Progesterone and Breast Cancer: an NCI Workshop Report

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Introduction

The National Cancer Institute (NCI) held a workshop on “Progesterone & Breast Cancer” in June 2018 that was co-chaired by Drs. Carol Lange (University of Minnesota) and Seema Khan (Northwestern University). The overarching goal of the workshop was to bring together major players in the area to discuss the role of progesterone (P4) in the mammary gland and clarify controversies surrounding its activity in breast cancer. While it is known that adding a progesterone receptor (PR) ligand, either an agonist or an antagonist, in the presence of estrogen most often inhibits tumor growth in breast cancer cell models, P4 has also been shown to drive tumor heterogeneity and stem cell outgrowth. Furthermore, while P4 reduces tumor size in the early stages of disease, the sustained presence of P4

causes breast tumors to become more heterogeneous, and more plastic with an increased ability to migrate. Hence, the NCI workshop focused on addressing the role P4/PR plays in regulating multiple signaling pathways and the context in which PR acts to inhibit tumor growth versus its ability to drive tumor heterogeneity and stem cell growth. Below is a summary of the discussion among experts in the field who attended the workshop. Herein, we convey the content of the workshop followed by the major points of discussion with the permission of the attendees and identify areas for future studies on the topic of P4/PR in ER+ breast, endometrial, and other cancers that are influenced by P4.

PR Isoforms

PR isoforms have different binding sites in chromatin, and the coregulators associated with PR isoforms are different (1); more recent studies have expanded the repertoire of isoform-specific PR binding partners as measured by Rapid Immunoprecipitation Mass Spectrometry of Endogenous proteins (RIME) (2). Distinct but overlapping gene sets are regulated by two major PR isoforms which can form either homodimers or heterodimers at hormone-responsive elements in chromatin. PR-B (but not PR-A) primarily cross-talks with ER at estrogen-regulated genes via direct association as measured by co-IP and ChIP assays performed in human breast cancer cell lines and breast tumors (3), as well as in intact cells by proximity ligand assays (TH Truong and CA Lange unpublished data). The ER/PR interaction with c-Src kinase and hormone-dependent activation of downstream MAPKs was first defined by seminal studies in 1998 (4) and a role for ER/PR transcriptional complexes in the regulation of global gene expression was later demonstrated by gene profiling and RIME studies performed in breast cancer models (2, 3, 5).

It is not known if both PR isoforms A and B are equally expressed in basal-like cancer stem cells in human breast cancer specimens. However, PR-B is a more potent proliferative isoform (in the absence of PR-A), while PR-A (in the absence of PR-B) is a more potent driver of stem cell-like phenotypes (6). While both PR isoforms can drive the formation of secondary

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mammospheres (also called tumorspheres) that are CD44+/CD24-low and ALDH+ in vitro, PR-A+ breast cancer cell lines grown as spheres appear more basal-like (i.e., are more CD49f+), while PR-B+ breast cancer cell lines form fewer spheres that are ~30% larger and more luminal-like (CD49f-low) (6). Evidence suggests that the ratio of PR-A:PR-B is disrupted in early carcinogenesis; atypical ductal hyperplasia, DCIS, and invasive disease all have higher PR-A expression relative to PR-B (7–9). Imbalanced PR isoform expression is also linked to poor endocrine therapy response. For example, the trans ATAC (Arimidex, tamoxifen alone, or in combination) cohort microarray showed that PR-A predominance predicted resistance to tamoxifen, but not anastrozole (10).

Although IHC-based detection of PR-A relative to PR-B isoforms in clinical samples is challenging (see the “Discussion” section), recent studies performed by Rojas et al. (11) reported that luminal A tumors tend to be PR-A rich, while more aggressive/endocrine-resistant luminal B tumors are PR-B rich. PR-A predominance may reflect the more rapid ligand-dependent downregulation of activated PR-B and/or greater sumoylation and increased stability of PR-A (12). The majority of cancer cells within a PR+ breast tumor express both isoforms (13); a nuclear aggregated or focal pattern of PR expression (i.e., activated PR) was associated with higher tumor grade/more advanced disease. Of note, the Clarke lab (Sydney) has published that certain anti-PR antibodies that detect both PRA and PRB on Westerns were unable to detect PR-B (and were therefore PR-A-specific) on IHC/IF of FFPE tissue. However, uncertainty may arise in staining of samples that are not fixed and processed in the same way as human tissues handled in a routine clinical pathology setting (e.g., cells fixed on slides, mouse tissues, fixed but not processed in the same way). Thus, it is true that a subset of PR monoclonal antibodies recognizes only the PR-A isoform in archival FFPE tissue (14). Additionally, PR monoclonal antibodies may preferentially recognize PR-A isoforms (15, 16) and weakly recognize selected post-translationally modified PR isoforms in archival formalin-fixed paraffin-embedded (FFPE) breast cancer tissues (CA Lange, unpublished results).

