Progestins counteract estrogens to shift breast cancer cell metabolism

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Abstract

Progesterone receptors (PR) profoundly influence breast cancer biology by modifying estrogen receptor (ER) gene regulation, and, under some contexts, increasing populations of cancer stem cells. Abnormal metabolism is a cancer hallmark that has largely been understudied in relation to hormones in ER+PR+ breast disease. In this study, we investigated how progestins, in the absence or presence of estrogens, affect breast cancer cell metabolism. We measured metabolites using ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS) in three ER+PR+ breast cancer cell lines (T47D, UCD4, and UCD65) treated with vehicle, estrogen only, progestin only, or the combination. Progestins, in the absence or presence of estrogens, largely downregulated metabolites, particularly those involved in tricarboxylic acid (TCA) cycle and amino acid metabolism. Seahorse metabolic analysis found progestins (alone or plus estrogens) generally shifted cells towards glycolysis with reduced ATP production. Transmission electron microscopy in cell lines and patient-derived xenograft tumors found that estrogens produced an elongated mitochondrial morphology, while estrogen plus progestin treatment reversed this trend. Using the photoconvertible MitoTimer reporter, progestins reduced both baseline and estrogen-induced mitochondrial turnover. Progestins blocked the estrogen-induced expression of mitochondrial biogenesis regulators PGC1 α and PGC1 β and their downstream targets. These findings indicate that progestins dominantly affect cell metabolism to shift cells to a more glycolytic phenotype with reduced mitochondrial function and amino acid pools; this transition is indicative of less proliferative, but also more dedifferentiated cells. Our results highlight potential benefits and detriments of current clinical studies testing selective PR modulators in ER+ breast cancers.

Background

In the normal breast, the female hormones estrogen and progesterone act in coordination to facilitate growth, development, and cyclical changes in mammary ducts. In breast cancer, however, hormone signaling becomes aberrant and uncoupled from its normal tissue function. Nearly three-quarters of breast cancers are estrogen receptor alpha (ER) and usually progesterone receptor (PR) positive and depend on estrogens for continuous growth (1). As most diagnoses occur in postmenopausal women with low circulating estrogen levels, tumor growth in these women relies on local adipose tissue production of estrogen (2, 3). Progesterone dissipates more completely at menopause (4), and the reintroduction of synthetic progestins in combination with estrogens in menopausal hormone therapy (MHT) increases breast cancer incidence (5). Paradoxically, in established breast cancers, these same synthetic progestins can be as effective as tamoxifen at improving progression free survival and are sometimes still used upon endocrine failure (6-8). Moreover, antiprogestins such as onapristone are in Phase II clinical trials for women with advanced breast cancer. The divergent actions of PR ligands in breast cancer remains an impediment to their guided use in treatments.

Receptors for estrogens and progestins, ER and PR, are closely related members of the nuclear receptor transcription factor family. Crosstalk between family members is emerging as prototypical in controlling gene regulation and cell phenotype (9, 10). Proteomic and genomic studies in ER+PR+ breast cancer cell lines and patient-derived xenografts (PDX) have found that unliganded PR is a cofactor in the ER transcription complex, but that progestins redirect ER-PR chromatin binding away from core estrogen-responsive to progestin-responsive genes (11-13). Thus, under conditions of estrogens plus progestins in breast cancer cells, PR target genes are predominately regulated. The consequences of this switch remain controversial. In many cases, progestins quell the mitogenic activities of ER (11-13). However, progestins alone in a low estrogen background can have modest mitogenic effects (reviewed in 14). Furthermore, progestin-activated PR transitions a subpopulation of breast cancer cells to a more stem-like phenotype (15-17). The downstream consequences of ER-PR crosstalk on breast cancer cell physiology, particularly cellular bioenergetics and metabolism, are relatively unexplored. Understanding these changes is important since cancer cells adopt distinct metabolic phenotypes that modulate their growth, survival, and stemness (reviewed in (18)).

