Title:

Progestin-mediated activation of MAPK and AKT in nuclear progesterone receptor negative breast epithelial cells: the role of membrane progesterone receptors

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Abstract

Progesterone (P4), a steroid produced during estrous cycles and gestation for maintenance of pregnancy, also plays key roles in breast development to allow lactation post-parturition. Progestins (P4 and related steroids) are also implicated in breast cancer etiology. Hormone replacement therapy containing both estrogen and progestins increases breast cancer incidence while estrogen hormone therapy lowers breast cancer risk. P4 signaling via nuclear P4 receptors (PRs) has been extensively studied in breast cancer, however, progestin signaling via non-classical membrane bound progestin receptors (MPRs and PGRMC1) remains unclear. Moreover, P4 metabolites and synthetic progestins may bind membrane progestin receptors. We hypothesized that PR-negative breast epithelial cells express non-classical progestin receptors, which activate intracellular signaling pathways differently depending on nature of progestin. Therefore, our objectives for the current study were to determine expression of MPRs and PGRMC1 in two PR-negative non-tumorigenic breast epithelial cell lines, assess progestin-mediated signaling and biological functions. We determined five MPR isoforms and PGRMC1 were present in MCF10A cells and all progestin receptors but MPR β in MCF12A cells. MCF10A and MCF12A cells were treated with P4, select P4 metabolites (5 α P and 3 α HP), medroxyprogesterone acetate (MPA), or a specific MPR-Agonist (MPR-Ag) and phosphorylation of ERK, p38, JNK, and AKT was characterized following treatment. To our knowledge this is the first report of ERK and JNK activation in MCF10A and MCF12A cells with P4, P4 metabolites, MPA, and MPR-Ag. Activation of ERK and JNK in cells treated with MPR-Ag implicates MPRs may serve as the receptors responsible for their activation. In contrast, p38 activation varied with cell type and with progestin treatment. P4 and MPA promoted AKT phosphorylation in the MCF12A cell line only whereas no activation was observed in MCF10A cells. Interestingly, cellular proliferation increased in MCF10A cells treated with MPA or 5 α P, while MPR-Ag tended to slightly decrease proliferation. Collectively, our data highlights the importance of investigating the effects of synthetic progestins in breast cancer biology. Our results add to the understanding that various progestins have on breast epithelial cells and underscores the importance of considering both membrane bound receptors and progestin type in breast cancer development.

Keywords

Medroxyprogesterone acetate, membrane progesterone receptors, breast cancer, 5 α -dihydroprogesterone, 3 α -dihydroprogesterone

1. Introduction

Progesterone (P4) is a steroid produced predominantly by the corpus luteum during estrous cycles and pregnancy, as well as the placenta during gestation. P4 elicits a number of different biological responses in females principally associated with establishment and maintenance of pregnancy, influencing several target tissues. In breasts, P4 promotes formation of lobular-alveolar structures during pregnancy, regulating normal breast physiology (Topper and Freeman, 1980). Specifically, P4

stimulates cell proliferation through regulation of cell cycle genes, synthesis of growth factors, and growth factor receptors; during lactation, P4 stimulates differentiation. Progestins (P4 and related steroids) are also implicated in breast cancer etiology. The Women's Health Initiative randomized controlled trials of combined estrogen and progestin hormone therapy (CHT) and unopposed estrogen hormone therapy (EHT) clearly demonstrates CHT use increases breast cancer risk while EHT lowers risk (Rossouw, Anderson, Prentice et al., 2002, Anderson, Limacher, Assaf et al., 2004). Schairer and colleagues demonstrated breast cancer risk increased 8% per year in women taking CHT compared to 1% per year in women receiving estrogens alone in a study conducted with 46,355 women (Schairer, Lubin, Troisi et al., 2000). Many similar, large cohort studies have confirmed that combined estrogen-progestin treatment increases breast cancer risk compared to estrogen alone (Li, Weiss, Stanford et al., 2000, Persson, Weiderpass, Bergkvist et al., 1999, Ross, Paganini-Hill, Wan et al., 2000). One of the largest studies conducted, the "Million Women Study" involving 1,084,110 women (50-64 years old) in the United Kingdom revealed an increased risk of incidence and fatal breast cancer for women taking CHT compared to women using EHT (Bliss and Gray, 2003). These studies strongly indicate progestins play crucial roles in breast cancer etiology and progression.