PR Mutations

PR mutations in both the PR-B promoter and PR primary sequence have been described in the literature primarily for reproductive cancers. For example, *PGR* gene polymorphisms (namely +331G/A and V660L SNP or PROGINS) that alter PR isoform expression or activity are associated with increased endometrial and ovarian cancers, while effects on breast cancer risk appear modest (17–20). It should be noted that results reported in the greater literature are relatively inconsistent, suggesting that more study is needed, especially in light of newer deep-sequencing technologies.

PR splice variant receptors have been described that exhibit constitutive DNA-binding activity (12, 21). Similar to ER, PR mutations have been identified in metastatic breast cancers (S Fuqua, unpublished data). Dr. Fuqua’s team (Baylor College of Medicine, TX) has performed exon sequencing for all steroid receptors and their co-activators in paired primary tumors and metastatic lesions and found several novel as well as at least one previously published mutations in PR, namely the *PGR* haplotype known as PROGINS that results in a V660L substitution in the PR hinge (H) region that confers increased PR stability and expression (22). Both ER and PR expression varies widely in luminal breast cancer. LOH of the *PGR* gene locus occurs with high frequency in breast cancer, as does loss of ER (23). Thus, while approximately 70% of newly diagnosed luminal type breast tumors are ER+/PR+, about 40% and 25% of these tumors exhibit loss of heterozygosity (LOH) at either the *PGR* or *ESR1* gene locus, respectively (5); ER and PR LOH are positively correlated. Despite this genetic loss, ER and PR mRNA levels remain very similar to that of diploid luminal tumors suggesting that other compensatory factors may exist in these tumors to maintain some level of ER and PR expression.

Epigenetic Effects

Differential methylation of the *PGR* gene has also been reported. There are published studies showing that the PR-A promoter region was more frequently methylated (i.e., in a gene-repressed state) than the PR-B promoter region in TAM-resistant patients (24). This finding is inconsistent with reports of PR-A dominance in luminal breast cancers and may reflect a change in PR isoform expression that occurs during late stages of endocrine-resistant tumor progression.

PR Ligands

Both E2 and P4 are effective (direct or indirect) breast mitogens and breast cancer risk is closely related to a woman’s exposure to ovarian hormones (25). The role of PR and its ligands in breast cancer is also highly context-dependent. In terms of using P4 as opposed to synthetic progestins, researchers have used R5020 for two decades and have not observed any major differences between this synthetic progestin and P4 in their laboratories. R5020 is a more stable PR ligand relative to P4 since P4 is actively metabolized within the cell and R5020 is not. Deep sequencing studies have established that the effect of P4 is identical to that of R5020 (2). For the most part, P4 and progestins have similar biological effects except for medroxyprogesterone 17-acetate (MPA) which can also activate either GR (26) or AR (27) in biochemical assays conducted in vitro (as do many synthetic

antiprogestins). Many of the synthetic progestins used clinically have androgenic effects, including those used as oral contraceptives. There is also a complete overlap in the transcriptional profiles of the high-affinity PR ligand ORG2058 (16 α -ethyl-21-hydroxy-19-norprogesterone) versus P4 in T-47D breast cancer cells (28).

Data indicates that norgestrel is also a dangerous progestin (i.e., agonist). A Finnish study (29) carried out a retrospective analysis of women diagnosed with breast cancer and their use of intrauterine norgestrel preparation for the treatment of menorrhagia (i.e., heavy menstrual bleeding). The study found that women who had used a norgestrel-releasing device had a higher and dose-dependent increase in risk. The precise health record maintained in the country lends considerable power to the study. Another large Danish study involving 1.8 million women, followed on average for about 10.9 years, demonstrated that oral contraceptives containing synthetic progestins increased breast cancer risk (30); this is dependent on the type of progestin and duration of use. This study has major implications for the role of progestins in breast cancer and may, in fact, be in part due to the androgenic effects of synthetic progestins that are widely reported (31, 32). However, a recent meta-analysis of worldwide epidemiological evidence supports the finding of increased breast cancer risk for women taking any form of progestin-containing HRT (33).