Estrogens are generally reported to stimulate breast cancer cell metabolism to satisfy the increased energy and biomass needs of growing cells. Energy-generating processes of oxidative phosphorylation (oxphos) and glycolysis are reportedly enhanced in the presence of estrogen through genomic and non-genomic mechanisms (reviewed in (19)). Both ERa and ERb can localize to the mitochondrial matrix in MCF7 cells; however, whether they directly regulate mitochondrial (mt) DNA is unclear (20, 21). Estrogens do, however, foster mitochondrial biogenesis machinery by directly regulating nuclear respiratory factor 1 (NRF-1) (22). NRF-1 in turn upregulates the mitochondrial transcription factor TFAM that then targets mitochondrial genes. Likewise, estrogen treatment increases expression of mitochondrial-encoded electron transport chain subunits, including NADH dehydrogenase subunit 1 (ND1) and cytochrome oxidases I and II (COI, COII) (23-25). Glutamine uptake and consumption were found to increase with estrogen treatment in MCF7 breast cancer cells (26). Collectively, studies support that ER targets bioenergetics processes to meet the demands of continuous breast cancer cell growth.

In contrast to estrogens, progestin effects on breast cancer cell metabolism are less studied. In T47D cells, PR regulates genes involved in cholesterol and steroid, fatty acid and lipid, and nucleotide and amino acid metabolism (27). The synthetic progestin medroxyprogesterone acetate increases fatty acid synthase in T47D breast cancer cells, and leads to increased de novo lipogenesis and accumulation of lipid droplets (28). A truncated isoform of PR (termed PR-M) was reported to localize to the mitochondria of T47D and MCF10A cells, and potentially mediate progesterone effects on mitochondrial membrane potential (29, 30). Thus, while progestins and PR clearly affect breast cancer cell metabolism, the scope of their impact remains largely untested, particularly in the context of estrogen-progestin combinations, which is the typical physiological and clinical context.

In this study, we evaluated the effect of estrogens, progestins, and the combination on modulation of cell metabolism in ER+PR+ breast cancer cells. We found that progestins perturb estrogen-driven increases in metabolites and metabolic processes. Progestins cause a small but consistent shift towards glycolysis, potentially reverse estrogen-induced mitochondrial elongation, and deplete cell amino acid pools. Overall, we conclude that progestins exert a dominant effect on estrogen-driven energy production in breast cancer cells, prospectively as part of a broader transition from rapid tumor growth to cytostasis, metabolic flexibility, potentially favoring a cancer stem cell phenotype. The consequences of a progestin-induced metabolic shift are unclear but could have implications for the targeted use and long-term efficacy of progestin and anti-progestin based breast cancer therapies.

Methods

Cell lines and cell culture

The breast cancer cell line T47D was obtained from the University of Colorado Cancer Center Tissue Culture core (RRID: CVCL_0553) and was maintained in minimal Eagle's medium, 5% fetal bovine serum (FBS), 1X non-essential amino acids, 1×10^{-9} M insulin, 0.1 mg/mL sodium pyruvate, and 2 mM L-glutamine. Development of ER+PR+ breast cancer PDX UCD4 and UCD65 has been previously described (13, 31). The UCD4 and UCD65 cell lines were derived from their corresponding PDXs and remain ER+PR+ (32). The UCD65 and UCD4 cell lines were maintained in DMEM/F-12 1:1 with 10% FBS, 1×10^{-9} M cholera toxin, 1×10^{-9} M hydrocortisone, and 1×10^{-9} M insulin. Cell lines were authenticated using short tandem repeat (STR) analysis using the University of Arizona Genetics Core (University of Arizona, Tucson, AZ). For UCD65 and UCD4, cells were matched to the original PDX and not to any other cell lines in the database. All cell lines were routinely tested for mycoplasma contamination using the MycoAlert mycoplasma detection kit (Lonza, Basel, Switzerland). In vitro hormone experiments were performed using phenol red-free media with the same additives described above. Hormone treatment was used as follows: 17- β -estradiol (E2), 10^{-8} M (Sigma-Aldrich, St. Louis, MO); R5020, 10^{-8} M (PerkinElmer, Waltham, MA); or progesterone (P4), 10^{-7} M (Sigma-Aldrich), or the combination of E2 plus R5020 (both 10^{-8} M) for 24 h unless otherwise indicated. PR expression was induced in UCD65 and UCD4 cells by E2 pre-treatment for a minimum of 24 h prior to experiment start.

Animal experiments

All animal experiments were performed under a protocol approved by the University of Colorado Institutional Animal Care and Use Committee. For T47D xenografts, 1×10^6 cells were injected into the mammary fat pad of female NOD/SCID/IL2rg^{-/-} (NSG) mice supplemented with E2 or E2+P4 pellets as

previously described (15). PDX tumors were partitioned and grown in female NSG mice supplemented with subcutaneous silastic pellets containing E2 or E2+P4 as previously described (33, 34).