Conflicting reports suggest progestins either induce or inhibit cell death in breast cancer cells (Formby and Wiley, 1998, Kandouz, Lombet, Perrot et al., 1999, Moore, Spence, Kiningham et al., 2006, Ory, Lebeau, Levalois et al., 2001). Fundamentally, progestins must bind and activate specific receptors to cause biological responses. Historically, most actions attributed to progestins involve binding to the nuclear progestin receptors (PRs), known as (PR-A) or (PR-B); research focused on nuclear receptors has yielded critical biological information. However, substantial evidence of progestin-initiated events in PR-negative cells exists. Progestins also bind and activate distinct membrane bound proteins such as the membrane P4 receptors (MPRs), which have 7-8 transmembrane domains and P4 receptor membrane component-1 (PGRMC1), which possesses a single transmembrane domain (Peluso, Romak and Liu, 2008, Thomas, 2008, Zhu, Bond and Thomas, 2003). The MPRs are members of the progestin and adipoQ receptor (PAQR) family and consist of 5 different subtypes (MPR α , - β , - γ , - δ , - ϵ) (Pang, Dong and Thomas, 2013, Tang, Hu, Arterburn et al., 2005, Thomas, Pang, Dong et al., 2007). The existence of non-classical P4 receptors (MPRs and PGRMC1), distinctive in protein sequence, function, and localization from PRs, increases the complexity of progestins signaling in breast cells and demands further investigation into progestins and breast cancer biology.

A notable attribute of mammary tissue and breast cancer cells is their ability to convert P4 to biologically active metabolites 5 α -dihydroprogesterone (5 α P) and 3 α -dihydroprogesterone (3 α HP) that cause opposing actions on cell proliferation and apoptosis (Wiebe, 2006, Wiebe, Beausoleil, Zhang et al., 2010, Wiebe, Muzia, Hu et al., 2000) in cells lacking PR (Weiler and Wiebe, 2000). Cell proliferation is increased by 5 α P and decreased by 3 α HP, (Wiebe, 2006, Wiebe et al., 2000); while apoptosis is stimulated by 3 α HP with decreased Bcl2/Bax ratio while this ratio increases in 5 α P treated breast cells (Wiebe et al., 2010). Notably, the progestin metabolites initiate cell

signaling via receptors located in the plasma membrane and not through classical PRs (Weiler and Wiebe, 2000) yet identification of membrane receptors for progestin metabolites has not been revealed. Many conclusions based upon progestins signaling in breast cancer cells were made without the knowledge that MPRs and PGRMC1 existed or that P4 metabolites are biologically active. The lack of consistent findings with respect to progestins in breast cancer biology may in part be attributed to (1) the presence of different progestin receptors and (2) the potential biotransformation of P4 to bioactive metabolites, yielding varied cellular responses.

Limited studies suggest normal breast epithelial cells increase proliferation in response to synthetic but not natural progestins. In vitro experiments investigating the role of P4 regulating breast epithelial cell proliferation have produced inconsistent results, as reviewed by Clarke and Sutherland (Clarke and Sutherland, 1990). P4 either decreases or has no effect on proliferation of normal breast epithelium explanted into nude mice (Laidlaw, Clarke, Howell et al., 1995, McManus and Welsch, 1984). Treatment of MCF10A cells, PR-negative benign breast epithelial cells, with the CHT P4-derivative medroxyprogesterone acetate (MPA) with growth factors increased proliferation whereas P4 had no effect (Kramer, Seeger, Kramer et al., 2006, Kramer, Seeger, Kramer et al., 2006). These data suggest differences in the type of progestins may account for the discrepancies noted in cell proliferation of benign human breast epithelial cells. Indeed, the pharmacological properties of progestins vary depending on their parent compound in which they were synthesized from, leading to disparities in biological activities (Schindler, Campagnoli, Druckmann et al., 2003, Stanczyk, 2003). MPA is often used in contraception and CHT. Intriguingly, increased risk of breast cancer in women taking CHT depends on the type of progestin used, as women taking natural P4 had no statistically significant increase in breast cancer risk whereas an increased risk was noted in women taking synthetic progestins like MPA (Fournier, Berrino and Clavel-Chapelon, 2008, Fournier, Berrino, Riboli et al., 2005).

