

Clonal ES-2 cells stably expressing GFP-tagged EV (clone #3), PR-A (clone #7), and PR-B (clone #1) and breast cancer cells T47D Y, YA, and YB originally described by Sartorius *et al*[86] were hormonally starved in modified improved MEM (IMEM) (Gibco, catalog #A10488) plus 5% DCC for 24 hr. Afterwards, cells were treated with R5020 (10 nM) or vehicle control for 24 hour prior to RNA extraction using the RNeasy kit (QIAgen, #74104). DNase I treated (QIAgen, #79254) triplicate RNA samples were prepared for expression analysis using the Illumina HT-12v4 bead chip platform according to the manufacture's protocols. Data were analyzed within R software[153] using the Bioconductor[154] package, lumi[155] where raw intensities were $\log_2$ transformed and quantile

normalized. Differentially expressed genes were analyzed using the limma package[156], where empirical Bayes was used to better estimate the variance of the genes. Gene expression data presented contain $\log_2$ normalized intensities and biological comparisons presented (e.g. R5020/vehicle) contain $\log_2$ fold change with the Benjamini and Hochberg (BH) adjusted *P* value.[157] Heat maps were generated by unsupervised hierarchical clustering of genes via the 'aheatmap' function in the NMF R package.[158] Clustering was performed using Pearson distance and average linkage. Rows were scaled to have mean zero and standard deviation equal to one. All gene expression data is available in the NCBI Gene Expression Omnibus (GEO) database (accession number: GSE69296).

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from cells in triplicate wells using TriPure Isolation Reagent (Roche, #11667165001) and isopropanol precipitation. RNA (1.0 μg) was reverse transcribed to cDNA according to manufacturer's instructions using the qScript cDNA SuperMix (Quanta Biosciences, #95048-100). qPCR was performed using Light Cycler® FastStart DNA Master SYBR Green I (Roche, #12239264001) on a Light Cycler® 480 II Real-Time PCR System (Roche). qPCR cycling conditions with primer sequences (available upon request) and concentrations were performed as described previously.[48]

Immunoblotting

Western blots were performed as previously described.[48] Western blots were probed using the following primary antibodies: PR-A/B (H-190) and p21 (C-19) were purchased from Santa Cruz Biotechnology; FOXO1 (#2880) was purchased from Cell Signaling Technologies; and Actin (Sigma-Aldrich, #A4700). Phospho-Ser190 PR anti-sera was purchased from NeoMarkers (#MS-1331-P1). Custom made phospho-Ser294 PR antibodies (clone 8508) were commissioned from Thermo Fisher Scientific designed to recognize the following phospho-specific peptide sequence: C-PMAPGR(pS)PLATTV-amide. HRP-conjugated goat anti-rabbit and goat anti-mouse (Bio-Rad, #170-6515 and #170-6516) secondary antibodies were used to detect their respective primary antibodies, and immunoreactive proteins were visualized on Kodak X-OMAT LS film (Carestream Health, #864-6770) following ECL detection with Super Signal® West Pico Maximum Sensitivity Substrate (Pierce, #34087). All Western blotting experiments were performed at a minimum in triplicate, and representative experiments are shown in each respective figure.

Flow Cytometry

Cells were treated in 5% DCC with either 10 nM R5020 or equal volume vehicle (ethanol) for 96 hrs. Cells were collected, fixed and stained as described previously.[48] Propidium iodide staining was detected using a LSRII (BD Biosciences, #H4760). Cells were gated for cell cycle phases using BD

Biosciences FACSDiva 8.0 software. Cell cycle profiles were analyzed using FlowJo vX software (Tree Star Inc.).

Chromatin Immunoprecipitation (ChIP)

Cells were treated for 1 hr with either R5020 (10 nM) or equal volume vehicle (ethanol) in 5% DCC and cell samples were fixed, harvested, and lysed according to optimized manufacturer's instructions using the ChIP-IT™ Express Magnetic Chromatin Immunoprecipitation Kit (Active Motif, #53008). Samples were homogenized using a Bioruptor sonicator (Diagenode, Inc.). ChIP reactions were incubated overnight on an end-to-end rotator using 100 µL of isolated chromatin and either 2 µg of PR-A/B antibody (Ab-8), FKHR (H-128) antibody (Santa Cruz Biotechnology, #sc-11350), or 0.4 µg of normal mouse or rabbit IgG (Santa Cruz Biotechnology, #sc-2025 # sc-2027). Samples were washed, eluted, reverse cross-linked, and treated with Proteinase K according to manufacturer's instructions (Active Motif). DNA was analyzed by RT-qPCR as described above.

5-bromo-2'-deoxyuridine (BrdU) Assay

Cells were plated in 96-well plates in IMEM plus 5% DCC. Twenty-four hours later, cells were treated with either R5020 (10 nM) or equal volume vehicle (ethanol) in IMEM plus 5% DCC for 96 hr. Three hours prior to the 96 hr timepoint, 10X BrdU from the BrdU Cell Proliferation Assay Kit (Cell Signaling, #6813) was prepared in IMEM and then added to each well for a final concentration of 1X. Plates were returned to a 37°C incubator with 5% CO_2 for 3 hr. Cells were fixed, DNA was denatured, and detection of BrdU incorporation

into cellular DNA during cell proliferation was conducted according to manufacturer's protocol.

Senescence Associated-β-galactosidase (SAβGal) Activity Assays

Cells were continuously treated for 96 hr in IMEM plus 5% DCC. Cells were washed, fixed, and stained for SAβGal activity according to manufacturer's instructions using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, #9860). Staining of cell nuclei was achieved with DAPI containing ProLong® Gold antifade reagent (Invitrogen, #P-36931). All bright-field and fluorescent cell images described herein were acquired with a Leica DM4000B microscope (Leica Microsystems, Inc.) and captured using the Leica Application Suite software (version 4.2). Percentage of positive SAβGal cells (blue staining with enlarged nuclei) was determined from quantifying three fields at 100X magnification using ImageJ software. Values were normalized to total nuclei present per field from DAPI staining.

Statistical Analysis

All reported values represent the mean ± the standard deviation (SD). Data shown are representative of the indicated replicates of each experiment. Statistical analyses were performed using a Student's *t*-test where significance was determined with 95% confidence (*$p \leq 0.05$, **$p \leq 0.01$).

3.3 RESULTS

Creation of PR isoform specific ovarian cancer cell models.

Cell line models derived from human tumors frequently lose steroid receptors (SR) expression when propagated in monolayer tissue cultures. We detected low to negligible levels of PR mRNA and protein in a panel of well-characterized ovarian cancer cell lines including the clear cell carcinoma cell line, ES-2.[48] PR expression in these models was only modestly induced by estrogen. Thus, to study the impact of progesterone/PR signaling on ovarian cancer cell biology without the added complexity of estrogen addition (PR is an ER target gene), we generated ES-2 cell models stably expressing either PR-A or PR-B. We selected ES-2 cells due to their inherent aggressive nature and rapid growth rate[159], low endogenous PR mRNA levels[48], and molecular and genomic characteristics that are similar to primary tumors of high-grade serous ovarian carcinomas (the most common cancer of the ovary).[160]

Pooled populations of PR-expressing cells as well as multiple clones of stable ES-2 cell lines expressing GFP-tagged PR-A, GFP-tagged PR-B (with the PR-A start site mutated), or GFP-only empty vector (EV) control were created (Figure 1A). GFP-tagged PRs are fully functional in COS and HeLa cell models.[87, 161] To verify that GFP-tagged PR isoforms behave as ligand-activated transcription factors in progestin-treated ES-2 cells, PR transcriptional activity in pooled populations of ES-2 cells expressing EV control, PR-A, and PR-B was evaluated using PRE-luciferase reporter assays. Similar to results obtained in

breast cancer cell models[79], PR-B was more transcriptionally active (~5-fold) relative to PR-A as measured using this minimal (2X-PRE-driven) promoter; the synthetic progestin R5020 (10 nM; for 18hr) significantly increased luciferase expression in cells expressing PR-A (1.8 fold) and PR-B (9.5 fold) relative to vehicle or EV (GFP-only) controls (Figure 1A). Both isoforms underwent a slight up-shift in gel mobility upon R5020 treatment (Figure 1A inset), indicating direct PR phosphorylation (further addressed below). Endogenous PR-A (*HEF1*)[34] and PR-B (*BIRC3*)[76, 162] target genes were also selectively upregulated in an isoform-specific manner in pooled cell populations relative to vehicle and EV controls (Figure 1B).

Multiple clonal PR+ ES-2 cell lines were isolated and expanded after selection for PR protein expression levels comparable to PR expressed in T47D breast cancer cell lines stably expressing either PR-A only (YA), PR-B only (YB), or constitutively expressing both endogenous isoforms in the absence of estrogen (CO)[86] (Figure 1C). One GFP-only EV control clone (EV #3), four GFP-PR-A-expressing clones (PR-A #1, #5, #4, #7) and two GFP-PR-B-expressing clones (PR-B #1, #3) were selected for further study. PR phosphorylation status was examined in PR-containing clonal cell lines following R5020 (10 nM for 1 hr) treatment. Similar to pooled populations (Figure 1A inset), R5020 induced a subtle up-shift in PR gel mobility in studies with clonal cell lines as measured by Western blotting (Figure 1D). PR Ser190 is a constitutive phosphorylation site similarly present in each clone. PR Ser294 is a proline-directed (i.e. primarily

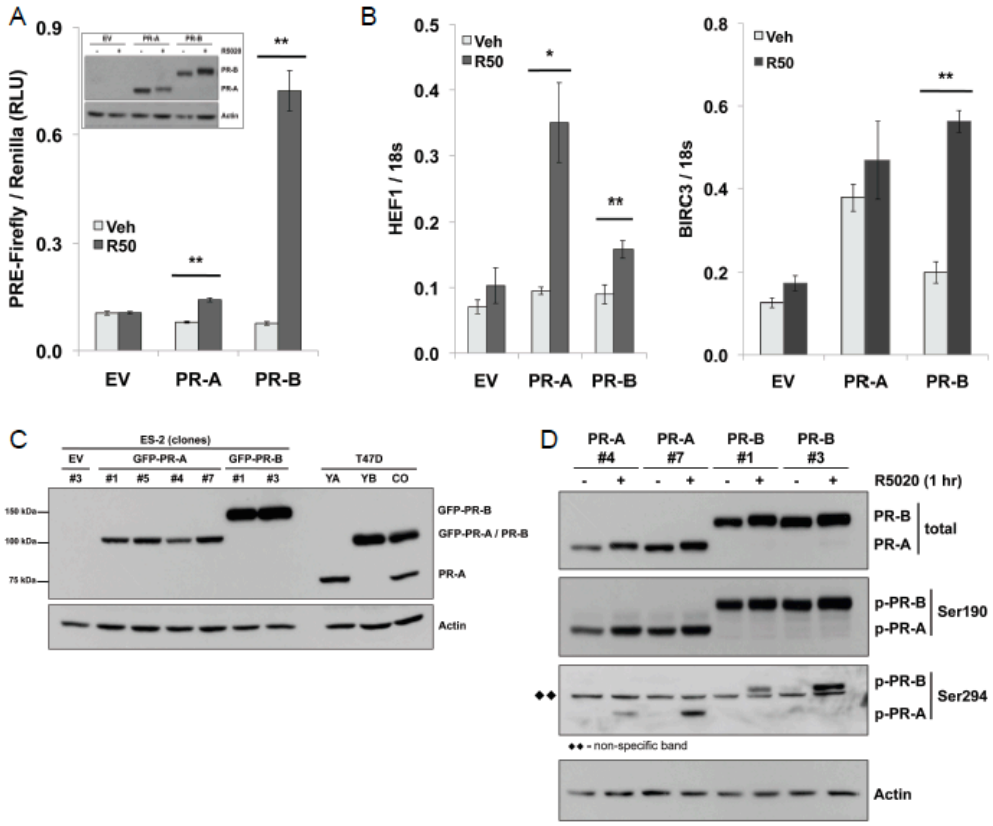


Figure 3-1: Stable expression of PR isoforms in ES-2 cells. (A) *Inset*, Western blot analysis showing total PR expression in ES-2 cell pools expressing GFP-tagged empty vector control (EV), GFP-tagged PR-A (PR-A), or GFP-tagged PR-B (PR-B) and treated without or with R5020 (10 nM) for 24 hr. ES-2 cells expressing EV, PR-A, or PR-B were transiently transfected with a progesterone response element (2X-PRE) containing luciferase reporter gene and treated for 18 hr with R5020 (10 nM). Relative luciferase units (RLU) were normalized to the mean result ± standard deviation (SD) for Renilla luciferase expression (n=3, **p≤0.01). (B) RT-qPCR analysis of HEF1 and BIRC3 mRNA expression after 24 hr R5020 (10 nM) treatment in ES-2 cell pools expressing EV, PR-A or PR-B (n=3, *p≤0.05 **p≤0.01). (C) Western blot analysis of total PR expression in ES-2 cells stably expressing GFP-tagged EV control (clone #3), GFP-tagged PR-A (GFP-PR-A clone #1, #5, #4, #7), or GFP-tagged PR-B (GFP-PR-B clone #1, #3) relative to T47D breast cancer cells stably expressing PR-A-only (YA), PR-B-only (YB), and both endogenous PR isoforms (CO). Actin served as a loading control. (D) Western blot analysis of PR-A and PR-B phosphorylation at Ser294 and Ser190, and total PR protein expression in ES-2 PR-expressing cells treated with R5020 (10 nM) for 1 hr. ◆◆ denotes a non-specific band present in the phospho-PR Ser294 blot. Actin served as a loading control.

phosphorylated by CDKs and/or MAPKs)[163-165] phosphorylation site robustly phosphorylated in PR-B-expressing cells in the presence of R5020 (a non-specific protein just below PR-B was detected in all lanes). Notably, PR-A-expressing clones exhibited varied ligand-dependent phosphorylation of Ser294, with robust levels detected in PR-A-expressing clone #7 cells relative to clone #4 cells. Phosphorylation of Ser294 occurs on PR-B, but not PR-A, in breast cancer cell models.[79, 166] Together, these data confirm that GFP-tagged PR-A and PR-B stably expressed in ES-2 cells can bind ligand, undergo phosphorylation at well-characterized sites, and selectively regulate isoform-specific endogenous target genes.