PRs in Luminal and Lobular Breast Cancer

Invasive lobular breast cancer (ILC) is the most frequent histological subtype after invasive ductal carcinoma (IDC). Compared with IDC, ILC tend to be (1) larger at diagnosis, (2) difficult to diagnose by mammography, (3) metastasize to unusual sites like the ovary and gastrointestinal tract, (4) poorer long-term outcomes, and (5) more highly ER+ and PR+ (34, 35).

Several lines of evidence suggest that PR is a driver of ILC. First, a study of 144 ILC tumors examining gene expression identified two main subtypes: an immune-related subtype and a hormone-related subtype. The latter had high expression of PR and its downstream signaling pathway is the main driver of this subtype (36). Another study examined the risk of different histological subtypes of breast cancer in women using hormone replacement therapy containing estrogen alone or E + a synthetic progestin (P). The relative risk of ILC was higher in women taking E + P versus E alone (2.0 versus 1.4, respectively) (37). This indicates that progestins specifically increase the risk of ILC. Whether oral contraceptives increase the risk of ILC in women is controversial, however, the above-mentioned Danish study shows increased risk of breast cancer in women using contraceptives containing progestin (30).

To date, all studies examining PR function in breast cancer have used IDC models. Two ILC cell lines (BCK4 and MDA-MB-134) that are ER+/PR+ have been

used to study the function of PR in ILC where P4 increased estrogen-stimulated growth in vivo as measured by tumor volume and Ki67. Unlike in IDC, PR was not downregulated by P4 either in vitro or in vivo in models of ILC (37). P4 also influenced the organ sites to which ILC cells metastasized, as compared to IDC ((38); Britta Jacobsen, unpublished results). These studies reveal clear differences in biology between ILC and IDC.

Researchers have developed elegant breast cancer patient-derived xenograft (PDX) models to test progestin therapies and mechanisms of ER or PR action (39, 40). ER+ PDX grown in Nod/SCID mice have a 20% take rate, and all require E2 to grow. Addition of P4 + E2 is typically inhibitory to PDX growth (39, 41). Single-cell RNAseq to segregate high PR-expressing from low PR-expressing cells demonstrated that most of the cells were of the luminal A subtype (C Sartorius, unpublished results). This is interesting because a common criticism of the PDX models is that they do not represent luminal A tumors. This may be a product of sequencing type and tumor heterogeneity. It also raises the issue as to whether the effects of progestins and/or anti-progestins are effective in the context of endocrine resistance? In the intraductal model, ER+ PDX grown in NOD/SCID mice have a 100% take rate and all of them grow without E2 supplementation (42) and the models reflect the biology of their clinical counterparts (43).

Stem Cells

PR-A is a potent driver of stem cell expansion in breast cancer models relative to PR-B, while of the two isoforms, is a stronger driver of proliferation in soft agar (6). These recently published data demonstrated that phosphorylation of PR Ser294 is required for breast cancer stem cell outgrowth as measured in tumorsphere assays and using a variety of breast cancer stem cell markers including CD44+/CD24- and ALDH (6). PR Ser294 phosphorylation is elevated when the same cells are subjected to 3D relative to 2D culture, as measured using specific antibodies and flow cytometry of permeabilized cells to detect minority PR+ subpopulations (AR Dwyer, TH Truong, and CA Lange, unpublished results). PR Ser294 phosphorylation is mediated by either P4 or growth factor (i.e., EGF) induced activation of MAPKs. Exogenously added P4 is not required for tumorsphere (mammosphere) formation (i.e., number) but contributes to increased tumorsphere size in defined media (6). While the combination of E2 + P4 inhibited cell growth in soft agar, these hormones (alone or in combination) have no effect on secondary tumorsphere formation or the expression of stem cell markers (i.e., the ALDH+ and CD44+/CD24-low cell populations). While it is important to note that mammosphere media contains trace amounts of P4, this finding is relevant to postmenopausal women since both estrogen and P4 levels are low (i.e., women with ER+ breast

cancer typically undergoes hormone-ablation). This contrasts with what is observed during pregnancy when levels of both hormones are high.