Overall, the functions of progestins on breast cells appear to depend on progestins present and receptors expressed. We hypothesize that PR-negative non-tumorigenic breast epithelial cells express non-classical progestin receptors, which function differently depending on nature of progestin. Therefore, the objectives of this study were to examine MPRs and PGRMC1 in two PR-negative non-tumorigenic breast epithelial cell lines and assess signaling, and biological functions using P4, MPA, P4 metabolites and a MPR agonist (MPR-Ag). In this study we used MCF10A and MCF12A cell lines to analyze the effects of P4, MPA, 5 α P, 3 α HP, and MPR-Ag on ERK, p38, JNK and AKT signaling and cell proliferation. Treatment with progestins resulted in varying levels of kinase activation depending on cell line and progestin. Cell proliferation was stimulated by MPA and 5 α P in MCF10A cells, while MCF12A cells did not differ in cell proliferation with any progestin treatment. These results highlight the importance of elucidating progestin function in benign breast epithelial cells. Because natural P4, its metabolites, and synthetic progestins can initiate different biological functions, these data may have relevant clinical implications as well as advancing understanding of progestin functions in breast cancer biology.

2. Materials Methods

2.1. Cell Culture: MCF12A and MCF10A human epithelial breast cells were purchased from ATCC. Cells were maintained in a humidified incubator at 37°C with 5% CO₂ in DMEM: F12 media (Life Technologies, Grand Island, NY) containing 100 units/mL penicillin/streptomycin (Life Technologies), 5% horse serum (Life Technologies), 20 ng/mL of epidermal growth factor (Life Technologies), 0.1 µg /mL cholera toxin (Sigma Aldrich, Saint Louis, MO), 10 µg /mL insulin (Sigma Aldrich, Saint Louis, MO), and 0.5 µg /mL hydrocortisone (Sigma Aldrich). Experiments were conducted in cells that were under passage 14 for all studies. For protein phosphorylation experiments, 500,000 cells were plated per 60 mm tissue culture dish in complete medium. Twenty-four hours after plating, cells were changed to serum-free medium and incubated for another 24 h prior to treatment. Treatments included 100 nM P4, 100 nM MPA, 1000 nM 3αHP, 1000 nM 5αP, 100 nM MPR-Ag (Org OD-02-0) or vehicle for 5, 10, 30, 60, or 120 min. To confirm progestin-induced phosphorylation of ERK and p38, cells were treated for 2h in the presence of ERK inhibitor (50 µM U0126; Sigma) or p38 inhibitor (50 µM SB203580; Sigma) prior to progestins treatment. Protein was harvested as previously described (Coleson, Sanchez, Ashley et al., 2015), transferred into a fresh tube and stored in -20°C. All experiments were repeated a minimum of three times.

2.2. Western Blot: Protein concentrations were determined using the Pierce BCA Protein Assay (Thermo Scientific) per manufacturer's instructions. Fifteen or twenty micrograms of protein lysate was loaded into 10% acrylamide gels. Gels were subjected to SDS-PAGE and transferred onto ImmunBlot PVDF membrane (BioRad) using the Semi-Dry transfer method. Membranes were blocked for an hour at room temperature in 5% non-fat milk or 5% BSA (Santa Cruz Biotechnologies, Santa Cruz, CA) in Tris buffered saline with Tween (TBS-T), the primary antibody was applied in blocking solution and incubated overnight at 4°C with agitation. Antibodies were purchased from Santa Cruz Biotechnologies, Aviva Systems Biology (San Diego, CA), or Cell Signaling Technology (Beverly, MA). Primary antibodies against MPRα (Santa Cruz, sc-134815), MPRβ (Santa Cruz, sc-134814), MPRγ (AbCam, 79517) MPRδ (Aviva, ARP49900_P050), MPRε (Santa Cruz, sc-99578), PGRMC1 (Santa Cruz, sc-98680), pERK (Santa Cruz, sc-7383), ERK (Santa Cruz, sc-93), pAKT (Cell Signaling 9271L), AKT (Cell Signaling 9272S), pJNK (Cell Signaling 9251L), JNK (Cell Signaling 9252L), p-p38 (Cell Signaling 4511L), p38 (Cell Signaling 9212S) and appropriate secondary antibodies goat anti-rabbit (Santa Cruz, sc-2004) or goat anti-mouse (Santa Cruz, sc-2005) were used according to manufacturer's recommendations. Blots were washed in TBS-T then incubated with secondary antibody at room temperature for an hour. Membranes were washed with TBS-T, and then exposed to Clarity Western ECL Substrate (BioRad) per manufacturer's instructions. Chemiluminescent images were collected on a ChemiDoc XRS+ using Image Lab 5.2.1 software (BioRad).