PR isoforms regulate unique gene sets in ovarian relative to breast cancer cells.

To assign isoform-specific transcriptional actions to PR-A and PR-B stably expressed in ovarian cancer cells, we performed genome-wide transcriptional profiling of representative clonal ES-2 cell lines expressing either EV (clone #3), PR-A (clone #7), and PR-B (clone #1) following treatment without or with R5020 (24 hr) and created representative heat maps (Figure 2A) and Venn diagrams (Figure 2B-C) illustrating isoform-specific gene regulation (>2-fold up or downregulated genes are shown). Cells expressing either PR-A-only or PR-B-only regulate distinct gene clusters relative to the same parental cells expressing EV (GFP-only). Simply the expression of PRs in the absence of exogenously added ligand dramatically altered the transcriptome. Strikingly, in the absence of

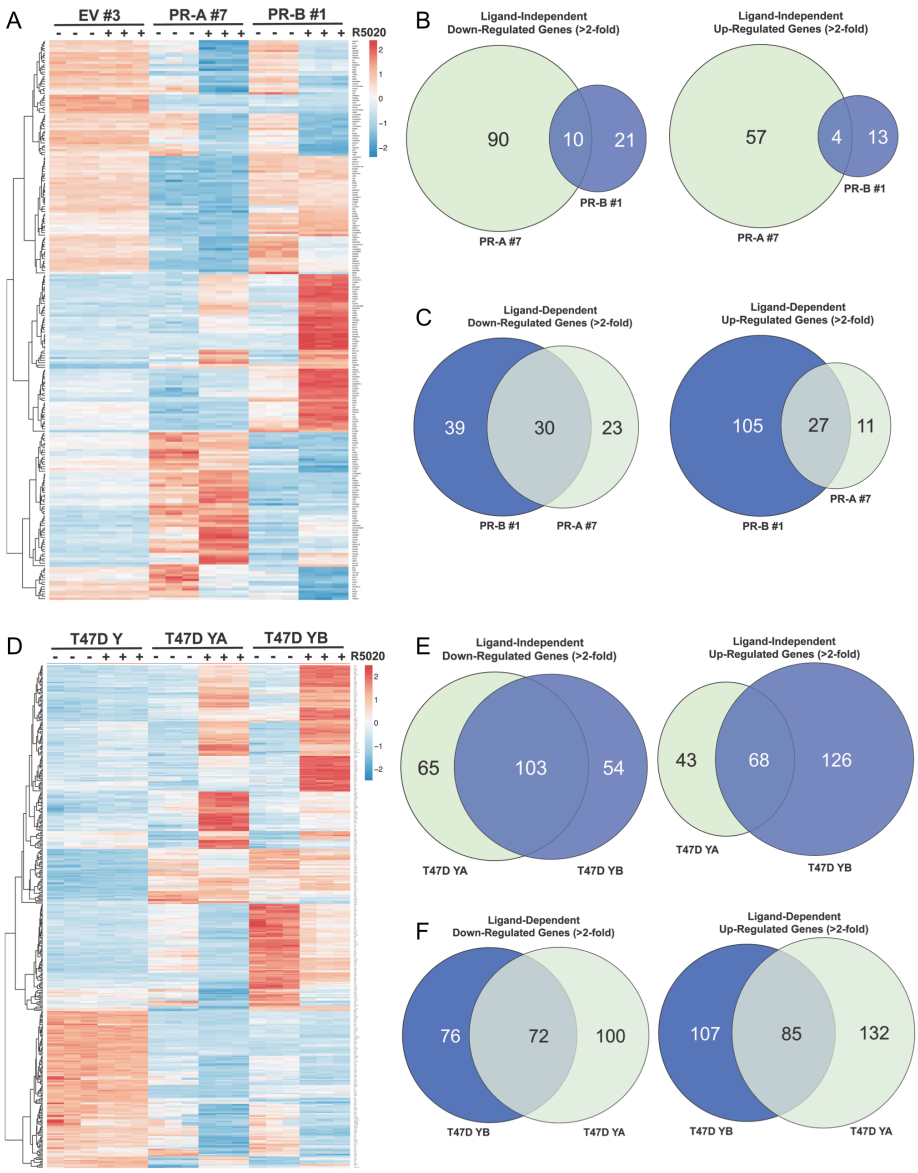


Figure 3-2: Gene expression profiling of PR-A and PR-B transcriptional activity in ovarian and breast cancer cells. (A) Heat map highlighting the transcriptional profiles between ES-2 ovarian cancer cells stably expressing EV control (clone #3), PR-A (clone #7), and PR-B (clone #1). Cells were treated with vehicle or R5020 (10 nM) for 24 hr and harvested RNA was subjected to Illumina gene profiling as described in Methods. Genes differentially expressed >2-fold are displayed for each treatment group. The experiment was performed in triplicate. (B) Venn diagrams showing the number of unique genes downregulated or upregulated >2-fold in the absence of R5020 treatment in PR-A- (clone #7) and PR-B-expressing (clone #1) cells. (C) Venn diagrams depicting the number of unique genes downregulated or upregulated >2-fold with R5020 treatment in PR-A- (clone #7) and PR-B-expressing (clone #1) cells. (D) Heat map highlighting the transcriptional profiles between breast cancer cells expressing EV control (T47D Y), PR-A (T47D YA), and PR-B (T47D YB). The cells were treated with vehicle or R5020 (10

nM) for 24 hr and harvested RNA was subjected to Illumina gene profiling as in part A. Genes differentially expressed >2-fold are displayed for each treatment group. The experiment was performed in triplicate. (E) Venn diagrams depicting the number of unique genes downregulated or upregulated >2-fold in the absence of R5020 treatment in T47D YA and YB cells. (F) Venn diagrams depicting the number of unique genes downregulated or upregulated >2-fold with R5020 treatment in T47D YA and YB cells.

ligand, PR-A altered the expression of more genes (100 genes downregulated and 61 genes upregulated) relative to PR-B (31 genes downregulated and 17 genes upregulated; Figure 2B). In contrast, in the presence of ligand, PR-B (132 genes upregulated and 69 genes downregulated) was the more active transcriptional regulator relative to PR-A (53 genes downregulated and 38 genes upregulated). These data suggest that PR-B is the dominant hormone-sensitive PR isoform in ovarian cancer cells, while PR-A may mediate significant ligand-independent actions, but functions predominantly (in the absence or presence of ligand) via repressive actions (further addressed below).

Regulation of isoform-specific PR target genes in ES-2 cells suggests important functional differences between PR isoforms (Figure 2B-C). To understand what specific cellular pathways may be isoform-specifically regulated, we performed Gene Ontology (GO) analysis for gene terms significantly regulated by either PR-A or PR-B (Figure 3). In the presence of progestin, gene terms significantly upregulated by PR-A (38 genes, 27 of which were also upregulated by PR-B) were enriched in cell adhesion, regulation of cell-substrate adhesion, extracellular matrix organization, and growth factor binding. Gene terms significantly downregulated by PR-B in response to progestin (69 genes,

PR-A Ligand-dependent Upregulation (Biological Process)					
GO.ID	Term	Annotated	Significant	Expected	Fisher p-value
GO:0007155	cell adhesion	274	14	2.23	3.4e-05
GO:0022610	biological adhesion	274	14	2.23	3.4e-05
GO:0010810	regulation of cell-substrate adhesion	47	7	0.38	0.00019
GO:0031589	cell-substrate adhesion	91	8	0.74	0.00098
GO:0030155	regulation of cell adhesion	108	8	0.88	0.00294
GO:0030198	extracellular matrix organization	117	8	0.95	0.00387
GO:0043062	extracellular structure organization	117	8	0.95	0.00387
GO:0010812	negative regulation of cell-substrate ad...	15	4	0.12	0.00561
GO:0009749	response to glucose	38	5	0.31	0.01152
GO:0009746	response to hexose	40	5	0.33	0.01221
GO:0042221	response to chemical	920	19	7.49	0.01221
GO:0034284	response to monosaccharide	43	5	0.35	0.01610
GO:0010038	response to metal ion	79	6	0.64	0.02381
GO:0071363	cellular response to growth factor stimu...	215	9	1.75	0.02381
GO:0010035	response to inorganic substance	120	7	0.98	0.02386
GO:0070848	response to growth factor	220	9	1.79	0.02502
GO:0009743	response to carbohydrate	52	5	0.42	0.02606
GO:0036293	response to decreased oxygen levels	84	6	0.68	0.02606
GO:0070887	cellular response to chemical stimulus	643	15	5.23	0.02606
GO:0010811	positive regulation of cell-substrate ad...	28	4	0.23	0.02861
GO:0045785	positive regulation of cell adhesion	54	5	0.44	0.02861
GO:0048731	system development	1024	19	8.33	0.02861
GO:0070482	response to oxygen levels	91	6	0.74	0.03060
GO:0051272	positive regulation of cellular componen...	95	6	0.77	0.03737

PR-A Ligand-dependent Upregulation (Cellular Component)					
GO.ID	Term	Annotated	Significant	Expected	Fisher p-value
GO:0031012	extracellular matrix	102	8	0.82	0.001
GO:0005578	proteinaceous extracellular matrix	83	6	0.66	0.023

PR-A Ligand-dependent Upregulation (Molecular Function)					
GO.ID	Term	Annotated	Significant	Expected	Fisher p-value
GO:0019838	growth factor binding	35	5	0.29	0.020

PR-A Ligand-Independent Upregulation (Biological Process)					
GO.ID	Term	Annotated	Significant	Expected	Fisher p-value
GO:0040011	locomotion	392	17	4.89	0.021

PR-A Ligand-Independent Upregulation (Cellular Component)					
GO.ID	Term	Annotated	Significant	Expected	Fisher p-value
GO:0031012	extracellular matrix	102	9	1.34	0.0059
GO:0005615	extracellular space	263	13	3.44	0.0129
GO:0005576	extracellular region	1099	28	14.39	0.0248

PR-B Ligand-dependent Upregulation (Biological Process)					
GO.ID	Term	Annotated	Significant	Expected	Fisher p-value
GO:0070482	response to oxygen levels	91	12	2.36	0.027

PR-B Ligand-dependent Downregulation (Biological Process)					
GO.ID	Term	Annotated	Significant	Expected	Fisher p-value
GO:0005578	proteinaceous extracellular matrix	83	9	1.11	0.0012
GO:0031012	extracellular matrix	102	9	1.36	0.0035
GO:0005604	basement membrane	37	5	0.49	0.0425

PR-B Ligand-dependent Downregulation (Molecular Function)					
GO.ID	Term	Annotated	Significant	Expected	Fisher p-value
GO:0004222	metalloendopeptidase activity	11	4	0.13	0.013
GO:0008237	metallopeptidase activity	31	5	0.36	0.031
GO:0004175	endopeptidase activity	58	6	0.68	0.040

Figure 3-3: Gene Ontology (GO) analysis of significantly regulated genes in PR-expressing ovarian cancer cells. Genes regulated >2-fold relative to vehicle treatment were analyzed for GO term enrichment as previously described.[78] Analysis of the top ranked and significant GO terms are listed for PR-A and PR-B in a ligand-dependent and –independent manner. Significant terms have a Benjamini and Hochberg adjusted Fisher p-value of $p \leq 0.05$.

30 of which were also down-regulated by PR-A) were also associated with regulation of the extracellular matrix, but significantly included genes associated with regulation of the basement membrane. Gene terms associated with pathways regulating the cellular response to oxygen levels were significantly upregulated by ligand-bound PR-B. Other PR-isoform specific regulated gene terms significantly associated with known pathways as defined by GO are listed in Figure 3.

Differential regulation of PR isoform-specific target genes identified using the Illumina platform (Figure 2) was validated by RT-qPCR in multiple ES-2 clones (Figure 4). Progestin-induced expression of selected PR-A and PR-B target genes was evaluated at 24 hr and 96 hr (R5020; 10 nM). PR-A target genes, *CRISPLD1* and *WISP1*, known to be involved in the regulation of the extracellular matrix, were robustly induced following R5020 treatment of PR-A expressing clones (#4 and #7) relative to modest or insignificant regulation in PR-B expressing clones (#1 and #3) (Figure 4A). CRISPLD1 belongs to a super-family of proteins that have extracellular endocrine or paracrine functions and are also involved in the regulation of extracellular matrix and in cell-cell adhesion during fertilization.[167] WISP1 binds to proteoglycans, decorin and biglycan, present in the extracellular matrix of connective tissues.[168] PR-B target genes *BIRC3* (a mediator of cancer cell pro-survival) and *GZMA* (a novel PR-B target gene identified herein and a mediator of cytotoxic T-cell responses significantly elevated in the serum of women with ovarian cancer[169]) were robustly induced

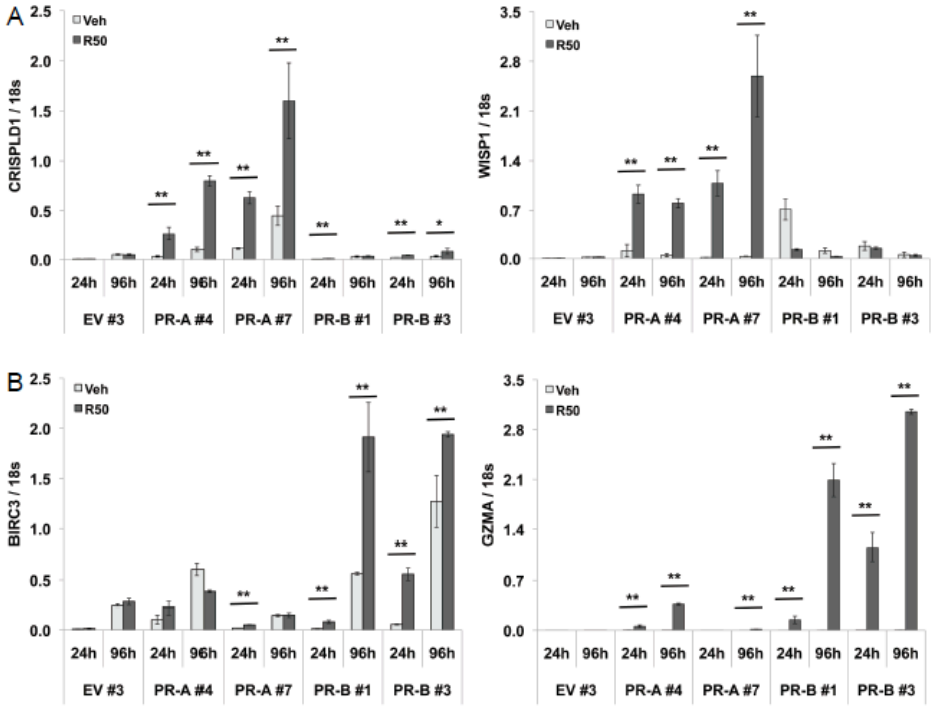


Figure 3-4: Validation of PR isoform-specific gene targets in ovarian cancer cells. RT-qPCR analysis of PR-A selective target gene mRNAs (A) encoding CRISPLD1 and WISP1 (A) or PR-B selective target gene mRNAs encoding BIRC3 and GZMA (B) following 24 hr and 96 hr R5020 (10 nM) treatment in ES-2 cells stably expressing EV control (clone #3), PR-A (clone #4, #7), or PR-B (clone #1, #3) (n=3, *p≤0.05, **p≤0.01).

in PR-B-expressing clones (#1 and #3) relative to weak or insignificant induction in PR-A-expressing clones (#4 and #7) or controls (Figure 4B).