Metabolomics

Metabolites were extracted from T47D, UCD65, and UCD4 cell pellets in quadruplicate using ice-cold lysis/extraction buffer (5:3:2 methanol:acetonitrile:water) at a concentration of 2×10^6 cells per mL. Metabolomics and analyses were performed using the University of Colorado Cancer Center's Mass Spectrometry Metabolomics Shared Resource essentially as described (35, 36). Briefly, after sample randomization, 10 μ L of extracts were injected into a Thermo Vanquish UHPLC system (San Jose, CA) and resolved on a Kinetex C18 column (150×2.1 mm, 1.7μ m, Phenomenex, Torrance, CA) at 450 μ L/min through a 5 min gradient from 5 to 95% organic solvent B (mobile phases: A = water, 0.1% formic acid; B = acetonitrile, 0.1% formic acid in positive ion mode or mobile phases: A = 18 m Ω H₂O, 1 mM ammonium acetate; B = acetonitrile, 1 mM ammonium acetate for negative ion mode). Untargeted data acquisition, quality control, and targeted data analysis were performed as previously described (37). Precipitated protein was reconstituted in PBS and measured using BCA protein assay (Pierce, Thermo Fisher, Waltham, MA). Metabolomics intensity signals were normalized to sample protein concentration. The metabolomics dataset supporting the conclusions of this article has been deposited to the MetaboLights database (RRID:SCR_014663), with the identifier MTBLS2138. The complete dataset can be accessed here: <https://www.ebi.ac.uk/metabolights/index>.

Normalized data was imported into MetaboAnalyst software (RRID:SCR_015539) (38), where data was log-transformed and autoscaled (39). Partial least squares discriminant analysis (PLS-DA) was performed on all samples within cell lines for visual inspection of clustering patterns and outlier detection. Heatmaps were constructed using Pearson distance with average linkage and depict non-scaled PLS-DA variable importance in projection (VIP) averaged across replicates (N=4) within treatment groups.

For pathway analysis, pairwise comparisons of E2-treated vs. E2+R5020-treated cells were used. Metabolites from random forest variable importance analysis with mean decrease in accuracy >0 were evaluated for fold-change direction (lower in E2+R5050 vs. E2, called "down"; higher in E2+R5020 vs. E2, called "up"). These subsets of metabolites were submitted to MetaboAnalyst Pathway Analysis (MetPA) and can be found in **Supplementary Table S1** for T47D cells, **Supplementary Table S2** for UCD65 cells, and **Supplementary Table S3** for UCD4 cells. Pathways were identified using default settings; specifically, the hypergeometric test for overrepresentation analysis and relative betweenness centrality was used for pathway topology analysis, with pathways mapped to the *Homo sapiens* KEGG reference library.

Seahorse metabolic phenotyping

Metabolic phenotype was determined using the Seahorse XFe96 Extracellular Flux Analyzer (Agilent, Santa Clara, CA). T47D, UCD65, and UCD4 cells were cultured in the presence of vehicle (0.2% ethanol), E2 (10^{-8} M), R5020 (10^{-8} M), or E2 plus R5020 (10^{-8} M each) for 24 h and assessed via the Mito Stress Test kit (Agilent). Samples were analyzed with 7-8 replicates per treatment. Cell count at time of assay was used for data normalization and obtained using the Cytation 1 Cell Imaging Multi-Mode Reader (BioTek) with Hoescht 33342 (Sigma) fluorescent staining.

ATP assay

Intracellular ATP was quantified using the ATP Fluorometric Assay from Novus Biologicals (#NBP2-54855, Biotechne, Cambridge, MA) according to the manufacturer's protocol. Briefly, cells treated with hormones for 24, 48, or 72 h were lysed in ice-cold assay buffer and deproteinized using the Deproteinizing Sample Preparation Kit (#K808-200, BioVision, LLC, Milpitas, CA) according to the manufacturer's protocol and assayed under fluorimetric conditions (Ex 535/Em 587) in triplicate. Results are representative of at least 3 experiments.