2.3. Cell viability: MCF10A and MCF12A cells (2,000 cells/well) were plated onto 96-well white clear-bottom plates (Costar, Washington DC) in complete media. The following day, media containing charcoal-stripped serum replaced complete media and cells were incubated for 24 h. Next, cells were treated with 100 nM of P4, MPA, 5αP,

3 α HP, MPR-Ag, or vehicle, added in serum-free media and allowed to incubate for an additional 72 h. The CellTiter Glo assay (Promega, Madison, WI) was used per manufacturer's instructions to determine cell viability.

2.4. Statistical Analysis: All experiments were repeated a minimum of three times. Graph-Pad Prism was used to compare treatments to control using a student's *t* test.

3. Results

Protein levels of MPR α , MPR β , MPR γ , MPR δ , MPR ϵ , and PGRMC1 were determined in 15 μ g total MCF10A and MCF12A cell lysate (Figure 1). Additional positive and negative controls were used to assure the fidelity of our immunoblotting (data not shown). MPR α , MPR γ , MPR δ , MPR ϵ , and PGRMC1 were all similarly produced in MCF10A and MCF12A cells, whereas MPR β was expressed only in MCF10A cell lines. Notably, MCF10A and MCF12A cell lines are triple negative and therefore do not express PR.

To determine if progestin compounds could activate key intracellular signaling pathways, we treated cells with 100 nM P4, 100 nM MPA, 1000 nM 3 α HP, 1000 nM 5 α P, 100 nM MPR-Agonist (MPR-Ag) or vehicle and protein was collected at 5, 10, 30, 60, or 120 min after treatment. For all experiments, images are representative of three separate experiments. Phosphorylation of ERK1/2 was assessed via western blot analysis and compared to total ERK1/2 (Figure 2). In both cell lines, all treatments promoted transient phosphorylation of ERK1/2. We next assessed phosphorylation of JNK using the treatments listed above. As with ERK1/2, phosphorylation of JNK was observed in all treatment conditions, with levels decreasing to baseline phosphorylation by 120 min following exposure (Figure 3). Phosphorylation of p38 was also evaluated. In contrast to ERK1/2 and JNK, p38 activation was observed following exposure to P4 and MPA in both cell lines, whereas the metabolites 3 α HP and 5 α P induced phosphorylation only in MCF10A cell lines (Figure 4). Exposure to the agonist MPR-Ag elicited a very modest response in p38 phosphorylation in MCF10A but not MCF12A cell lines. Activation of AKT was ascertained, and P4 and MPA promoted AKT phosphorylation in the MCF12A cell lines only (Figure 5); no activation was observed in MCF10As with any treatment. To confirm activation of MAPK pathways were specific to treatment with progestins, inhibitors of ERK, JNK, or p38 were utilized prior to treatment. As shown in Figure 6, phosphorylation of ERK by P4, MPA, 3 α HP, 5 α P, or MPR-Ag was abrogated when MCF10A cells were pre-treated with the ERK inhibitor U0126. Phosphorylation of p38 by P4, MPA, 5 α P, or MPR-Ag was also abolished when pre-treated with the p38 inhibitor SB203580 (Figure 6).

As we observed alterations in pathways known to modulate cell growth, we determined cell viability following treatment. No significant cell viability changes were observed in the MCF12A cells following 72 h exposure to any treatment. In contrast, proliferation of MCF10A cells increased significantly ($P < 0.05$) following exposure to MPA and 5 α P, but not P4 or 3 α HP (Figure 7). Treatment with MPR-Ag tended ($P = 0.08$) to decrease cell viability in the MCF10A cells.