In contrast to its protective role in reproductive tissues, progesterone is a potent mitogen in the mammary gland.[170] PR-B but not PR-A is required for ductal side-branching and alveologenesis that occurs during mammary gland development. Breast cancer cell models expressing PR-B, but not PR-A, grow in soft agar in response to progestin treatment.[37, 76, 171] Indeed, both overlapping and isoform-specific PR target gene up- and downregulation have been extensively reported in breast cancer models.[34, 35] To illustrate potentially distinct

transcriptional actions of PR isoforms in ovarian relative to breast cancer models, we repeated whole genome profiling studies in T47D cells stably expressing either PR-A-only (YA) or PR-B-only (YB), using the naturally occurring PR-null variant parental T47D cell line (Y) as a control. As above for ES-2 cells, T47D cells were treated with R5020 (10 nM) for 24 hr, and global gene expression profiles were measured using whole genome gene expression bead (Illumina) arrays. As in our ovarian cancer cell models, transcriptional differences between PR-null T47D cells and parental T47D cells stably expressing either PR-A or PR-B were readily observed (Figure 2D). However, in sharp contrast to hormone-naive ovarian cancer models, in the absence of progestin, breast cancer cells expressing PR-B upregulated more genes (194 genes) relative to cells expressing PR-A (111 genes) (Figure 2E). In the absence of ligand, expression of PR-A (168 genes) or PR-B (157 genes) resulted in downregulation of a similar number of genes from distinct but largely overlapping subsets (103 genes in common) (Figure 2E). In the presence of progestin, PR-A and PR-B both activated and repressed numerous genes from distinct but largely overlapping subsets. Thus, in contrast to PR expression in ovarian cancer models (where there is modest overlap between PR-A and PR-B regulated gene sets, and PR-B appears to be the dominant hormone-regulated isoform) PR expression in breast

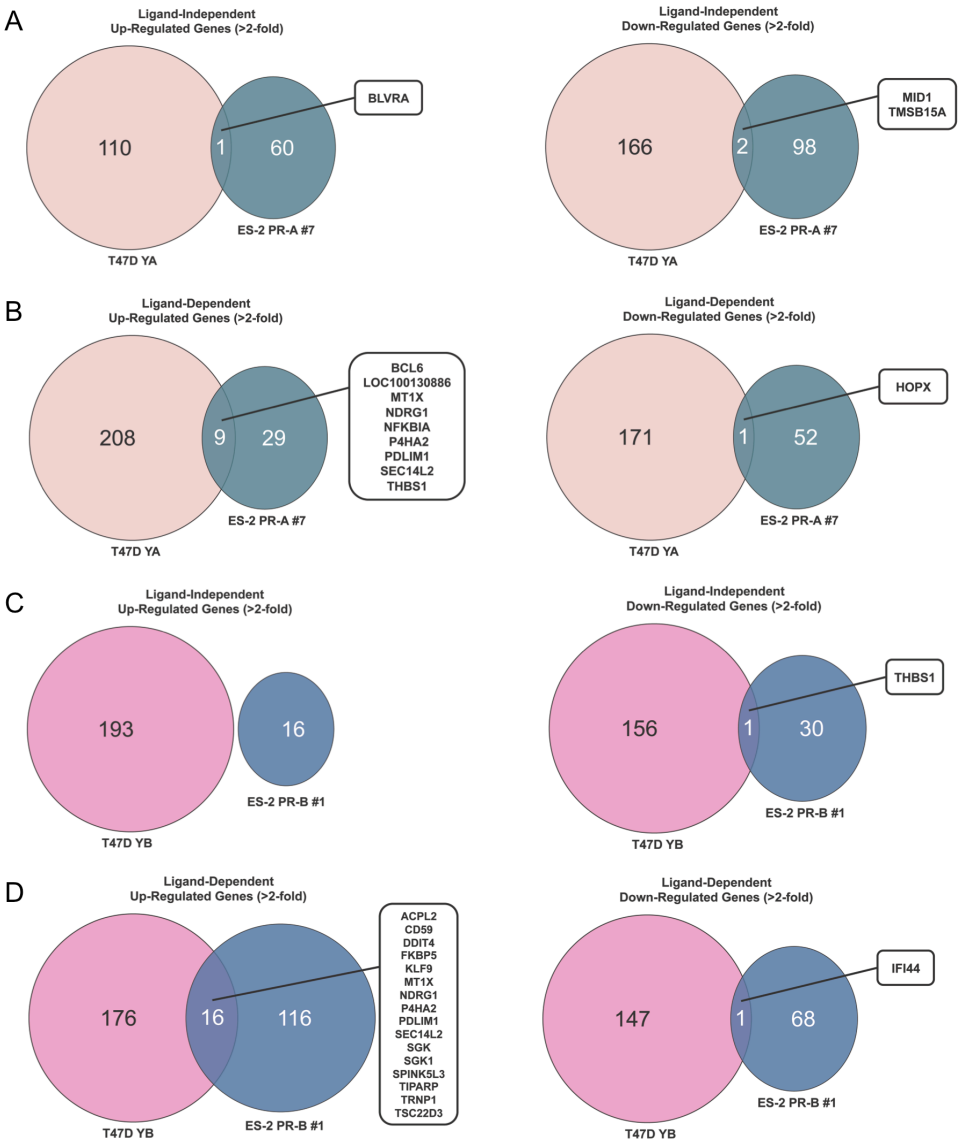


Figure 3-5: Differential transcriptional regulation of PR isoform-specific target genes in ovarian and breast cancer cells. (A) Venn diagrams depicting the number of unique genes upregulated or downregulated >2-fold in the absence of R5020 treatment in PR-A-expressing T47D YA and ES-2 PR-A #7 cells. (B) Venn diagrams depicting the number of unique genes upregulated or downregulated >2-fold with R5020 treatment in PR-A-expressing T47D YA and ES-2 PR-A #7 cells. (C) Venn diagrams depicting the number of unique genes upregulated or downregulated >2-fold in the absence of R5020 treatment in PR-B-expressing T47D YB and ES-2 PR-B #1 cells. (D) Venn diagrams depicting the number of unique genes upregulated or downregulated >2-fold with R5020 treatment in PR-B-expressing T47D YB and ES-2 PR-B #1 cells. The genes from the shared Venn category are listed.

cancer models exhibits moderate overlap between PR-A and PR-B regulated gene sets and both isoforms are highly responsive to hormone when these models are assayed using similar conditions (i.e. compare Figure 2 to Figure 5). We detected no differences in transcriptional activity between GFP-tagged PRs and untagged PRs in multiple cell types (data not shown).

PR-B activates a FOXO1/p21 senescence program repressed by PR-A in ovarian cancer cells.

To more fully understand the basis of PR isoform-specific gene regulation in ovarian cancer cells, we considered the largely repressive actions of (primarily unliganded) PR-A relative to the predominantly hormone-sensing actions of (liganded) PR-B (compare Figure 6A, left to right Venn diagram). Only four genes were significantly repressed by PR-A and activated by liganded-PR-B: *KYNU*, *FOXO1*, p21, and *ZDHHC9* (Figure 6A). KYNU is involved in the biosynthesis of NAD cofactors from tryptophan catabolism through the kynurenine pathway. The expression of KYNU is significantly downregulated in several cancer types, such as in invasive ductal breast carcinomas[172], pediatric acute myeloid leukemias harboring IDH mutations[173], and highly aggressive osteocarcinoma cell lines.[174] ZDHHC9 is a palmitoyltransferase specific to HRAS and NRAS[175] and reported to be widely overexpressed in human cancers.[176] Notably, we previously identified both FOXO1 and p21 as required factors for progestin-mediated cellular senescence in PR-B+ ovarian cancer cells.[48] Our microarray experiments

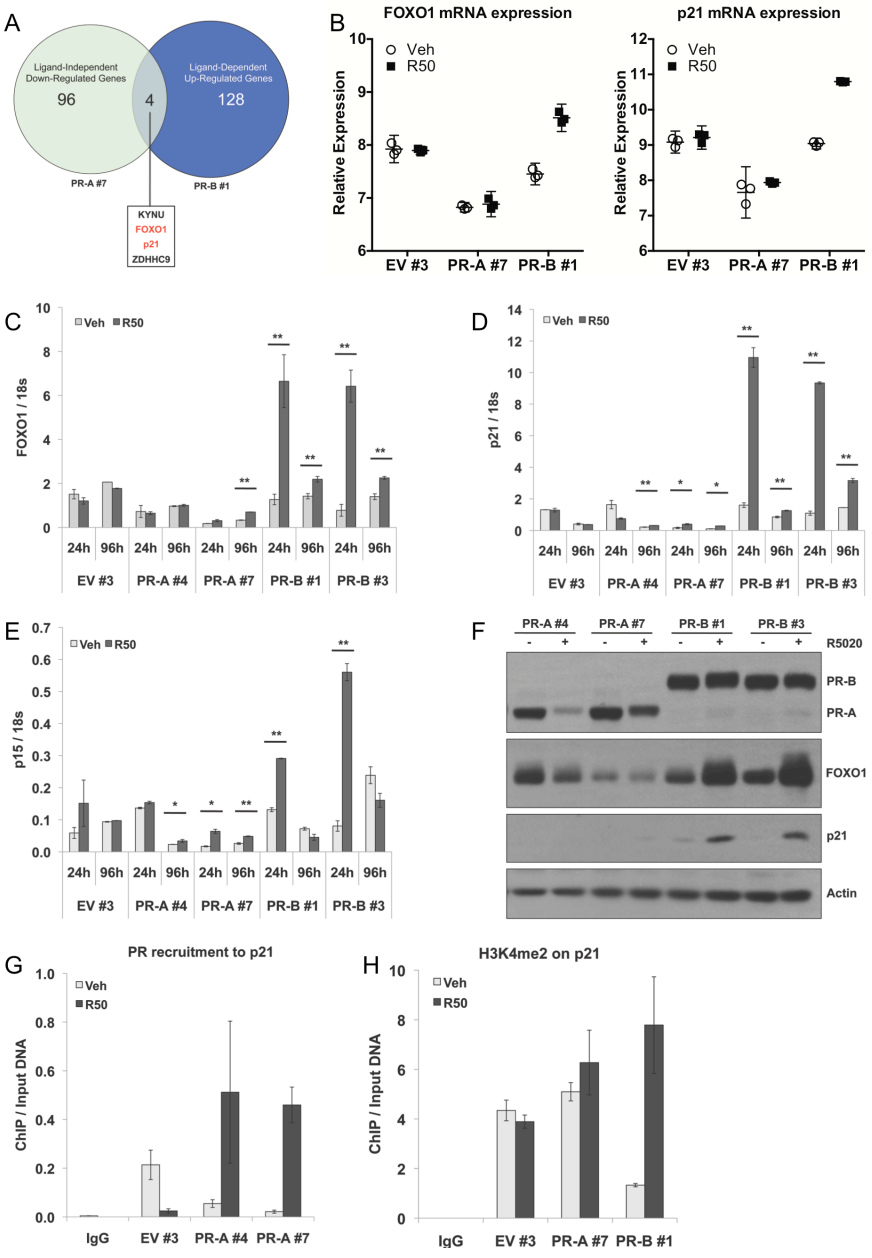


Figure 3-6: Progestin treatment induces isoform-specific differential expression of cellular senescence mediators in ovarian cancer cells. (A) Venn diagram describing the number of unique genes downregulated in the absence of R5020 treatment in ES-2 cells stably expressing PR-A (clone #7) and upregulated in ES-2 cells stably expressing PR-B (clone #1) with R5020 treatment. Four genes from the shared Venn category are listed. (B) Relative gene expression of FOXO1 and p21 in ES-2 cells stably expressing EV control (clone #3), PR-A (clone #7), or PR-B (clone #1) treated with vehicle or R5020 (10 nM) for 24 hr as determined by Illumina microarray experiments (Figure 2A). RT-qPCR analysis of (C) FOXO1, (D) p21, and (E) p15 mRNA expression following 24 hr and 96 hr R5020 (10 nM) treatment of ES-2 cells stably expressing EV control (clone