In primary cultures of normal breast epithelia, P4 stimulates cell growth by increasing progenitors. Primary breast cultures were sorted for enriched populations and it was found that PR transcripts were higher in the bipotent progenitor-enriched compartment (44). Furthermore, P4 increases stem and luminal compartment, but decreases basal compartment, as measured by gene signature. Interestingly, while E2 also increases stem/luminal lineages and stem MetaPCNA index, combining E2 + P4 attenuates expansion of the stem cell compartment in cultures of normal breast epithelia (D Graham, unpublished results).

Both RANKL and Wnt4 are essential mediators of PR signaling during mammary gland development. Elegant data links Wnt4/RANKL and PR to stem cell signaling (45, 46). PR knockout mice form a ductal tree but no side branching; Wnt4 is required for side branching. Stem cell function can be assessed by serial transplantation into cleared mammary fat pad for seven generations. PR knock-out cells were only efficient to the first generation and Wnt4 knock-out cells were even less efficient than PR^{-/-}, while RANKL^{-/-} do not exhibit any alterations in mammary gland growth, side branching is inhibited. This was also seen in ex vivo experiments with tissue microstructures treated with P4 alone (46). Similarly, blocking RANKL signaling reduced P4-driven carcinogenesis in vivo, specifically in the early stages of tumorigenesis (47). P4 dramatically increased mammary stem and progenitor cell activity in the adult mammary gland (48). Further, RANK^{-/-} mice or denosumab-treated mice do not exhibit P4-induced mammary stem/progenitor cell expansion. They also fail to elicit a mammary Wnt response (47).

Dosage and Route of Administration

The dosage and route of P4 administration are very important and unfortunately, not always considered. Since the natural hormone, progesterone, is rapidly metabolized, researchers prefer to use R5020, a long-lived synthetic progestin that may provide more reproducible results.

Another key element of experimental design is the use of low- vs. high-dose P4 or progestins in in vitro studies—low-dose P4 can be proliferative and activate PRs, while at a higher dose P4 is inhibitory and may inhibit PRs or cause their rapid downregulation/degradation. This phenomenon is true for all steroid hormone receptors and was the reason for the early use of high-dose DES or testosterone to treat breast or prostate cancer respectively, prior to the routine use of the appropriate anti-hormones for these cancers. It is thus difficult to interpret studies that exceed the range of 1 nM E2 and 1–10 nM P4 (or R5020).

Hormone Replacement Therapy

An important fact that needs to be considered is that testosterone levels can vary among women. There is data to suggest that there is a difference in the androgenic hormone levels between premenopausal and postmenopausal women and the impact of P4 is influenced by the hormonal milieu.

The French E3N cohort study demonstrated an unequal risk for breast cancer associated with different postmenopausal hormonal therapy (49); there was an increased incidence seen in women undergoing hormone replacement therapy (HRT) that contained any synthetic progestin, but not natural P4. A caveat to this study was that increased duration of exposure to E2 + natural P4 also showed a trend towards increased BC incidence (49). A limitation of the study as stated by the investigators themselves was that the study was statistically underpowered.

There is no strong epidemiological data showing association between hormone (P4) exposure and breast cancer outcomes. However, P4 has a protective role for ovarian and endometrial cancers. Conversely, local hormone delivery (Mirena IUD) to fallopian tube results in increased proliferation of ovarian epithelial cells, similar to the situation in an ovulating woman, indicating that they may still have an increased risk of ovarian cancer (comments of Malcolm Pike). Both E and P4 are greatly elevated in BRCA mutation carriers, especially P4, due to a defect in hormone biosynthesis within the ovaries of germline BRCA mutation carriers (50). This is true in humans and has been recapitulated in BRCA-mutant mouse models (51).

Progesterone Levels

Epidemiological data in postmenopausal women have indicated that P4 levels are low in post-menopausal women. However, this is based on data collected from a small sample size and there have been questions raised regarding the accuracy of the antiquated assays that were used. The role of circulating hormone levels relative to local or intratumoral (i.e., autocrine) hormone levels in disease progression remains controversial (i.e., few studies have measured P4). However, one needs to acknowledge that the predominant source of circulating P4 in non-pregnant women is cyclical production by the corpus luteum, no longer active after menopause. Although it may be that the production of P4 by the postmenopausal adrenal gland has been underestimated. In addition, while either E2 or P4 alone are proliferative, the fluctuating high levels of P4 (as ovulation ceases and estrogen levels drop) during perimenopause have the potential to drive the proliferation of existing lesions in the breast (comments of J Dinny Graham).