4. Discussion

Progestins are clearly involved in breast cancer biology, especially in light of the WHI and million women's studies demonstrating increased breast cancer risk in women receiving CHT compared to EHT alone. In fact, women receiving EHT alone demonstrated a reduced risk (Rossouw et al., 2002, Anderson et al., 2004). Fundamentally, progestins induce biological response(s) by interacting with a receptor. Extensive study of the nuclear PR has provided a wealth of information. The recent discovery of other progestin receptors confounds interpretation of progestin intracellular signaling, as functions previously attributed to nuclear receptors may be partially or wholly due to non-nuclear receptor activation. Studying MPRs and PGRMC1 in breast cancer biology is needed to advance understanding of progestin functions. Progestins, including natural P4, biologically active metabolites, and synthetics such as MPA vary in their pharmacology and induce differing biological outcomes. Moreover, progestins are metabolized to bioactive metabolites that have distinctive biological responses further complicating the understanding of progestins in breast cancer biology. Several studies have reported progestin effects in breast cancer cells, but it is important to determine effects of progestins in benign breast epithelial cells. Presently, we lack clear understanding of how non-classical progestin receptors function in nontumorigenic breast cells as most research has been conducted in breast cancer cells. The objectives of the current study were to determine how natural P4, select progestin metabolites, MPA and the MPR-agonist alter signaling and cell proliferation in nontumorigenic breast cells and the involvement of non-classical progestin receptors.

While strong data exist implicating MPA in the etiology of breast cancer, much of the underlying biology remains poorly understood and the work completed largely focused on nuclear PR. Progestin-induced functions have been reported in the nontumorigenic breast cells, MCF10A and MCF12A, despite lacking PR (Kramer et al., 2006, Kramer et al., 2006, Behera, Dai, Garde et al., 2009). We determined that MCF10A and MCF12A cells express non-classical progestin receptors, which may account for progestin functions in breast epithelial cells (Figure 1). To our knowledge, this is the first report of MPRs and PGRMC1 production in MCF10A and MCF12A cells. MCF10A cells are positive for all membrane-bound progestin receptors assayed for, whereas the MCF12A express all but MPR β (Figure 1). MPR α , the first cloned MPR (Zhu et al., 2003, Ashley, Clay, Farmerie et al., 2006, Zhu, Rice, Pang et al., 2003) is expressed in breast cancer cells such as MB468 and SKBR3 (Dressing, Alyea, Pang et al., 2012, Zuo, Li and You, 2010) and based on our results, the nontumorigenic MCF10A and MCF12A cell lines. We observed benign breast epithelial cells also express MPR β and MPR γ , yet expression differs between the two lines with MCF12A being MPR β null. More recently, two new isoforms MPR δ and MPR ϵ , were discovered, and it was suggested only neural tissues produced these isoforms (Pang et al., 2013). We are the first to demonstrate MCF10A and MCF12A cells produce MPR δ and MPR ϵ , and MPR δ appears to be the most highly expressed MPR in breast epithelial cells. The function of MPR δ and MPR ϵ are unknown. PGRMC1 is produced in both breast epithelial cell lines, in agreement other reports indicating PGRMC1 and MPRs are often expressed in similar mammalian tissues with overlapping distributions (Thomas, 2008, Tang et al., 2005, Cahill, 2007).

PGRMC1 may enhance cell surface expression and functions of MPR α in breast cancer cells (Thomas, Pang and Dong, 2014), but if PGRMC1 performs similar functions in breast epithelial cells as well as with other MPRs is not known. Collectively, these data demonstrate nontumorigenic breast cells express a number of different progesterin receptors, which likely are responsible for progesterin-induced functions in cells lacking PR.