Transmission electron microscopy

Cells were cultured on PermaNox 60-cm dishes (VWR, Radnor, PA). Excised tumors were cut into approximately 1 mm³ pieces. Cultured cells and tumor pieces were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer and then post-fixed with reduced osmium (1.5% potassium ferrocyanide + 1% osmium tetroxide) followed by 2% osmium tetroxide. Samples were dehydrated with a graded series of ethanol and embedded in a thin layer of Epon. Following Epon curing, small pieces were cut out and re-embedded in blocks that were sectioned at 65 nm on an ultramicrotome, collected on formvar coated slot grids, and post-stained with 2% osmium tetroxide and lead citrate.

At least 10 fields per treatment were imaged and blinded prior to analysis. Mitochondrial length along the longest axis was measured using Fiji and plotted via histogram, with bin mode indicated on the X-axis. Outliers greater than 3 standard deviations outside the mean of the full dataset were excluded. Differences in distributions were analyzed using the Kolmogorov-Smirnov test for frequency distributions; comparisons for cells were 1) vehicle vs. E2 and 2) E2 vs. E2+R5020, while tumor samples were pooled by hormone treatment and compared E2 pellets vs. E2+P4 pellets.

MitoGFP and MitoTimer

T47D cells were labeled overnight with CellLights BacMam 2.0 MitoGFP (Thermo Fisher, Waltham, MA) according to the manufacturer's protocol. Cells were treated for 24 h with vehicle (EtOH), E2 (10^{-8} M) or P4 (10^{-7}), or E2 + P4 for 24 h then were fixed in 4% paraformaldehyde, counterstained with DAPI, and mounted on coverslips. Images were collected using confocal laser scanning microscopy (Zeiss LSM 780) with 40X objective.

T47D cells were transduced to express the MitoTimer construct from Addgene (Watertown, MA) as previously described (40). Following stable transduction with the pLenti-CMV-rtTA3 Blast (w756-1) (Plasmid #26429, RRID:Addgene_26429) with >2 weeks blasticidin selection, cells were transiently transfected with pTRE-Tight-MitoTimer (Plasmid #50547, RRID:Addgene_50547) using Lipofectamine 3000 (Thermo Fisher, Waltham, MA). MitoTimer expression was induced by treatment for 1 hour with doxycycline (4 ug/ml, Cayman Chemical, Ann Arbor, MI), followed by washout and hormone treatment for 48h, at which point most mitochondria should be yellow to red. A second 1h dox pulse, followed by washout, was used to label a new wave of mitochondria corresponding to cells undergoing active biogenesis. Following an additional 6 h of hormone treatment, live cells (via IncuCyte ZOOM at 20X magnification) or fixed cells (4% paraformaldehyde with DAPI counterstain) were imaged for analysis. Ten fields/condition (>200 cells each) were quantitated for green/red intensity using ImageJ/Fiji in a blinded manner and plotted as green:red ratio for each cell.

Real-time quantitative PCR (qPCR)

RNA was harvested using QIAzol lysis reagent (Qiagen, Venlo, the Netherlands) and converted to cDNA using the Verso cDNA kit (Thermo Fisher, Waltham, MA). qPCR was performed on cDNA using Absolute Blue Sybr Green (Thermo Fisher, Waltham, MA) and normalized to β -actin using the Pfaffl method (41). qPCR primers are provided in **Supplemental Table S4**. Results are representative of 3-4 experiments.

L-amino acid assay

Amino acids were measured using the L-Amino Acid Quantitation Kit (#MAK002, Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. Briefly, T47D cells treated with hormones for 24 h were homogenized in ice-cold assay buffer, diluted within linear range of the assay, and colorimetric absorbance at 570 nm measured in triplicate. Results are representative of at least 2 experiments.

Statistical analyses

Statistics were performed using GraphPad Prism 8.3.0 (GraphPad Software, San Diego, CA), with the exception of metabolomics data, which was analyzed using MetaboAnalyst (see Metabolomics section for details). Two-tailed Student's t-tests, one-way ANOVA followed by Tukey multiple comparison tests were used to compare groups where noted. For comparisons with unequal variance, data were log-transformed prior to one-way ANOVA testing. Significance at $P < 0.05$ is indicated in figures and legends.