PR and Immune System

Dr. Christy Hagan's lab (University of Kansas Medical Center, KS) showed that PR promotes an immunosuppressive environment through downregulation of interferon signaling. PR and STAT1 interact; PR-mediated inhibition of interferon signaling may provide a mechanism for ER+/PR+ tumors to escape from immune surveillance (52). Further analysis demonstrated that interferon gene sets are downregulated by PR via the activation of STAT1. PR+ breast tumors show decreased expression of interferon-stimulated genes. R5020 attenuates phosphorylated STAT1 (pSTAT1) levels, suggesting that PR downregulates inputs to pSTAT1. The interaction between PR and STAT1 was robust, but not isoform-specific.

Antibodies

Christine Clarke's group (University of Sydney) has characterized PR-A and PR-B directed antibodies in human tissue and confirmed their isoform-specific detection using cell lines that express either PR-A or PR-B alone (15). However, other groups (Dean Edwards, Carol Sartorius) have been unable to consistently reproduce these findings, and this may be explained by differences in IHC staining protocol, tissue fixation, and even isolation/age of patient tumor samples. There is also some frustration that some of the existing antibodies do not work ideally for a wide variety of assays (comments of Carol Sartorius). The detection of PR isoforms in tissues remains a challenging and controversial area of study.

Innovative Technology

Recent advances in technology offer new opportunities to define the molecular status of steroid receptor complexes, particularly DNA bound forms and the potential to impact the clinical translation of these findings. Dr. Gordon Hager and colleagues (National Institutes of Health, MD) employ a technique known as the number and brightness (N&B) method that allows the quantification of the oligomeric state of proteins in living cells (53). N&B provides a read-out of the size of the molecule that moves through the focus of the microscope. These experiments show that PRs, glucocorticoid receptor (GR), and androgen receptor all adopt multimeric structures when examined in living cells (54). Findings with GR are particularly interesting. GR transitions from a dimeric state to a tetrameric state upon binding to glucocorticoid response elements. Using a mutation that mimics the DNA bound state, it was subsequently shown that the tetrameric state confers on the receptor the ability to access or "pioneer" into closed chromatin, thus activating response elements not available to the dimeric receptor (55). In aggregate, these results open a new

chapter in the biology of steroid receptors, raising serious questions concerning the standard homo- or heterodimeric receptor model that has prevailed for more than 30 years.

Dr. Teresa Woodruff (Northwestern University, IL) and her collaborators have innovated the use of a tissue chip *in vitro* model, called the EVATAR, which uses 3D reproductive tissues and a microfluidic system to recapitulate hormonal changes during the human menstrual cycle over 28 days (56). Tissues from the ovary, fallopian tube, uterus, cervix, and liver were connected in a microfluidic system allowing for the dynamic sharing of media and paracrine factors. The pituitary influence was achieved by manual addition of follicle stimulating hormone and luteinizing hormone receptor ligand. The microfluidics system promoted viability of the 3D tissue-mimics in culture, as fresh nutrients flowed in and waste products were eliminated. Microfluidics also allowed for sharing of paracrine factors between tissues that preserved the biology of each tissue in a systematic fashion. EVATAR was originally designed to test drugs to aid in predicting success in clinical trials but can also be used for research purposes when studying endocrine mechanisms of action of hormones and compounds in multi-organ systems that require long-term treatment times (comments of Julie Kim).

New Models to Study Progesterone Action in Ductal Carcinoma In Situ

Dr. Dean Edwards presented a ductal carcinoma in situ (DCIS) model using MCF10 DCIS.COM cells engineered with lentiviral vectors to stably express ERalpha, PR-A, and PR-B. Based on gene expression profiling, these cells resemble luminal breast cancer subtypes and are robustly responsive in cell culture to either estrogen or progesterone in terms of regulation of known target genes (57). The cells have also been used successfully in the mouse mammary intraductal xenograft (MIND) system and are responsive to estrogen and progesterone *in vivo*. In the MIND system, these cells form pure DCIS lesions with histopathological characteristics similar to human disease and eventually undergo breakdown of myoepithelium and basement membrane and invade into stroma. Combined E2 and P4 treatment stimulated up-regulation of a NEMO/NF- κ B/IL-6 pro-inflammatory pathway in these cells as xenografts in the MIND system. Furthermore, this pathway maintained expression of the PML tumor suppressor, implicating NEMO as a potential tumor suppressor regulated by E2 and P4 in the transition of DCIS to invasive cancer (58). These ER/PR+ DCIS.COM cell lines appear to be a physiologically relevant model to study molecular mechanisms and the role of progesterone in the transition of DCIS to invasive breast cancer which is largely unknown (59).