Interestingly, phosphorylation of ERK occurred in both MCF10A and MCF12A nontumorigenic cells following treatment with all progestins tested (Figure 2). To our knowledge, this is the first report of progestins activating the ERK pathway in PR-negative benign breast epithelial cells. MCF10A cells are a spontaneously immortalized, non-malignant cell line obtained from a patient with fibrocystic breast disease (Dawson, Wolman, Tait et al., 1996) and are considered “normal” because they do not form tumors in immunodeficient mice. The MCF10A cells represent a unique model to study breast cancer progression and many groups have generated a series of cell lines originating from MCF10A cells that form tumors in immunodeficient mice as recently reviewed (So, Lee, Kramata et al., 2012). Using transformed MCF10A lines that caused tumors in immunodeficient mice, it was noted that pERK was highly phosphorylated in the lines that formed tumors quickly and it was suggested that ERK activation might be critical for developing malignant breast cancer (Lee, Liu, Goodman et al., 2006). We observed pERK activation with natural P4 and the synthetic progestin MPA, as well as the metabolites 3 α HP and 5 α P and the MPR-Ag. Thus, (1) the ERK pathway is stimulated by varying progestins and (2) MPRs are involved in pERK stimulation. It is important to note that MCF10A and MCF12A cells are PR null, yet we established MPRs and PGRMC1 are produced and may be responsible for ERK activation. The fact that MPR-Ag was able to induce pERK in both MCF10A and MCF12A cells indicate the MPRs may serve as the receptors for ERK activation. Comparable experiments using the MPR-Ag demonstrated activation of ERK in breast cancer, fish granulosa, and neuronal cells (Pang et al., 2013, Dressing et al., 2012, Dressing, Pang, Dong et al., 2010) similar to our results in nontumorigenic breast cells. Moreover, phosphorylation of ERK with all progestins tested was abolished when cells were pre-treated with the ERK inhibitor (Figure 6) further implicating MPRs and/or PGMRC1 as receptors responsible for ERK activation. Progesterin-induced activation of ERK has not been previously reported in either MCF10A or MCF12A cell lines. Taken together, our findings provide a foundation to explore the potential involvement of progestins and ERK activation in breast cancer development.

Similar to ERK stimulation, all progestins induced JNK phosphorylation in both MCF10A and MCF12A cells (Figure 2). Progesterin-induced JNK stimulation has not been reported in MCF10A or MCF12A cells to our knowledge. The effects of P4 or related progestins on JNK activation are not well understood. Expression of MPR α , - β , - γ was demonstrated in a variety of ovarian cancer cell lines lacking PR and in agreement with our studies, P4 treatment resulted in JNK activation (Charles, Thomas and Lange, 2010). As the ovarian cancer cells tested lack PR, the authors suggest P4 is functioning through MPRs. Treatment of human umbilical vein endothelial cells with supraphysiological levels of P4 was able to induce JNK phosphorylation, but JNK

activation was not evident at physiological concentrations (Powazniak, Kempfer, de la Paz Dominguez et al., 2009). Studies evaluating P4 effects on epidermal growth factor (EGF) signaling in breast cancer cells revealed treatment of T47D-YB cells with R5020, a P4 agonist, for 48 h prior to EGF treatment resulted in JNK phosphorylation (Lange, Richer, Shen et al., 1998). However it should be noted that R5020 itself did not induce pJNK, but rather sensitized the breast cancer cells to the effects of EGF. Notably, unlike PR, MPRs do not bind R5020 (Kelder, Azevedo, Pang et al., 2010). Results from the current study indicate progestins induce pJNK activation directly in breast epithelial cells. As MCF10A and MCF12A are PR null and the MPR-Ag activates JNK, we suggest the progestin-induced effects are mediated through MPRs. The consequence of pJNK upon progestin stimulation requires further investigation, and we are currently exploring downstream functions.

Activation of p38 following progestin treatment, varied by cell type, with MCF10A cells demonstrating strong induction of phosphorylated p38 with P4, MPA, 3 α HP and 5 α P treatment and modest p38 activation with MPR-Ag administration (Figure 4). When cells were pre-treated with p38 inhibitor, activation by P4, MPA, 5 α P, or MPR-Ag was diminished (Figure 6) suggesting membrane progestin receptors are responsible for the progestin-induced activation of p38. P4 and MPA only induced modest p38 activation in MCF12A cells and other progestins elicited no effect. It is intriguing that the various progestins tested would induce differences in intracellular signaling in two different breast epithelial cell lines. Currently, the reason is unclear, though only MPR β was not detected in MCF12A cells, which may account for differences noted. Treatment of PR-negative ovarian cancer cells expressing MPR α , MPR β , MPR γ with P4 results in p38 activation (Charles et al., 2010). Similar to our results, p38 activation occurs in human myometrial cells treated with P4 (Karteris, Zervou, Pang et al., 2006). The myometrial cells expressed MPR α and MPR β . Though myometrial and breast cells differ, use of non-tumorigenic cells provides an advantageous model to study progestin actions. Our data provide novel information on how progestins can elicit differing p38 activation in benign breast epithelial cells.