#3), PR-A (clone #4, #7), PR-B (clone #1, #3) (n=3, *p≤0.05, **p≤0.01). (F) Western blot analysis of PR, FOXO1, and p21 protein expression in ES-2 cells stably expressing PR-A (clone #4, #7) or PR-B (clone #1, #3) after 96 hr treatment of R5020 (10 nM). Actin served as a loading control. (G) ChIP assays showing PR recruitment to a PRE-containing region of the p21 promoter. EV control and PR-A-expressing cells (clone #4, #7) were stimulated with vehicle or R5020 (10 nM) for 1 hr. Fixed lysates were subjected to ChIP assays as described in Methods using specific antibodies targeting PR (or IgG control). (H) ChIP assays demonstrating detection of H3K4me2 at the PRE-containing region of the p21 promoter. EV control, PR-A- (clone #7), and PR-B-expressing (clone #1) cells were stimulated with vehicle or R5020 (10 nM) for 1 hr. Fixed lysates were subjected to ChIP assays as described in Methods using specific antibodies targeting H3K4me2 (or IgG control).

revealed repression of both p21 and FOXO1 mRNA expression in untreated or treated PR-A-expressing (clone #7) ES-2 cells relative to EV (clone #3) controls (Figure 6B). In contrast to PR-A+ cells, R5020 treatment (24 hr) significantly upregulated p21 and *FOXO1* mRNA in PR-B+ cells (clone #1). These results (i.e. robust PR-B-selective upregulation of FOXO1, p21, and the FOXO1 target gene, p15) essentially repeated by RT-qPCR in multiple clones of ES-2 cells stably expressing either PR-B-only or PR-A-only and treated with R5020 for 24-96 hrs (Figure 6C-E). *FOXO1*, p21, and p15 mRNA levels were either slightly upregulated or unchanged in PR-A+ cells relative to their pronounced induction in PR-B+ cells (24 hr). These genes were also insensitive to progestin in EV+ (clone #3) controls (Figure 6C-E). Western blot analysis confirmed these results (Figure 6F), demonstrating robust upregulation of FOXO1 and p21 protein within 96 hr R5020 treatment in PR-B+ clones (#1 and #3) but not in PR-A+ clones (#4 and #7). Taken together, these data indicate that while progestin-treated PR-A+ cells are capable of modestly inducing FOXO1 and p21 at the mRNA level, changes in protein expression do not persistently manifest (i.e. by as late as 96

hrs). In the presence of progestin, PR-B is a stronger transcriptional activator of known senescence mediators relative to PR-A.

To demonstrate the repressive action of PR-A on the p21 promoter, we performed chromatin immunoprecipitation (ChIP) assays to determine if liganded PR-A binds to PRE-containing regions of the endogenous p21 promoter. Indeed, in the presence of progestin, PR-A was significantly recruited (9- and 21-fold in clones #3 and #7, respectively) to a PRE-containing region downstream of the p21 transcriptional start site previously identified by ChIP-Seq studies conducted in PR+ breast cancer models[102] and validated in our previous study[48]; PR recruitment to this region in EV control (clone #3) cells was minimal and similar to background (IgG control) levels (Figure 6G). We previously reported[48] similar recruitment (5-fold relative to controls) of PR-B to the same PRE-containing region following R5020 treatment (data not shown).

Taken together, our data suggest that PR-B-containing transcriptional complexes activate p21 transcription, while PR-A-containing complexes are repressive. Changes in gene expression are linked to epigenetic histone tail modifications, such as methylation and acetylation. Histone H3 Lys4 dimethylation (H3K4me2) is an epigenetic modification associated with transcriptional activation. To measure the level of H3K4me2 (i.e. as a surrogate marker of p21 gene activation) at the same PRE/PR-containing p21 promoter region, EV control (clone #3), PR-A-expressing (clone #7), and PR-B-expressing (clone #1) cells were treated with vehicle control or R5020 (10 nM for 1 hr) and

the degree of histone H3 Lys4 dimethylation was measured by ChIP assay (Figure 6G). H3K4me2 levels were significantly elevated (6-fold) only in progestin-treated ES-2 cells stably expressing PR-B (clone #1) relative to ES-2 cells stably expressing PR-A (clone #7) and EV (clone #3) vehicle controls. These results suggest that PR-A is recruited to the p21 promoter but is incapable of mediating robust hormone-dependent p21 gene activation.

PR-B robustly induces cellular senescence relative to PR-A in ovarian cancr cells.

To account for the differences in PR isoform-specific regulation of senescence mediators (Figure 6), we evaluated the senescence phenotype in PR-A and PR-B expressing ES-2 cells. Our previous study demonstrated that in the presence of progestin, PR-B promoted cellular senescence, a cell fate wherein cells remain viable and long-lived, but are non-proliferative.[48] A common marker of cellular senescence is the accumulation of endogenous lysosomal β-galactosidase. We estimated the percentage of senescent ES-2 cells via measurement of the activity of senescence-associated β-galactosidase (SAβGal) at pH 6[90] (i.e. blue stained cells with enlarged nuclei). Representative images showing SAβGal and DAPI staining performed in clonal ES-2 cell lines expressing EV (clone #3), PR-A-only (clone #7), or PR-B-only (clone #1) and treated (96 hr) with either R5020 or vehicle control are depicted (Figure 7A) along with quantitation of SAβGal positive cells in five clonal cell lines (Figure 7B). As expected, R5020 (96 hr) significantly induced senescence as measured

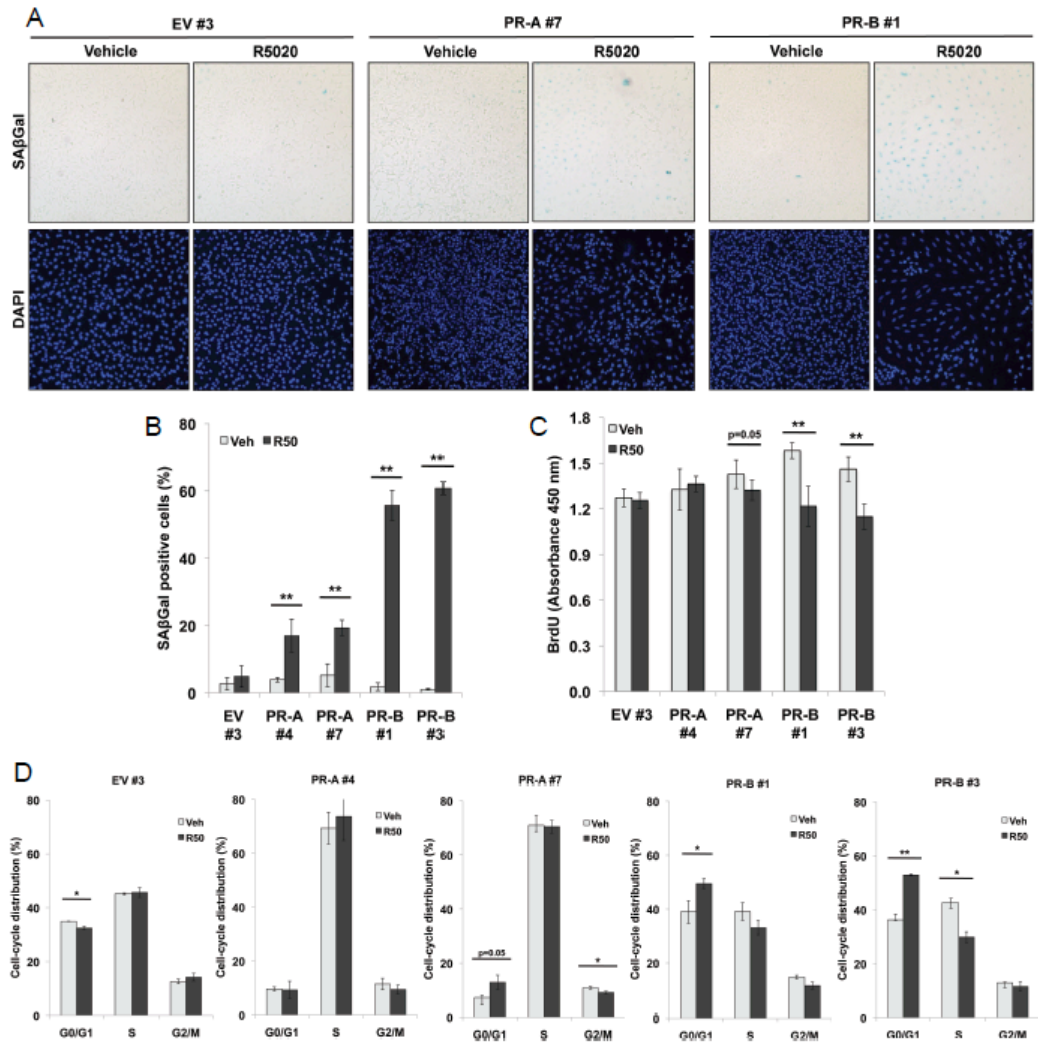


Figure 3-7: PR isoform expression and activity induces cellular senescence.
(A) Representative staining for SAβGal activity in EV control (clone #3), PR-A- (clone #7) or PR-B-expressing (clone #1) ES-2 cells treated with R5020 (10 nM) for 96 hrs (magnification = 100X). Cell samples were mounted onto glass slides using ProLong® Gold Antifade Reagent with DAPI (Invitrogen) for bright-field and fluorescent microscopy. B) Percentage of positive SAβGal cells in EV control (clone #3) and PR-A- (clone #4, #7) and PR-B-expressing (clone #1, #3) cells treated with R5020 (10 nM) for 96 hr was determined by quantifying three fields at 100X magnification. Values were normalized to total nuclei present in each field as determined by DAPI staining (n=3, **p≤0.01). (C) BrdU incorporation analysis of EV control (clone #3), PR-A- (clone #4, #7) or PR-B-expressing (clone #1, #3) cells continuously treated with R5020 (10 nM) for 96 hrs. BrdU was added to the wells, and cells were incubated for 3 hr prior to fixing the cells and denaturing the DNA according to manufacturer's protocol (n=2, **p≤0.01). (D) Cell cycle analysis by propidium iodide staining of EV control (clone #3) PR-A- (clone #4, #7) or PR-B-expressing (clone #1, #3) cells treated with R5020 (10 nM) for 96 hr (n=2, *p≤0.05 **p≤0.01).

by SAβGal in PR-B-expressing ES-2 cells (clones #1 and #3 exhibited 51% and 59% positive cells, respectively) relative to vehicle controls. In contrast, PR-A-expressing ES-2 cells (clones #4 and #7) exhibited significantly less SAβGal (21% and 22%, respectively) relative to PR-B-expressing ES-2 cells. Minimal SAβGal levels were observed in EV #3 (2%) and vehicle-treated cells (Figure 7A-B).

Cellular senescence is often associated with a decrease in cell proliferation that is accompanied with cell cycle exit (i.e. cells accumulate in G1). Thus, we examined BrdU incorporation to detect altered proliferation following progestin treatment of PR-expressing ovarian cancer cells. After 96 hr R5020 treatment, BrdU incorporation was significantly inhibited in PR-B-expressing cell clones, indicating that R5020 attenuated DNA synthesis and halted proliferation. R5020 treatment did not significantly alter BrdU incorporation in EV (clone #3) and PR-A-expressing (clone #4) cells, and only weakly inhibited PR-A+ clone #7 cells relative to that observed in PR-B-expressing clones (Figure 7C). Finally, cell cycle (FACS) analysis of PR-B-expressing cells, as measured by propidium iodide staining, confirmed a significant increase in the percentage of cells in the G0/G1 phase of the cell cycle accompanied by a decrease in the percentage of cells in S phase following 96 hr of R5020 exposure relative to vehicle controls (Figure 7D). Similar to the above findings using BrdU, progestin treatment elicited minimal or insignificant effects on the cell cycle distribution of ES-2 cells expressing either EV (clone #3) or PR-A (clones #4 and #7; weak but significant

effects were again observed in PR-A clone #7). Taken together, our data suggest that in the presence of progestin, PR-B is a stronger promoter of cellular senescence relative to PR-A in ovarian cancer cells.

Active FOXO1 expression confers PR-B-like behavior to PR-A-containing cells.