Endometrial Cancer

PR is protective of E2-induced endometrial hyperplasia and cancer. Thus, another important area of research is the response of endometrial hyperplasia and cancer to P4 or progestin therapy (comments of Sanaz Memarzadeh). In the case of PTEN null endometrial tumors, wherein PTEN was only lost in the endometrial epithelia, the tumors were hormone-sensitive and had abundant expression of stromal PR. Stromal deletion of PR as a single genetic change in these tumors induced P4 resistance indicating that paracrine signaling through the stroma was essential for the P4-induced therapeutic effects (60). Work by many investigators has shown that levonorgestrel (LVG) can be an appropriate therapy for localized endometrial hyperplasia and tumors (60). However, patients presenting with metastatic disease are not good candidates for treatment with an IUD and systemic therapy with P4 can be considered. A clinical challenge is that reliable predictors of response to P4 therapy are not readily available and need to be explored more extensively. The expression of PR in the stroma may serve as a discriminatory biomarker of P4 sensitivity in endometrial hyperplasia based on analysis of a small cohort of patients (60) but needs to be validated in a larger cohort.

Discussion

Below are additional discussion points from workshop attendees that have been captured verbatim or revised by the speaker contributors herein.

Epidemiology

There is speculation about the mechanism of action of MPA in the Women's Health Initiative and Million Women's studies. Is the effect of MPA in altering ER signaling accomplished via its agonistic effect on AR in normal breast tissue to increase breast cancer risk? This is an area that requires further examination. While the Women's Health Initiative and Million Women Study found a significantly increased risk with MPA and other synthetic progestins (61–63), two studies in France and Finland reported no association between use of the natural hormone P4 and breast cancer risk (29, 49). It appears that the type of progestin, dose, and patient age seem to all affect the risk and we do not know enough yet.

The postmenopausal estrogen/progestin intervention (PEPI) study shows that P4 at 200 mg per day did increase density although not as much as MPA (64). However, use of a 200 mg dose of P4 may not prevent endometrial cancers, while 200 mg was effective in decreasing endometrial proliferation (65, 66), the studies that use “micronized” P4 reported that it was not protective. The “micronized” form needs to be

absorbed/has low bioavailability relative to other forms (i.e., of PR agonists) which is a whole different story (i.e., a further complication with regard to sources of variability and data interpretation (67)).

Estrogen replacement therapy is very similar to estrogen plus P4 therapy—this is because the P4 dose is too low; the low dose was chosen as it prevents endometrial hyperplasia. However, endometrial hyperplasia is not a good model. This tissue can exhibit high levels of cell proliferation without hyperplasia; we also have strong evidence of no hyperplasia in E2-treated premenopausal woman. Most PR researchers like the idea of using P4 (i.e., progesterone). Related to this topic, a recent meta-analysis of the worldwide epidemiological evidence reports findings of an increased breast cancer risk in women taking menopausal hormone therapy (i.e., as E2 alone, E2 with daily P4, or E2 with intermittent P4); risk increased with each preparation, but varied by type of preparation and duration of use (33).

PR Antibodies

Dr. Greene's group has successfully used the commercially available KD68 antibody for ChIP analyses. Santa Cruz replacement antibody (F4) was also used successfully in Lange lab for ChIP, but this antibody favors PR-A isoform over PR-B. There was consensus that there should be greater attention paid to validating the antibodies and publishing details regarding the protocols used so that it is reproducible in different laboratories.

Dr. Khokha indicated that they had issues when they tried the commercially available RANK antibody. In addition, RANKL mRNA is difficult to measure.

Dr. Sartorius: We have used Thermo AB-8 and other of the Graham/Clarke antibodies for ChIP with success. There are production and consistency problems with the Thermo antibodies, so it has been challenging.