Recent studies provide evidence progestins can regulate AKT activation, potentially through MPR α (Zuo et al., 2010, Tan and Thomas, 2014, Vares, Sai, Wang et al., 2015), yet most results are based on indirect evidence. In Atlantic Croaker sperm, stimulation with endogenous progestin results in AKT activation, a function believed to be induced via MPRs activation (Tan and Thomas, 2014). MPR-Ag treatment induced sperm hypermotility, though MPR-Ag induction of AKT activation was not reported. In our current study we did not observe AKT phosphorylation with any of the progestins tested, including MPR-Ag in MCF10A cells (Figure 5). This is contrary to a recent report by Vares and colleagues, in which the authors report AKT stimulation in MCF10A cells with P4 and MPR-Ag (Vares et al., 2015). However, AKT activity was monitored by inversely correlating to FOXO transcriptional activity whereas direct evidence of pAKT activation was not shown. We did not measure FOXO transcriptional activity in the current study. In MCF12A cells we observed AKT stimulation with P4 and MPA. In support, MPA induced pAKT activation in PR positive T47D cells (Saitoh, Ohmichi, Takahashi et al., 2005), but it is not known if T47D cells express MPRs. Similarly, MPA induces pAKT in

C4HD cells, a progestin-dependent murine tumor line (Salatino, Beguelin, Peters et al., 2006, Salatino, Schillaci, Proietti et al., 2004), yet if MPRs or PGRMC1 are expressed in these cells is unknown.

Our results indicate MPA and 5 α P increase cell proliferation in MCF10A cells, yet natural P4 does not (Figure 7), further highlighting the divergent effects progestins can elicit. In support, Kramer and colleagues reported MPA increased cell proliferation in MCF10A cells yet P4 did not alter proliferation, and authors suggested this was mediated through MAPK pathways (Kramer et al., 2006, Kramer et al., 2006). We observed ERK activation with all progestins tested and activation was abrogated upon pre-treating cells with ERK inhibitor (Figure 6), yet only MPA and 5 α P increased cell proliferation. As natural P4 differs in chemical structure compared to its metabolites, synthetic MPA, and MPR-Ag, its possible the binding and subsequent activation of downstream pathways also differ. Whether other signaling cascades such as NF κ B, Jak/Stat are activated by P4 metabolites, MPA or MPR-Ag is unclear, but an area we are investigating. Our data revealing 5 α P increasing cell proliferation in MCF10A cells confirms several other reports in breast epithelial as well as cancer cells (Wiebe, 2006, Wiebe et al., 2010, Wiebe et al., 2000, Pawlak, Zhang and Wiebe, 2005). Treatment with MPR-Ag on the other hand tended ($p=0.08$) to decrease cell proliferation. Because MCF10A cells lack PR but express all membrane bound receptors assayed for, it is difficult to identify which receptor(s) is key in the MPA and 5 α P induced effects. Studies to decipher these questions are currently underway in our laboratory.

5. Conclusions

As noted, MCF10A and MCF12A cells are triple negative suggesting progestins effects occur through other receptors and not PR. Our data indicate two benign breast cell lines express several MPRs and PGRMC1 and respond differently to various types of progestins. The fact that MPA increased cell proliferation in MCF10A cells yet P4 did not underscores the importance of investigating the roles synthetic progestins play in breast cancer biology compared to natural P4. Concurrently, our data demonstrate membrane bound progestin receptors are crucial to progestin signaling and activate MAPK and AKT pathways. Yet, because we also established MPR δ and MPR ϵ are synthesized in both cell lines, this highlights the complexity of progestin signaling in breast epithelial cells and underscores the necessity for future research to delineate receptor specific pathway activation. The various family members of the MAPK family and AKT play several multifaceted roles in cell signaling such as cell growth, differentiation, and apoptosis, yet the cellular decision involves a balance among competing pathways (Denhardt, 1996). We suggest the overall biological results of progestin signaling is a summation of signaling induced by binding of various progestins to receptors and cross-talk between receptors. Our results add to the understanding that various progestins have on breast epithelial cells and emphasizes the importance of considering both membrane bound receptors and progestin type.