Similar to cell cycle studies performed in breast cancer models,[37, 124] relative to PR-B, PR-A often fails to elicit robust induction of cell cycle mediators (p21) and their associated biological responses (i.e. biphasic regulation of cell proliferation in breast models), but may weakly mimic PR-B. Progestin treatment weakly induced *FOXO1* mRNA expression (Figure 6C) but did not modulate protein levels (Figure 6F) in FOXO1-expressing PR-A+ clones (#4 and #7) relative to PR-B+ clones (at 96 hr). We thus evaluated if FOXO1 activity was required for the weak but significant induction of cellular senescence in PR-A-expressing clone #7 using the selective FOXO1 small molecule inhibitor, AS1842856 (AS)[48, 103] (Figure 8). R5020 treatment of PR-A+ (clone #7) ES-2 cells for 96 hr modestly induced FOXO1 protein and mRNA expression relative to vehicle controls (Figure 8A-B). Treatment with AS alone did not affect basal PR-A or FOXO1 protein and mRNA expression, but AS blocked R5020-induced FOXO1 protein and mRNA expression (Figure 8A-B). *FOXO1* gene expression is known to be auto-regulated (i.e. FOXO1 contributes to regulation of its own gene expression).[177] Similar results were observed for R5020-induced p21 and p15

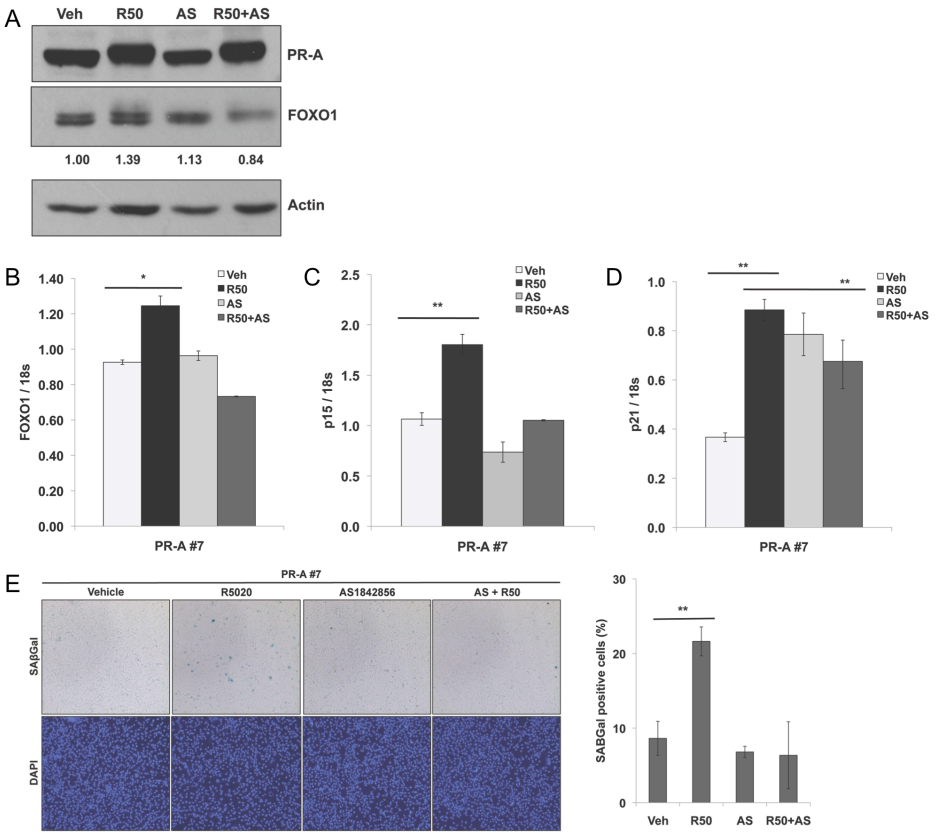


Figure 3-8: **PR-A-induced cellular senescence is dependent on FOXO1 activity.** (A) Western blot analysis of PR and FOXO1 protein expression in PR-A-expressing (clone #7) cells treated with vehicle control, R5020 (10 nM), AS1842856 (AS, 100 nM), or the combination of AS1842856 and R5020 for 96 hrs. Densitometry of FOXO1 protein expression in each treatment group is included in the figure. RT-qPCR analysis of (B) FOXO1, (C) p15, and (D) p21 mRNA expression in PR-A-expressing (clone #7) cells treated with R5020 (10 nM), AS1842856 (AS, 100 nM), or the combination of AS1842856 and R5020 for 96 hrs. (E) Representative staining for SAβGal activity of PR-A-expressing (clone #7) cells treated with R5020 (10 nM), AS1842856 (AS, 100 nM), or the combination of AS1842856 and R5020 for 96 hrs (magnification = 100X). Cell samples were mounted onto glass slides using ProLong® Gold Antifade Reagent with DAPI (Invitrogen) for bright-field and fluorescent microscopy. Percentage of positive SAβGal cells was determined from quantifying three fields at 100X magnification. Values were normalized to total nuclei present in each field from DAPI staining (n=3, **p≤0.01).

mRNA expression (Figure 8C-D). Consistent with these results, progestin-induced senescence, as measured by SAβGal activity, was significantly reduced upon inhibition of FOXO1 by AS (100 nM) in combination with R5020 for 96 hr relative to R5020-only treated controls (Figure 8E). Together, these data

implicate FOXO1 as a required mediator of senescence-associated gene expression in PR-A+ ES-2 cells treated with progestin.

We speculated that overexpression of FOXO1 in PR-A-expressing cells would confer PR-B-like behavior and thereby "rescue" hormone sensitivity and senescence-associated gene regulation similar to that induced in PR-B+ cells. PR-A-expressing ES-2 clones were screened for low to undetectable FOXO1 protein levels by Western blot (Figure 9A). These clones were fully capable of inducing robust expression of the PR-A target gene WISP1, but failed to upregulate PR-B-selective genes including FOXO1 following 96 hrs R5020 treatment (data not shown). The PI3K/AKT pathway is activated in a majority of ovarian cancers[178] and inactivates forkhead family transcription factors. Phosphorylation of FOXO1 at Thr24, Ser256, and Ser319 negatively regulates its nuclear localization and transcriptional activity[152, 179]; we therefore utilized the constitutively active form of FOXO1 (FOXO1-AAA) that cannot be phosphorylated by AKT.[148] FOXO1-AAA or empty vector control (EV) were stably expressed by retroviral transduction of PR-A+/FOXO1-low (clones #1 and #5) ES-2 cells. Expression of total FOXO1 protein was confirmed by Western blotting (Figure 9A). Notably, R5020 only weakly increased p21 mRNA expression after 96 hr in PR-A+ (clone #1) cells expressing EV (FOXO1-null) control. However,

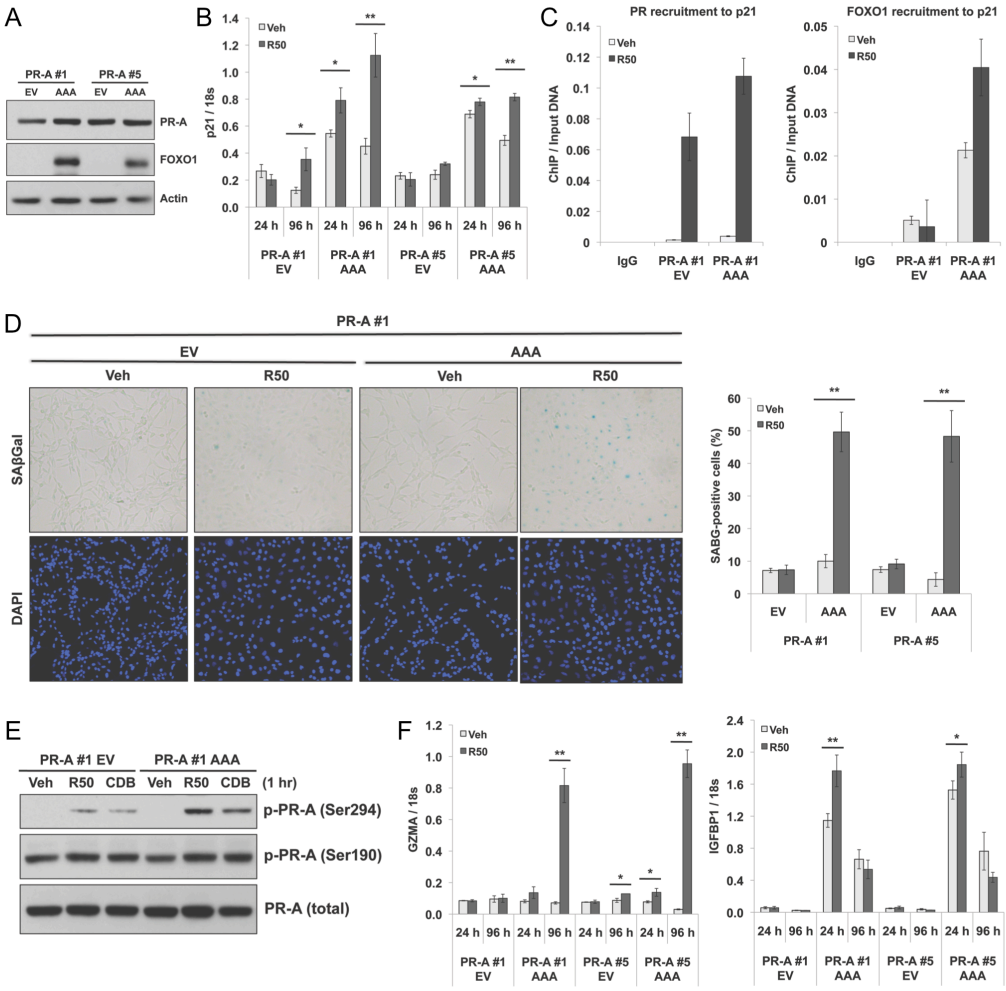


Figure 3-9: Active FOXO1 restores PR-A sensitivity to progestins and induces cellular senescence. (A) Western blot analysis of PR and FOXO1 protein expression in PR-A-expressing cells (clone #1, #5) stably expressing either EV control or constitutively active FOXO1 (AAA). Actin served as a loading control. (B) RT-qPCR analysis of p21 mRNA expression in cells expressing PR-A (clone #1, #5) and either EV control or constitutively active FOXO1 (AAA) and treated with vehicle or R5020 (10 nM) for 24 hr and 96 hr (n=3, *p≤0.05 **p≤0.01). (C) ChIP assays showing PR and FOXO1 recruitment to the p21 promoter. PR-A-expressing (clone #1) cells stably expressing either EV control or constitutively active FOXO1 (AAA) were stimulated with vehicle or R5020 (10 nM) for 1 hr. Fixed lysates were subjected to ChIP assays as described in Methods using antibodies targeting PR, FOXO1, or IgG control and RT-qPCR was performed on isolated DNA. (D) Representative staining for SAβGal activity in PR-A-expressing cells (clone #1) expressing either EV control or constitutively active FOXO1 (AAA) and treated with vehicle or R5020 (10 nM) for 96 hrs (magnification = 100X). Cell samples were mounted onto glass slides using ProLong® Gold Antifade Reagent with DAPI (Invitrogen) for bright-field and fluorescent microscopy. Percentage of positive SAβGal cells in PR-A-expressing cells (clone #1, #5) expressing either EV control or constitutively active FOXO1 (AAA) and treated with vehicle or R5020 (10 nM, 96 hr) was

determined from quantifying three fields at 100X magnification. Values were normalized to total nuclei present in each field from DAPI staining (n=2, **p≤0.01). (E) Western blot analysis of PR phosphorylated on Ser294 or Ser190 and total PR in PR-A+ (clone #1) cells stably expressing either EV control or constitutively active FOXO1 (AAA) treated with either vehicle control, R5020 (R50, 10 nM) or CDB-4124 (CDB, 1 μM) for 1 hr. (F) RT-qPCR analysis of GZMA and IGFBP1 mRNA expression in PR-A-expressing cells (clone #1, #5) co-expressing either EV control or constitutively active FOXO1 (AAA) and treated with vehicle or R5020 (10 nM) for 24 hr and 96 hr (n=3, *p≤0.05 **p≤0.01).

forced FOXO1-AAA expression in these cells significantly further increased p21 mRNA induction in the presence of R5020 relative to vehicle controls at both 24 hr and 96 hr (Figure 9B). These results essentially repeated in an additional ES-2 cell line co-expressing PR-A (clone #5) and FOXO1-AAA (Figure 9B). Similar results were observed in HeLa cells transiently expressing PR-A and FOXO1 as measured via 2X-PRE-luciferase reporter assays and RT-qPCR analysis of p21 expression (Figure 10).

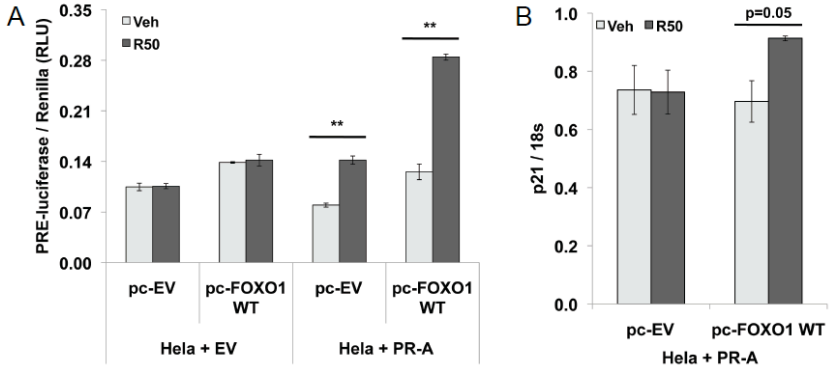


Figure 3-10: FOXO1 enhances progestin-dependent PR-A transcriptional activity in Hela cells. (A) HeLa cells were transiently cotransfected with GFP-tagged PR-A, either the pcDNA3.1 empty vector (pc-EV) control or FOXO1 WT (pc-FOXO1 WT) vector, and a progesterone response element (2XPRE) containing luciferase reporter gene construct. Cells were treated for 18 hr with R5020 (10 nM). Relative luciferase units (RLU) were normalized to the mean result ± standard deviation (SD) for Renilla luciferase expression (n=3, **p≤0.01). (B) RT-qPCR analysis of p21 mRNA expression after 24 hr R5020 (10 nM) treatment in HeLa cells transiently cotransfected with GFP-tagged PR-A and either the pcDNA3.1 empty vector (pc-EV) control or FOXO1 WT (pc-FOXO1 WT) vector (n=3).

ChIP assays revealed that upon R5020 treatment (10 nM; 1 hr), PR-A was again recruited to the p21 promoter in PR-A+ EV-expressing (clone #1) cells, and significantly further recruited upon exogenous FOXO1-AAA expression (Figure 9C). FOXO1-AAA recruitment to this site also occurred in the absence of ligand but was significantly increased in response to R5020 treatment (Figure 9C). Consistent with these results, overexpression of FOXO1-AAA in PR-A+ cells (clones #1 and #5) conferred increased SAβGal activity upon R5020 treatment relative to vehicle and EV controls (Figure 9D). The level of SAβGal activity detected in FOXO1-AAA overexpressed PR-A+ ES-2 cells was similar to that of PR-B+ cells when treated with R5020 (Figure 5A-B). Induction of SAβGal in R5020-treated PR-A+ cells expressing FOXO1-AAA (but not EV) was accompanied by cellular senescence-associated changes in cell cycle distribution (i.e increased percentage of cells in G0/G1; data not shown).