Hormone Replacement Therapy

Dr. Lange: The progesterone or progestin effect is influenced by the model being used. While in one ER+/PR+ model (BT474 cells) onapristone is very effective, mifepristone (RU486) does little to nothing. This can be flipped in another model such as MCF-7 or T47D cells and is also dependent on the assay type or culture system (i.e. using 2D vs. 3D conditions) (CA Lange lab, unpublished data). Relevant to this discussion, ER and PR-B (but not PR-A) directly interact in co-IP or ChIP assays and E2 stimulates a number of genes only in PR-B+ (but

not PR-null or PR-A+) cells (3). We have observed that phosphorylation of PR Ser294 is important for the ER/PR-B interaction (TH Truong and CA Lange, unpublished data). Thus, selected antiprogesterin agents (such as onapristone or PRA-027) that block Ser294 phosphorylation may inhibit this interaction while others (RU486) may actually stimulate ER/PR binding. We also see stronger effects with agents (onapristone) in cell lines with high ER expression relative to cell lines with lower ER levels, suggesting that this agent/agents in this class are working in part via altering/disrupting PR cross talk with ER rather than by strictly inhibiting PR.

Dr. Rui: Going back to HRT data and the impact of chronic progestin, it is believed that the stem cells that express PR, and are believed to be the tumor initiating cells, are the target. However, it appears that progestins have an impact in only some women. Is it because the cells are heterogeneous?

Dr. Graham: That is the implication. We need to understand what the transcriptional data are telling us because PR is expressed in basal cells, but the expression is lower than in luminal cells. I think the expression of PR is real, is not an artifact. The staining is clean. The other evidence we have is the P4 effect on cellular proliferation.

Dr. Khokha: We have seen positivity in basal cells and it is very real.

Effect of Progesterone on Stem Cells

Most of the data so far suggest CSCs are ER and PR negative—or become that way once exposed to hormones. There are also differences in mice vs. humans (in humans, it appears that some basal progenitor cells are PR+). We also have to be careful distinguishing normal from CSCs.

Dr. Rui: I have a question for you Dinny. You used the terminology – “progesterone pushing cells into the stem cell phenotype”. I wasn’t clear if it was proliferative expansion or reprogramming?

Dr. Graham: We do not know yet. The treatment was done for 14 hours only; hence it is hard to believe it is the result of proliferation and suggests that the increase must at least in part reflect reprogramming, since what we are seeing is an increase in the proportion of cells expressing a stem/progenitor gene signature. If we extend the time we would see more. We need to do more work to understand why that is happening.

Dr. Rui: CK5-positive cancer cells have ‘progenitor-like’ features, for progesterone to stimulate their expansion, we imagine that is more reprogramming than proliferative expansion. Do you have a comment on that, Carol?

Dr. Sartorius: It’s hard to measure. The closest we got is we seeded single cells labeled with a CK5 promoter-GFP reporter then added progesterone. Some cells turned green when alone, but one could argue that in those cells an autocrine feedback loop was initiated, but we cannot know for sure unless we are able to immunostain the single cell for CK5 - it is difficult to go back and stain a single cell for that.

Dr. Graham: Our primary embedded 3D model of normal breast tends to support progenitors, because the culture model supports the growth and formation of new structures, and when we stain the structures there is a high level of CK5 positivity. This can mean that measuring CK5 in the model is not very informative. However, my main point was that we tend to see quite a high proportion of CK5 positivity in our model, which likely reflects the fact that the structures formed actively proliferate, unlike the majority of other primary normal breast in vitro models, where it is very difficult to see a proliferation effect because the samples do not actually proliferate. Our model supports proliferation of these structures, we have higher than normal proliferative components, CK5 expression is seen in a lot of cells in the culture, which is different than resting tissue. The size of the progenitor population is also greater than you would expect to see in a tissue sample that has not been in culture. We did not see progesterone upregulating CK5 in any of our clusters but would have to go back and look. I have checked in our data and confirm we do not see up regulation of CK5 in progenitor populations by P. However, we do see down regulation in the mature luminal population.

Dr. Khokha: What was striking from our global mammary cell profiling data (68), and we do not know what it means, but ATAC-seq and methylome data on primary cells shows that PR in basal compartment sits in ‘open chromatin’ region. This dataset is quite interesting, I wonder if people who are experts could examine it. Another piece of data looked at lineage-specific markers generated as a module. We look at module for methylation pattern, transcription, proteomes, and you see interesting shifts that occur in individual markers after progesterone stimulation.

When treated with P4, while it may be true that breast tumor cell proliferation is inhibited, the fact is that there is an increase in the stem cell population in these tumors, which can be dangerous.

Dr. Sartorius: There is an expansion of stem cells with every cycle – so is there a cumulative increase in the number of stem cells over a woman's life? If we could measure the number of bona fide stem cells over age, one might predict it would cumulatively increase.