List of abbreviations

P4: progesterone

MPR: membrane progesterone receptor

MPR-Ag: membrane progesterone receptor agonist

PR: nuclear progesterone receptor

MPA: medroxyprogesterone acetate

5 α P: 5 α -dihydroprogesterone

3 α HP: 3 α -dihydroprogesterone

PGRMC1: progesterone receptor membrane component-1

CHT: Combined hormone therapy (estrogen + progestin)

EHT: Unopposed estrogen hormone therapy

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Figure Statements

Figure 1. MPR β is present in MCF10A but not MCF12A cell lines, and all other progestin receptors are similarly expressed in both. Fifteen μ g of protein was subject to western blot analysis, and probed for MPR α , MPR β , MPR γ , MPR δ , MPR ϵ , and PGRMC1 protein production.

Figure 2. ERK1/2 is transiently phosphorylated in MCF10A and MCF12A cell lines following treatment with P4, MPA, 3 α HP, 5 α P, or MPR-Ag. Cells were treated with 100 nM P4, 100 nM MPA, 1000 nM 3 α HP, 1000 nM 5 α P, 100 nM MPR-Agonist (MPR-Ag) or vehicle (Veh) and protein was collected at 5, 10, 30, 60, or 120 min after treatment. Phosphorylation of ERK1/2 was assessed via western blot analysis and compared to total ERK1/2. Images are representative of 3 experiments.

Figure 3. Phosphorylation of JNK in MCF10A and MCF12A cell lines after exposure to P4, MPA, 3 α HP, 5 α P, or MPR-Ag. Cells were treated with 100 nM P4, 100 nM MPA, 1000 nM 3 α HP, 1000 nM 5 α P, 100 nM MPR-Agonist (MPR-Ag) or vehicle (Veh) and protein was collected at 5, 10, 30, 60, or 120 min after treatment.

Phosphorylation of JNK was measured via western blot analysis and compared to total JNK. Images are representative of 3 experiments.

Figure 4. Transitory phosphorylation of p38 in MCF10A and MCF12A cell lines following treatment with P4, MPA, 3αHP, 5αP, or MPR-Ag. Cells were treated with 100 nM P4, 100 nM MPA, 1000 nM 3αHP, 1000 nM 5αP, 100 nM MPR-Agonist (MPR-Ag) or vehicle (Veh) and protein was collected at 5, 10, 30, 60, or 120 min after treatment. Phospho-p38 was determined via western blot analysis and compared to total p38. Images are representative of 3 experiments.

Figure 5. AKT phosphorylation in MCF10A and MCF12A cell lines following treatment with P4, MPA, 3αHP, 5αP, or MPR-Ag. Cells were treated with 100 nM P4, 100 nM MPA, 1000 nM 3αHP, 1000 nM 5αP, 100 nM MPR-Agonist (MPR-Ag) or vehicle (Veh) and protein was collected at 5, 10, 30, 60, or 120 min after treatment. AKT phosphorylation was established via western blot analysis and compared to total AKT. Images are representative of 3 experiments.

Figure 6. Activation of pERK and p-p38 by progestins is abrogated when pre-treated with inhibitors to pERK or p-p38. Cells were pre-treated for 2h with ERK (U0126) or p38 (SB203580) inhibitors and then treated with 100 nM P4, 100 nM MPA, 1000 nM 3HαP, 1000 nM 5αP, or 100 nM MPR-Agonist (MPR-Ag) and protein was collected at 10 min after treatment. **(A)** Phosphorylation of ERK1/2 was assessed via western blot analysis and compared to total ERK1/2. **(B)** Phosphorylation of p38 was assessed via western blot analysis and compared to total p38.

Figure 7. Viability of MCF10A cells increased following treatment with either MPA or 5αP. Cells were treated for 72 h with 100 nM of P4, MPA, 5αP, 3αHP, MPR-Ag or vehicle (Veh) control. Viability was determined as percent vehicle for each treatment. Treatment with MPA or 5αP stimulated cell growth ($P < 0.05$), whereas MPR-Ag treatment tended ($P = 0.08$) to impede proliferation. For all experiments $n \geq 3$.

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