These data suggest that expression of activated FOXO1-AAA in PR-A+ cells confers PR-B-like behavior (i.e. hormone responsive induction of p21 and senescence). To understand the mechanism for this effect, we assayed ligand-dependent and independent PR phosphorylation as a surrogate marker of heightened progesterone responsiveness.[180] Notably, phosphorylation of PR-B Ser294 mediates changes in PR-B promoter selection and hormone sensitivity, primarily assayed in breast cancer models.[38, 78, 79, 181] We included both R5020 (a PR agonist) and the selective PR antagonist, CDB-4124 (CDB). Surprisingly, while PR-Ser190 phosphorylation remained unaltered across all conditions,

addition of either R5020 or CDB similarly induced robust PR-Ser294 phosphorylation in PR-A+ (clone #1) ES-2 cells expressing FOXO1-AAA relative to EV-expressing and vehicle-treated controls (Figure 9E). Similar results were observed in a separate PR-A+ ES-2 clone (#5; data not shown). These data suggest that overexpression of activated FOXO1-AAA enhances PR-A Ser294 phosphorylation and transcriptional activity in a manner similar to PR-B activity on PR-B-selective target genes (e.g. p21). Surprisingly, overexpression of FOXO1-AAA in PR-A+ (clones #1 and #5) cells also significantly increased mRNA expression of the PR-B-selective genes, *GZMA* and *IGFBP1* (Figure 9F). Together, these data suggest that FOXO1 is a key mediator of enhanced hormone sensitivity on selected PR target genes (p21, *GZMA*, *IGFBP1*). Ligand-bound PR-A is fully capable of undergoing phosphorylation at Ser294 and regulating PR-B-only target genes when activated FOXO1 is present.

PR-A is capable of inhibiting the transcriptional activity of PR-B, as well as other SRs, including glucocorticoid, mineralocorticoid, androgen, and estrogen receptors.[106] Trans-repression of PR-B by PR-A has been primarily measured using reporter-gene assays[182, 183] rather then on endogenous genes and requires PR-A SUMOylation.[184] Our data suggest that PR-A is primarily repressive on the p21 promoter, but high levels of activated FOXO1 reversed this effect and enhanced both PR-A Ser294 phosphorylation and hormone sensitivity (i.e. of PR-A on PR-B target genes). To address PR-B target gene expression when PR-A and PR-B are co-expressed, we transiently transfected PR-A into PR-B-

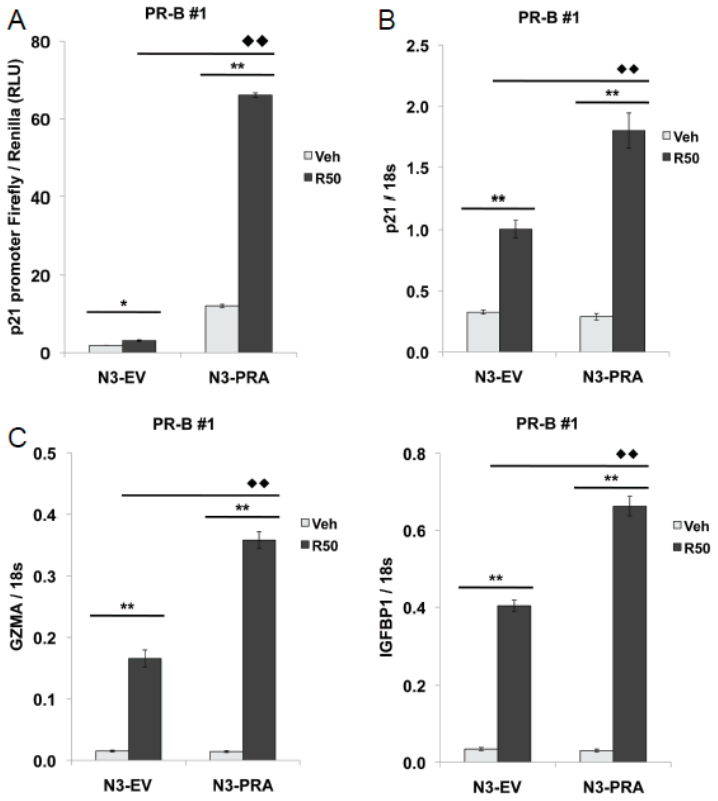


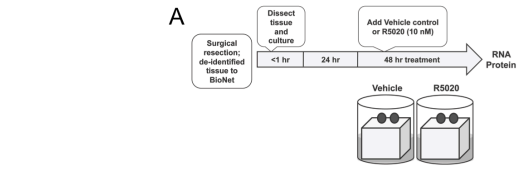
Figure 3-11: PR-A transactivates PR-B in PR-B-expressing ovarian cancer cells. (A) GFP-tagged empty vector (N3-EV) or GFP-tagged PR-A (4 µg) were transiently transfected into PR-B-expressing (clone #1) ES-2 cells with a p21 promoter-driven luciferase reporter construct and treated for 24 hr with R5020 (10 nM). Relative luciferase units (RLU) were normalized to the mean result ± standard deviation (SD) for Renilla luciferase expression (n=2, *p≤0.05, **p≤0.01, ◆◆p≤0.01). RT-qPCR analysis of (B) p21, (C) GZMA, and IGFBP1 mRNA expression in PR-B-expressing (clone #1) cells transiently transfected with either GFP-tagged empty vector (N3-EV) or GFP-tagged PR-A (0.5 µg). Cells were treated for 24 hr with R5020 (10 nM) (n=2, **p≤0.01, ◆◆p≤0.01).

expressing ES-2 cells. PR-B+ ES-2 cells (clone #1) were transiently transfected with either GFP-tagged EV (control) or GFP-tagged PR-A and a p21-promoter-driven luciferase reporter[48] (Figure 11A). As previously reported[48] R5020 (10 nM for 24 hr) significantly induced p21 promoter activity in PR-B-only expressing cells containing the EV control (~2-fold) as measured using p21-luciferase assays. Surprisingly, transient expression of PR-A further increased ligand-

induced p21 promoter activity (5-fold) in PR-B+ ES-2 cells. These results were confirmed at the level of transcription of the endogenous p21 promoter; addition of PR-A to PR-B+ ES-2 cells (clone #1) significantly increased p21 mRNA expression (i.e. from 3-fold in EV-expressing cells to 6-fold upon addition of PR-A; Figure 11B). Furthermore, in the presence of progestin, the PR-B-selective genes, *GZMA* and *IGFBP1*, were significantly increased upon expression of PR-A in PR-B+ ES-2 cells (clone #1; Figure 11C). Overall, our data suggest that when FOXO1 is present (a PR-B target gene), PR-A is equally hormone responsive (i.e. relative to PR-B) and capable of robust transactivation of selected PR-B target genes (p21, *GZMA*, *IGFBP1*).

Progestins upregulate p21 and FOXO1 mRNAs in PR-B positive ovarian tumors

To validate our novel *in vitro* findings in ovarian cancer cell line models defined herein, we assessed the impact of PR signaling in primary human ovarian tumors using an innovative *ex vivo* culture system.[149-151] Notably, tumor tissues cultured using this 3D system retain features of the original tumor, including SR expression. As illustrated in Figure 12A, *ex vivo* cultures of fresh primary ovarian tissue obtained from cytoreductive surgery were established using dissected tissues mounted on pre-soaked gelatin sponges equilibrated in culture media containing either vehicle control or R5020 (10 nM, 48 hr). A total of seven de-identified ovarian carcinoma tissues were obtained from the University of Minnesota's tissue procurement facility (BioNet) and cultured as described in



B

Tumor ID	Age	Final diagnosis	PR status		Response to R5020	
			PR-A	PR-B	FOXO1	p21
OVC-1	66	high-grade serous carcinoma		✔	↑	↑
OVC-2	54	borderline serous tumor or ovary	✔	✔	NS	NS
OVC-3	54	borderline serous tumor or ovary	✔	✔	NS	↓
OVC-4	71	mixed epithelial carcinoma: serous and mucinous		✔	↓	NS
OVC-5	48	differentiated seromucinous carcinoma		✔	NS	NS
OVC-6	52	high-grade serous carcinoma		✔	NS	↑
OVC-7	57	high grade serous carcinoma	✔	✔	↑	NS

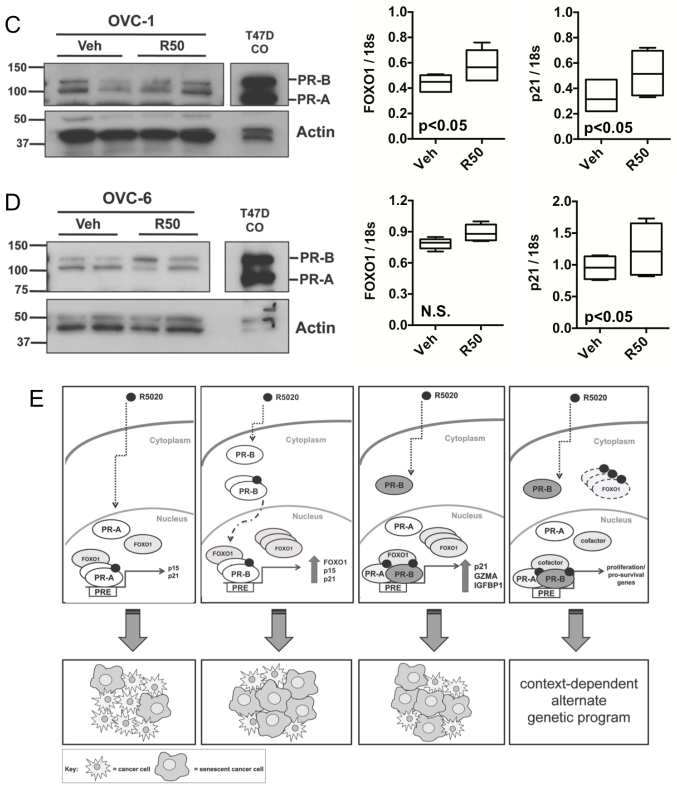


Figure 3-12: Progestin mediates p21 and FOXO1 expression *ex vivo* in PR+ human primary ovarian tumors. (A) Workflow schematic of *ex vivo* ovarian tumor explant assay as described in Methods. (B) Overview of patient age, final diagnosis, PR expression, and significant regulation of FOXO1 or p21 mRNA levels with R5020 (10 nM) treatment in seven ovarian cancer tumors collected in this study. ✔ denotes positive expression of PR-A or PR-B as determined by Western blot analysis (as in C-D). ↑, ↓, or NS denote significant upregulation, downregulation, or no significance, respectively, of R5020-induced changes in the expression of p21 and FOXO1 mRNA levels relative to same-tumor vehicle controls. (C-D) PR protein (Western blots; left) and p21 and FOXO1 mRNA (RT-qPCR; right) expression in human ovarian patient tumor OVC-1 and OVC-6 explants treated without (vehicle) or with R5020 (10 nM) for 48 hrs. In Western blots, PR isoforms expressed in explants are shown relative to T47D CO breast cancer cells

(positive control); actin served as a loading control. Data reflect the mean of duplicate samples. (E) Proposed model of PR-A- and PR-B-induced cellular senescence in ovarian cancer cells. PR-A primarily acts to repress expression of p15 or p21 and weakly induce cellular senescence in PR-A+ cells. In contrast, hormone-stimulated PR-B+ cells upregulate FOXO1, p21, and p15 expression. Elevated levels of FOXO1 (a PR-B target gene) then cooperate with PR-B to further upregulate FOXO1, p15, and p21 expression and robustly induce cellular senescence. Expression of PR-A in PR-B+/FOXO1+ significantly enhances progestin-dependent expression of PR-B target genes, p21, GZMA, and IGFBP1 and promotes cellular senescence. In the absence of FOXO1 expression, PR-A and PR-B regulate proliferative and/or pro-survival genes to promote alternate genetic programs.

Methods (Figure 12B). Tumors included in this study expressed the PR-B isoform only or both PR-A and PR-B (Figure 12B); we did not observe any tumors expressing PR-A only. A high-grade serous subtype tumor (OVC-1) retained expression of the PR-B isoform (two film exposures depicting PR isoform expression in tumor lysates and T47D CO whole cell lysate as a positive control are shown; Figure 12C). In the presence of progestin, *FOXO1* and p21 mRNA expression were significantly induced relative to vehicle controls (Figure 12C). A separate PR-B+ primary ovarian tumor (OVC-6) was similarly responsive to progestin treatment (48 hr); p21 mRNA but not *FOXO1* mRNA expression was significantly induced upon R5020 treatment (Figure 12D). Additional hormone-responsive tumors are indicated (Figure 12B). These data confirm that endogenously expressed PR-B induces p21 and FOXO1 expression in unmodified human primary tumors exposed to progestin. However, significant tumor heterogeneity in the response of ovarian tumors to progestins may relate in part to PR isoform expression as well as FOXO1 expression and activity (i.e.

as influenced by kinases that inactivate FOXO1 via phosphorylation events; see discussion).

Herein, our findings are consistent with a model that underscores the importance of activated (i.e. de-phosphorylated) FOXO1 as a mediator of PR hormone responsiveness (Figure 12E). In the absence of FOXO1 expression, PR-A primarily represses cellular senescence mediators, p21 and p15, and remains relatively insensitive to added progestin. In sharp contrast, PR-B-expressing cells induce abundant FOXO1, p21, and p15 expression in response to hormone. FOXO1 and PR-B further cooperate to promote a robust cellular senescent phenotype.[48] When both isoforms are present, active FOXO1 confers increased hormone responsiveness to PR-A at PR-B target genes, effectively "switching" the genetic programming to induce robust cellular senescence. Overall, we conclude that limiting expression of FOXO1 or AKT-driven FOXO1 phosphorylation and inactivation are important inputs to predicting progesterone/PR-driven responses in ovarian cancers.

3.4 DISCUSSION

PR isoform expression in ovarian tumors.