Dr. Khokha: There is not an easy way to answer that, because those kinds of experiments are not easy to do. The cells turnover, but what we did do is we looked at it in wild type mice in relation to age. In relation to other models, we have calculated in wild type mice the ones that have aged for 18 months and have tried to see how the different cell fractions changed. There is not a lot of net increase in total number of cells. It seems like they are expanding and then reverting to baseline.

Consensus on Experimental Conditions for the Study of PR Actions

Several researchers emphasized that under physiologically relevant conditions, a woman is never exposed to progesterone alone. Even in postmenopausal women, there are always endogenous estrogens present. Hence, any experiment that involves studying progesterone must be done in the presence of estrogen.

Dr. Sartorius: There was nothing happening in the breast cells, so after that we got rid of progesterone alone. We just use estrogen or progesterone and estrogen. That is why it is troubling to see titles talking about cancers being affected by estrogen alone and not progesterone. Dinny Graham's work supported this, showing that unopposed E2 stimulates proliferation but when P4 is also present the proliferative influence decreased. This is consistent with the cyclical pattern of proliferation in the normal breast through the menstrual cycle.

Dr. Lange: A reviewer at minimum should have asked to see the level of PR in that model. In a mouse model, did those mice have decent PR expression in the tissue to make that conclusion? At least for cells, we show the Western blots for both ER and PR. We used to call it priming, you prime with Estrogen then you get PR activity. It is a confounding thing to put all the hormones together. You have to do everything (i.e. each alone and then together). In addition, I would stress that if one is studying ligand-independent actions of either ER or PR, obviously the studies are completed in the absence of added hormones as an experimental variable, and compared to plus hormones (thus the study designs that exclude added

hormones are relevant to trying to understand how growth factors may activate or otherwise regulate steroid hormone receptors in little to no ligand, for example and controlled by showing the plus hormone condition).

Dr. Sartorius: When doing in vitro experiments, each of these conditions can be measured. One can add estrogen, progesterone, and combination. In vivo experiments almost always need estrogen. However, we then get criticized if we do not have a progesterone alone group, despite that most tumors do not then grow. It is confounding.

Dr. Lange: Those not familiar with the field ask why a Progesterone alone study was not done. The confusing area for me is that I do not think we really reached any consensus on adding estrogen to experiments on PRs or not - I think we do need to include all conditions so we can interpret the data and see what happens in each condition – but because at least in my hands and also Claudia Lanari's FGF2 work - we are seeing much ligand-independent ER and PR actions that are phosphorylation dependent - in which ER can act as a binding partner scaffold of liganded PR (Myc expression), and vice versa - PR and act as a scaffold for ligand-bound ER (CTSD gene) - we and the Lanari group see both ways - some genes respond to only one ligand - others need both - some only need a growth factor (EGF or FGF from stroma)- I think hormone-ablated women model the no/low hormone and high signaling or growth factor state so these experimental conditions are relevant to that - it is remarkable to me to see what these receptors can do without exogenous hormone - amazing really - they want to be on - and growth factors can sub during cancer contexts – at least growth factors very much lower the concentration of ligand needed to activate PR (i.e. 4-5 orders of magnitude lower when some EGF is around).

Critical Questions That Need to Be Addressed in the PR Field

Can the effects of a progesterone antagonist be reversed with an agonist?

Dr. Graham: Several mechanistic studies from the 1980s and 1990s have demonstrated that the ability to overcome antagonism was dependent on the strength of the binding affinity and relative dose. I think it's clear that antagonism can be overcome if there is the right context. For antagonists to really oppose strong synthetic agonists, they have to be present at 100-fold higher concentration than the agonist. So, I think the answer is yes, depending on antagonist dose, agonist can oppose its effects, but whether they will completely reverse the antagonism is concentration-

dependent, since at very high dose agonists will behave more like antagonist themselves.

Tools That the PR Research Community Needs for Progress

The scientific community engaged in P/PR research does not have the tools they need. There are good synthetic progesterone receptor agonists and antagonists but at present they are not easily available to everyone. Dr. McDonnell (Duke University, NC) offered that they would be willing to make these reagents for researchers. However, he stressed the need for a group that would work on validating the progestins.

Reagents/cell lines needed: There are very few cell line models to study invasive lobular cancer (ILC). Of the seven that are available, three are ER+ and two are ER+/PR+. There is a need for further studies to understand why P4/PR effects on proliferation *in vivo* are different in ILC and IDC. PR isoforms should also be considered in ILC. There is a need for more PR+ cell lines since MCF7 cells make very little PR even in the presence of estrogen.

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