Herein we modeled PR isoform expression in ovarian cancer cells to understand mechanisms of progesterone/PR-driven gene expression and cell biology. To date, two studies with large patient cohorts have examined the relative distribution of total PR levels within ovarian carcinomas. We first reported

that in a cohort of 504 ovarian tumors, 35% were PR+, with the highest PR expression in endometrioid (67%) and serous (35%; low grade serous, 64%) subtypes.[48] In accordance with our study, the international Ovarian Tumor Tissue Analysis (OTTA) consortium examined the association of ER and PR expression and survival in 2,933 invasive epithelial ovarian tumors with similar results.[49] Furthermore, the OTTA study confirmed the prognostic significance of PR expression in ovarian tumors; high PR expression (≥50% tumor cell nuclei staining) in high-grade serous ovarian carcinomas was associated with a significant improvement in survival.

PR isoforms are expressed from a single gene via alternate usage of at least two internal translational start sites (thus potentially creating full-length PR-B, truncated PR-A, or further truncated and transcriptionally inactive PR-C isoforms). Although numerous mRNAs capable of encoding both PR-A and PR-B have been characterized[185], isoform-specific expression is also regulated via two distinct upstream (i.e. distal and proximal) promoter regions.[186] At least three studies[8-10] have reported differential expression of PR isoforms in ovarian tumors. Akahira and colleagues[8] investigated PR-A and PR-B by immunohistochemistry of 107 patient tumors. They observed the dominant expression of PR-B across all of the histological subtypes, with PR-B frequently expressed in the serous subtype; PR-A was weakly expressed in mucinous followed by serous.[8] In a follow-up study, Akahira *et al* evaluated PR isoform expression via immunohistochemical staining in normal (n=8), benign serous

cystadenomas (n=10), borderline serous adenocarcinomas with low malignant potential (n=8), and malignant serous adenocarcinomas (n=24).[9] They reported that PR-A expression was decreased or absent in malignant tissues relative to normal, whereas PR-B expression was present across all tissue cases in that study. The authors concluded that the absence of PR-A expression in malignant ovarian tissues is associated with the development of ovarian tumors. Finally, Lenhard *et al*[10] examined the expression of PR isoforms in 155 ovarian patient cases, and reported PR-B expression in serous and endometrioid subtypes, while PR-A was present in serous followed by modest expression in endometrioid and clear cell.[10] Ultimately, these studies observed dominant expression of PR-B in ovarian tumors across all subtypes with reduced or absent expression of PR-A. The mechanism for loss of PR-A relative to PR-B in malignant ovarian tumors is unknown. However, in endometrial cancer cells, enhanced DNA methylation was detected at the PR gene locus when PTEN expression was deleted.[187] Approximately 7% of ovarian tumors evaluated in the 2011 TCGA analysis[41] harbored PTEN deletions, while 18% and 9% contained activating mutations of either PI3K or AKT, respectively.[41] Similar to these findings in ovarian cancer, methylation of the PR-A but not PR-B promoter upstream of the PR gene occurs in advanced breast cancers and is significantly associated with tamoxifen-resistance.[188]

To our knowledge, we are the first to examine the biological consequences of PR isoform-specific gene regulation in ovarian cancer models,

as well as to directly compare PR isoform-selective transcriptomes between ovarian and breast cancer models cultured under similar conditions (Figures 2, 5, and 6). Remarkably, in ovarian cancer models, we found that PR-A predominantly up- or down-regulates genes independently of ligand, while PR-B is the dominant hormone-responsive receptor; we were also surprised to discover relatively modest overlap between PR-A and PR-B target genes. In contrast, we observed much target gene overlap between PR-A and PR-B in similarly designed breast cancer models, where relative to PR-A, PR-B predominantly up-regulated target genes in the absence of progestin. Ligand-independent actions of PR-B have been well-characterized in breast cancer models[78, 171, 189] Surprisingly, there was also relatively little overlap between ovarian and breast cancer models with regard to hormone-regulated PR-target genes, with nine genes regulated by PR-A and 16 genes regulated by PR-B in both breast and ovarian cancer models (Figure 5). However, while PR-A- and PR-B-regulated genes were distinct in ovarian relative to breast cancer models, both isoforms regulated similar pathways involved in cell growth, proliferation, cell death, and survival (as predicted using Ingenuity Pathway analysis).

Progesterone is proliferative in breast, but inhibitory in reproductive tissues.

The paradoxical effects of progesterone observed in ovarian relative to breast cancer models may be attributed to differential co-factor availability[190] (discussed below) as well as differential cross-talk between PR and growth

factor-mediated signaling pathways (i.e. protein kinases). PR-B, but not PR-A, functions outside the nucleus to rapidly activate protein kinases (c-Src, MAPK, AKT) in part via ligand-induced interaction between a poly-Pro-rich region unique to PR-B and c-Src kinase (wherein c-Src is activated by SH3-domain ligation with PR).[31, 32, 36] In breast cancer models, rapid progesterone-induced MAPK activation (i.e. downstream of c-Src) or CDK2 robustly phosphorylates PR-B (but not PR-A) at Ser294.[37, 38] In this context, PR-B Ser294 phosphorylation is required for regulation of selected target genes in either the absence (ex. IRS-2, STC1) or presence (ex. MSX2, RGS2, MAP1A, PDK4) of hormone.[78] Thus, as expected, we observed robust ligand-induced phosphorylation of PR-B Ser294 in intact ES-2 cells (Figure 1D). However, in stark contrast to breast cancer models (i.e. where PR-A is not phosphorylated on Ser294[79, 166]), we were surprised to detect phosphorylation of PR-A Ser294 in intact ES-2 cells (Figure 1D). In breast cancer models, PR-B Ser294 phosphorylation confers hypersensitivity to progestin, increases the rate of ubiquitinylation of PR-B (an activation step for several SRs[191]), delays SUMOylation on K388[79], promotes ligand-independent transcriptional activity[110], and profoundly alters promoter selectivity.[78] Ligand- or growth-factor-induced PR-A Ser294 phosphorylation is generally undetectable.[79, 166] Consistent with these findings, we demonstrated that PR-A K388 SUMOylation (a transcriptionally repressive modification) occurs very rapidly (15 mins) and is sustained relative to PR-B in response to progestin or progestin plus growth factor (EGF) treatment.[79] Surprisingly, herein, we observed increased PR-

A Ser294 phosphorylation when FOXO1-AAA was expressed in ES-2 ovarian cancer models, suggesting that activated FOXO1 may serve to co-recruit protein kinases capable of phosphorylating Ser294 to PR-A-containing complexes. Taken together, our data suggest that increased PR-A Ser294 phosphorylation in the presence of activated FOXO1 may account for its ability to transactivate PR-B-specific target genes when these isoforms are co-expressed in PR-B+ cells (Figure 11). PR-A and PR-B cross-talk in the context of multiple post-translational modifications is a topic of further study.

FOXO1 as a critical cofactor in ovarian cancer biology.

Herein, our data reveal a mechanism whereby PR isoforms may maintain either distinct or overlapping transcriptional gene programs via context-dependent upregulation of key PR cofactors. PR-B but not PR-A induces FOXO1, the same pathway cofactor it requires to induce robust p21-dependent (i.e. via PR-B/FOXO1 complexes) cellular senescence in ovarian cancer cells.[48] In contrast, the relative in-availability of FOXO1 severely limits hormone-sensitivity in ovarian cancer cells expressing PR-A. In PR-A+ ES-2 cells, ligand-independent repression of FOXO1 mRNA may account for concomitant minimal regulation of p21 and p15 mRNAs and weak induction of cellular senescence when FOXO1 levels are also relatively low. Surprisingly, exogenous expression of active FOXO1 (FOXO1-AAA) restored hormone sensitivity in PR-A+ cells and conferred PR-B-like transcriptional activity at selected PR-B target genes (p21, *GZMA*, *IGFBP1*). In this context (PR-A plus activated FOXO1-AAA), progestin

induced a robust cellular senescence phenotype similar to that observed in PR-B+ cells. Ultimately, our study underscores the critical importance of FOXO1 as a required cofactor for PR-dependent actions in the ovary.

Steroid hormone action in ovarian cancer is grossly understudied relative to breast or prostate cancers. There is an urgent need to focus on the early events related to the contribution of hormones in the context of altered signaling events (loss of p53 or PTEN, activation of AKT signaling) that may predispose women to increased risk of ovarian cancer. Deregulation of FOXO1 is associated with tumorigenesis and cancer progression across multiple cancer types. A recent PAthway Representation and Analysis by Direct Inference on Graphical Models (PARADIGM) analysis of mRNA expression and copy number data revealed 80 significant pathways altered in primary ovarian serous cystadenocarcinomas; the FoxO family signaling pathway was identified as significantly altered in this subtype.[192] FOXO1 expression and activity is downregulated through alterations in upstream regulators, post-translational deregulation, or by genetic mutations[121]. Notably, AKT negatively regulates FOXO1 through phosphorylation and prevents FOXO1 nuclear accumulation, thus impairing target gene regulation.[121] Activating mutations of PI3Ks or inactivating mutations (i.e. functional loss) of PTEN are common early events in ovarian cancer. For example, activated AKT (i.e. downstream of these events) may impair PR-induced senescence signaling by nuclear exclusion of FOXO1 (Figure 8E). The early loss or inactivation of FOXO1 may render PR

"incompetent" at genes required for the induction of cellular senescence, leading to the loss of protective "sensing" by progesterone in ovarian tumors, and perhaps also in early PR+ breast tumors. Notably, analysis of The Cancer Genome Atlas data revealed significantly decreased FOXO1 mRNA expression in breast tumors relative to normal breast tissue ($p<2.2x10^{-16}$). Thus, we propose that FOXO1 acts as a "molecular switch" that confers highly hormone-sensitive cell growth inhibition/senescence to PR+ epithelial cells, but may allow progesterone to stimulate proliferation in a highly context-dependent manner. Upon loss of FOXO1, PR interaction with other available co-factors may mediate profound changes in gene expression and associated cell biology (i.e. from senescence induction to activation of proliferative/pro-survival programs). For example, STAT3 and STAT5 appear to be primary cofactors that direct PR-B-selective gene expression and increased breast cancer cell proliferation and survival.[193-195] Furthermore, in proliferating (S-phase) breast cancer models, CK2 confers ligand-independent PR-B target gene selection via phosphorylation of Ser81 (not found on PR-A) and recruitment of MKP3/DUSP6.[195] These examples illustrate how early genetic events (i.e. mutations that alter kinase signaling) have profound consequences on SR-mediated (i.e. epigenetic) regulation of gene programs and associated changes in cell and cancer biology.

Our data highlight the requirement of FOXO1 for PR transcriptional regulation and specificity. A previous study demonstrated that constitutively active FOXO1 enhanced the ligand-dependent transcriptional activity of PR-A in

PRE-luciferase assays.[147] One caveat of luciferase assays is that they primarily measure SR transcriptional activity when bound to minimal promoter elements in artificial contexts, limiting interpretations relevant to promoter selectivity during transcriptional regulation of endogenous gene promoters (i.e. in chromatin). Thus, while PR-A may appear to be "functional" in PRE-luciferase assays, it may repress selected endogenous genes in the context of chromatin. Herein, we validated that FOXO1 enhanced the ligand-dependent transcriptional activity of PR-A as measured via the levels of endogenous p21, *GZMA*, and *IGFBP1* mRNAs (ES-2 cells; and in HeLa cells for p21) and by readout of PRE-luciferase (HeLa cells). Furthermore, when we stably expressed the constitutively active FOXO1-AAA in PR-A+ ES-2 lacking detectable FOXO1, the sensitivity of PR-A to hormone was greatly enhanced and this formerly repressive isoform transactivated the p21 promoter (Figure 9). We conclude that constitutively active FOXO1 modulates PR-A hormone-sensitivity and influences the specificity of gene regulation, thereby mimicking the actions of PR-B.

In light of our findings herein, PR-B-dominance in ovarian tumors is intriguing. Early loss or inactivation of FOXO1 (i.e. via functional loss of PTEN and/or activation of PI3K/AKT signaling) is predicted to block induction of the senescence pathway, effectively "lifting" progesterone-mediated protection in PR-B+ ovarian cancer cells. It is tempting to speculate that in the absence of active FOXO1, PR-B acts as a driver of a proliferative/pro-survival transcriptional program similar to that observed in breast cancer models[78, 171, 189, 195] In support

of this concept, flow cytometry experiments revealed a large spike in the percentage of PR-B+ ES-2 cells in S phase upon FOXO1 knock-down relative to shRNA controls.[48] The context-dependent actions of PR-B in ovarian cancer models with chronically elevated AKT (i.e. inactivated FOXO1) signaling are a topic of further investigation.

While total PR levels are routinely measured in breast and endometrial cancers for clinical management and disease treatment, very few studies have examined the levels of PR isoforms in ovarian cancers. We collected a total of seven primary ovarian tumors and detected a dominance of PR-B-only in four tumors, while three tumors co-expressed PR-B and PR-A. In the *ex vivo* culture system, three high-grade serous ovarian tumors recapitulated our *in vitro* findings of PR-B-dependent induction of FOXO1 and/or p21 upon progestin treatment. As discussed above, aside from considerable tumor heterogeneity, several factors may account for lack of sensitivity to progestin in the other ovarian tumors; unresponsive tumors may harbor mutations that render FOXO1 inactive and unable to direct PR-dependent regulation of FOXO1 and p21 (i.e. required for senescence induction).

In summary, our data reveal distinct tissue-specific actions of PR-A and PR-B in ovarian relative to extensively studied breast cancer models. Our observations have important clinical implications with regard to the development of isoform-specific and/or tissue-selective progestins for endocrine therapies. The ability of PR-A to inhibit hyperplastic proliferation in the uterus, together with

reduced proliferative activity when PR-A is activated in ovarian and mammary gland tissue strongly indicates that targeted activation of PR-A with isoform-specific agonists will likely have a protective effect against uterine, breast, and ovarian carcinogenesis. Conversely, it may be desirable to inhibit the actions of PR-B, particularly when FOXO1 is inactivated in the context of heightened kinase signaling that is a hallmark of hormone-driven cancers. The quantification of PR isoforms and their key cofactors and target genes (i.e. FOXO1, p21) rather than total PR levels will be essential to improving the efficacy of disease management by isoform-specific selective PR modulators (SPRMs).

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3.6 CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

CHAPTER 4

CONCLUSIONS

4.1 SUMMARY

Mortality rates for ovarian cancer have remained largely unaffected despite clinical advances in detection methods, surgical techniques, and treatment regimens. Traditionally, ovarian cancer therapy has utilized extensive surgery followed by cytotoxic treatment strategies on the assumption that apoptotic tumor death increases the likelihood of increased patient survival. This strategy has limited treatment options to toxic compounds and high dose radiation for ovarian cancer patients. While these treatment strategies often yield partial to complete responses where the tumor shrinks or remains in remission, it can also cause severe side effects and toxicities in patients. Despite the initial treatment response, tumors often develop resistance and may reoccur or progress to advanced primary and metastatic tumors. An alternative strategy is the induction of cytostasis, where the proliferative capacity of tumor cells is permanently disabled without inducing apoptosis. Several clinical trials evaluating cytostatic treatments have yielded promising results,[196-198] suggesting that these

treatments may be as effective as traditional cytotoxic therapies in preventing tumor growth with fewer and less severe side effects.

Therapy-induced senescence is a promising approach to the induction of cytostasis in proliferative tumors. Senescent cells remain viable and metabolically active, but are permanently growth arrested. Of clinical interest, some tumor cells can be forced into senescence in response to stress and damage caused by chemotherapeutic drugs and/or radiation.[199] This suggests that many cancer cells posses intact, but repressed, signaling pathways that can be manipulated to undergo senescence. For example, low doses of doxorubicin induce senescence in numerous cancer cell lines, including those lacking tumor suppressors, p53 and p21.[200] This suggests that signaling pathways for senescence remain competent in cancers and can be activated in a context-dependent manner.

Herein, we have defined novel mechanisms for PR isoform-specific regulation of ovarian cancer cellular senescence in response to progesterone. We demonstrated that PR-A and PR-B-expressing ovarian cancer cells regulate distinct gene sets that differ from PR-driven gene sets in breast cancer cells. In ovarian cancer models, PR-A primarily regulates genes independently of progestin, while PR-B is the dominant ligand-dependent isoform. Ligand-activated PR-B acts through a FOXO1-dependent mechanism to upregulate p15 and p21 to robustly induce cellular senescence; PR-A represses FOXO1 and p21 resulting in weak induction of cellular senescence relative to PR-B. The

overexpression of constitutively active FOXO1 (FOXO1-AAA) in PR-A-only expressing cells conferred robust ligand-dependent upregulation of PR-B-target genes (e.g. p21) and induced robust cellular senescence similar observed in PR-B+ cells. Surprisingly, similar results were observed when PR-A was titrated into PR-B-containing cells, suggesting that FOXO1 confers transactivation of PR-B by PR-A. Finally, PR isoform-specific regulation of the FOXO1/p21 axis was recapitulated in seven human primary ovarian tumor explants treated with progestin. In summary, these data indicate a critical requirement for FOXO1 in progesterone signaling towards cellular senescence in ovarian cancer cells and reveals a novel mechanism for FOXO1 control of PR hormone sensitivity.

The dogma held that progesterone primarily acts through PR-B and is largely proliferative in the breast. However, in the reproductive track, progesterone was suspected to act primarily through PR-A isoforms as potent inhibitors of cell growth as PR-A is known to trans-repress the transcriptional activities of ER and PR-B. Our studies are the first to demonstrate that PR-B is a tumor suppressor and a mediator of ovarian cancer cellular senescence[48], yet the same receptor is clearly proliferative in the normal and cancerous breast.[170, 180] In addition, our studies are the first to demonstrate that PR-A trans-activates PR-B transcriptional activity in ovarian cancer cells, yet the same receptor trans-represses PR-B activity in breast cancer cells. The molecular mechanism of our findings involves the requirement for the interaction between FOXO1 and PR-A and PR-B in the ovary at tumor suppressor genes (i.e. p21, p15). The same

tumor suppressor genes are regulated in cancerous breast tissue, but to a weaker intensity; this weakened response may be due to post-translational modifications (i.e. phosphorylation) of PR that are specific to developing breast cancer.

In addition, the dogma held that PR-A and PR-B are differentially susceptible to post-translational modifications in response to the same kinase and hormone stimulation. This complexity contributes to the distinctions between the genes they activate. For example, PR-B, but not PR-A, is robustly phosphorylated on Ser294 in response to progestin and MAPK activation in breast cancer cells. In contrast, detection of PR-A Ser294 phosphorylation is low to unmeasurable in intact breast cancer cells, but exhibits increased K388 SUMOylation (a transcriptionally repressive modification).[79] Our studies are the first to demonstrate that PR-A can be robustly phosphorylated on Ser294 in response to progestin when FOXO1 is present and active. Increased PR-A Ser294 phosphorylation was detected when FOXO1-AAA was expressed in ES-2 ovarian cancer models relative to expression of the empty vector control, suggesting that activated FOXO1 may serve to co-recruit protein kinases capable of phosphorylating Ser294 to PR-A-containing complexes. Taken together, our data suggest that increased PR-A Ser294 phosphorylation in the presence of activated FOXO1 may account for its ability to transactivate PR-B-specific target genes when these isoforms are co-expressed in PR-B+ cells.

Collectively, our studies suggest FOXO1 is a critical determinant of PR isoform transcriptional activity as it acts as a rheostat and pioneer factor for progesterone and PR to inhibit cell growth in ovarian cancer. In PR-A-expressing cells, FOXO1 is repressed in a ligand-independent manner relative to PR-B-expressing cells; progestin treatment in PR-A+ cells minimally regulates FOXO1 mRNA levels at late timepoints (96 hr) but modestly induces senescence. When a certain threshold of FOXO1 expression achieved, either by basal expression in progestin-treated PR-B+ cells or by overexpression of constitutively active FOXO1 (FOXO1-AAA), PR-A sensitivity to progestin is restored and its transcriptional activity mimics PR-B on selectively PR-B target genes, such as p21, GZMA, and IGFBP1. FOXO1, like other members of the Forkhead domain family, may act as a pioneer factor in PR-expressing cells. As pioneer factors, FOX proteins can associate with compacted chromatin and modulate chromatin to facilitate accessibility for other transcription factors[201], such as PR. Previously, we presented evidence for the requirement of FOXO1 for the binding of PR-B to DNA on a commonly regulated PR-B/FOXO1 gene, p21. Stable knock-down of FOXO1 reduced PR-B recruitment to the p21 promoter and ablated progestin-dependent upregulation of p21, thereby decreasing induction of senescence. A recent study evaluating the co-occupancy of FOXO1 and PR cistromes in decidualized primary human endometrial stromal cells confirmed the intimate crosstalk between FOXO1 and PR, as 75% of FOXO1 binding sites overlapped with PR binding sites.[202] PR binding enrichment on shared PR and FOXO1

genomic sites was decreased when FOXO1 was silenced, suggesting that chromatin accessibility for PR was lost and FOXO1 is required for PR binding to target genes.

Similar to breast studies, we suspect that PR isoforms in ovarian cancer models are also exquisitely sensitive to kinase inputs that may alter this biological outcome. Both PR and FOXO1 are tightly regulated by phosphorylation events. Hormone-driven breast and gynecologic cancers frequently exhibit upregulated protein kinases, such as MAPK[164], CDK2[203], and CK2[76], which directly phosphorylate and modulate PR-B target gene selectivity. Notably, the same kinases that are recruited to PR-B in "rapid" signaling (i.e. extra-nuclear) complexes (i.e. CDK2 and MAPK) also inhibit FOXO1 via regulation of specific phosphorylation sites that favor nuclear export.[204] Deregulation of FOXO1 is associated with tumorigenesis and cancer progression. FOXO1 is downregulated in several carcinomas, including ovarian[101], through alterations in upstream regulators, post-translational deregulation, or by genetic mutations.[121] Specifically, AKT-mediated serine/threonine phospho-regulation of FOXO1 is well-defined and prevents FOXO1 nuclear accumulation, thus impairing target gene regulation.[121] As mutations of PI3Ks or PTEN are common early events in cancer (particularly in breast, uterine, and ovarian cancers), activated AKT and other mitogenic protein kinases may prevent PR-induced senescence signaling by nuclear exclusion of FOXO1. Thus, the early loss or inactivation of FOXO1 may render PR "incompetent" at genes required for the induction of cellular

senescence, leading to the loss of protective “sensing” by progesterone in ovarian tumors. Whether these events may redirect PR to “alternate” genes that instead favor tumor progression are unknown and a topic for further study.

4.2 FUTURE DIRECTIONS

Other treatment regimens, such as hormonal therapy have been evaluated for ovarian cancer. The use of progestins alone (megestrol acetate and medroxyprogesterone acetate) as ovarian cancer therapies have been examined in several relatively small phase II clinical trials with variable inclusion criteria and modest response rates.[129] However, retrospective studies evaluating the association of total PR expression and progression-free disease survival[8, 11-18, 49] support the concept that subsets of PR-positive ovarian tumors are highly sensitive to hormones and thus more likely to respond to endocrine therapy. It is likely that the ovarian tumors that exhibited modest response rates to progestin therapy in the clinical trials were PR-positive and became senescent over the course of treatment evaluation. Since growth arrested senescent cells cannot further divide, but depend upon specific kinase-mediated signal transduction pathways for prolonged survival, this population of cells may be more vulnerable to subsequent therapies that inhibit mitogenic protein kinases and thereby promote apoptosis. We hypothesize that senescent cancer cells become “oncogene addicted” to protein kinase-initiated signaling pathways, and the combination of kinase inhibitors with progestin treatment may cause a dramatic

increase in cell death in PR+ cells. The induction of senescence followed by targeting key effectors of pro-survival to apoptosis in senescent cancer cells may improve the efficacy of both treatment strategies as a cancer therapy.

While elegant models have recently emerged [205, 206], knowledge gaps still exist. What are the best methods and experimental models to elucidate progesterone-specific effects in hormone-responsive tumors? While breast and ovarian cancers are diagnosed in both pre- and post-menopausal populations, a majority of the current cell-based models were originally established from post-menopausal patients. To understand steroid receptor actions, cells are treated with varying concentrations of exogenous hormones that may or may not reflect true physiological levels experienced in a pre-menopausal (cyclical hormone exposure) or post-menopausal (constant/low hormone exposure) context. Are the hormone concentrations used in the laboratory relevant to these contexts and thus to the biology of the tumors that arise? In addition, decreased PR expression is associated with progression of disease in breast and gynecologic cancers,[207, 208] whereas over 50% of acquired endocrine-resistant breast tumors retain PR expression.[209, 210] How do breast and other tumors lose PR expression and/or regain it during extended periods of endocrine (anti-estrogen therapy)? How should we model these changes? Concerning *in vitro* models, PR expression is often lost when primary isolates or immortalized cell lines are continuously cultured on 2D surfaces. The development of co-culture or 3D models may more accurately reflect *in vivo* cellular architecture relevant to

paracrine signaling and tumor biology[211] and will allow a more accurate characterization of the mechanisms and biological effects of hormone and antitumor treatments. Finally, routine detection and quantification of individual PR isoforms in clinical samples may provide valuable information as potentially distinct biomarkers of tumor behavior that could be used to further guide endocrine therapy.

Understanding how PRs function differentially in each normal and neoplastic tissue type will reveal how these highly modified receptors can be therapeutically targeted, perhaps as separate isoforms, to favor one biological outcome (growth inhibition, senescence, apoptosis) over another (proliferation, survival). Ultimately, in order to effectively manipulate PR action pharmacologically to treat tumors arising from different tissue types we must first appreciate their mechanistic complexity. Isoform-specific ligands as activators or inhibitors would be a valuable set of tools to accomplish this goal. In the current age of cancer genomics and personalized medicine, clinical readouts of PR-driven gene signatures may provide an additional means to discern context-dependent protective versus deleterious PR actions present in individual tissues and tumors.

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APPENDICES

Contributions by author in Chapter 2:

Caroline H. Diep:
- Identified, characterized, and validated PR induction of cellular senescence in ovarian cancer cell models (Figures 1C-E, 4A-B, 4D, 5D-F)
- Characterized and validated the induction of cell cycle mediators in a time- and ligand-dependent manner in cell line models (Figures 5A, 7B-D)
- Identified, characterized, and validated the mechanism of PR induction of FOXO1-dependent cellular senescence in ovarian cancer cell models (Figures 8-12)
- Experimental design of study; analysis and interpretation of the data
- Drafted and revised the publication for intellectual content

Nathan J. Charles:
- Generated the ES-2 GFP-PR model (Figure 2A)
- Identified the cellular senescence phenotype in ES-2 GFP-PR upon exposure to the progestin, R5020 (Figures 2B-D, 4C, 4E, 6)
- Identified the R5020-dependent upregulation of p21 expression in ES-2 GFP-PR model (Figures 5B-C)
- Generated the ES-2 sh-p21 model (Figure 7A)

C. Blake Gilks:
- Provided data of Estrogen Receptor (ER) and Progesterone Receptor (PR) expression in 504 human ovarian tumors (Figure 1A)

Steve E. Kalloger:
- Provided data of Estrogen Receptor (ER) and Progesterone Receptor (PR) expression in 504 human ovarian tumors (Figure 1A)

Peter A. Argenta:
- Provided materials for primary ovarian tumor study (Figure 6)
- Critical revision of the publication for intellectual content

Carol A. Lange:
- Conception and experimental design of study
- Analysis and interpretation of the data
- Critical revision of the publication for intellectual content

Contributions by author in Chapter 3:

Caroline H. Diep:
- Experimental design of study; analysis and interpretation of the data in Figures 1, 4-12.
- Drafted and revised the publication for intellectual content

Todd P. Knutson:
- Analysis and interpretation of the gene microarray data (Figure 2)
- Gene Ontology analysis of significantly regulated genes (Figure 3)
- Critical revision of the publication for intellectual content

Carol A. Lange:
- Conception and experimental design of study
- Analysis and interpretation of the data
- Critical revision of the publication for intellectual content