Supplemental Materials

Supplemental materials are available at Figshare online data repository:
<https://dx.doi.org/10.6084/m9.figshare.13073390>

Results

Progestins counter-regulate estrogen-driven metabolic reprogramming

Progesterone and its synthetic analogs have context dependent effects on breast cancer cell proliferation, frequently counteracting the potent growth stimulating effects of estrogens, but also demonstrating occasional mitogenic activity in the absence of estrogens. These occur in a cell line and tumor-specific manner, underscoring the complexity of PR biology. We assessed the effects of vehicle, the synthetic progestin R5020, estrogen (E2), or the combination on the growth of three ER+PR+ breast cancer cell lines (T47D and PDX-derived cell lines UCD65 and UCD4 (32)). Progestin had variable effects, counteracting E2 in T47D cells, with no significant decrease in E2 effects in UCD65 or UCD4 cells over one week of growth (**Supplemental Fig. S1**). To globally evaluate the effects of progestins, alone or in the presence of estrogens, on the bioenergetics of breast cancer cells, we performed metabolomics on T47D, UCD65, and UCD4 cells given the same treatments for 24 h. PLS-DA scores plots show that the metabolite signatures of E2, R5020, and E2 plus R5020 were distinct compared to vehicle or each other in all three cell lines (**Fig. 1A**). Heatmaps of the top 50 features by variable importance (VIP) scores were generated using Metaboanalyst 4.0 (38) and demonstrate wide-scale reductions in metabolites in cells treated with R5020, either alone or in combination with E2 (**Fig. 1B**).

We used MetaboAnalyst Pathways Analysis (MetPA) to identify the most significant pathways altered in each cell line, shown in **Fig. 1C**. We focused our analysis on E2 vs. E2+R5020 which captures the most common physiological scenario. In all three cell lines, many metabolic pathways were down-regulated in E2+R5020 compared to E2, including the TCA cycle (2/3 cell lines), amino acid metabolism (all 3 cell lines), and glutathione metabolism (2/3 cell lines). Only the pentose phosphate pathway was stimulated by E2+R5020 vs. E2 in 2/3 cell lines (T47D and UCD65).

We noted a striking decrease in amino acid metabolites, and a decrease in multiple types of amino acid metabolism comparing E2+R5020 vs. E2 alone in all three ER+PR+ breast cancer cell line analyzed. Amino acids play a multifaceted role in cancer cells role as building blocks for protein biosynthesis, nucleotide and glutathione synthesis, and as metabolic substrates for the TCA cycle. To test the effect of progestins on intracellular amino acids we used colorimetric L-amino acid kits on cells given the different hormone treatments. Progestins elicited a striking reduction in the L-amino acid pool comparing R5020 to vehicle, or E2+R5020 compared to E2 alone (**Supplemental Fig. S2** and **Table S1-3**). Collectively, these results suggest progestins impact the overall metabolic phenotype of breast cancer cells, and trend towards downregulating metabolic pathways in the absence or presence of estrogens, regardless of their impact on cell growth.

Progestins shift cells to a more glycolytic phenotype

Since TCA cycle was a key affected pathway in two cell lines, and an obvious target for cell metabolism, we investigated how hormone treatments impacted the energetic profile of breast cancer cells using Seahorse metabolic analysis. Progestins did not significantly affect the oxygen consumption rate (OCR) of breast cancer cells. However, progestins (R5020 vs vehicle or E2+R5020 vs E2) moved cells toward a glycolytic phenotype and slightly reduced maximal OCR (**Fig. 2A** and **Supplemental Fig S3A-C**). When mitochondrial stress was induced by inhibiting ATP synthase (oligomycin) and uncoupling membrane potential (FCCP), R5020 alone or with E2 likewise shifted cells towards glycolysis. Thus, progestins effectively shift cells from an “Energetic” and “Aerobic” phenotype towards a “Glycolytic” phenotype. The OCR and ECAR profiles are depicted in **Fig. 2B** and show a minimal effect on OCR, with an increase in ECAR comparing R5020 to vehicle or E2+R5020 to E2 alone.

To test whether hormones affect energy production, we measured ATP production in T47D and UCD65 cells. We observed a time-dependent increase in ATP over 72 h with E2 treatment (**Supplemental Fig. S3D-E**). R5020 alone did not impact ATP in either cell line while the combination E2+R5020 blunted the E2 affect. These data suggest that progestin treatment modestly attenuates the E2-driven increase in energy production.

Progestin co-treatment blocks E2-driven mitochondrial elongation

To test whether progestins target mitochondrial function, we evaluated mitochondrial morphology using transmission electron microscopy (EM) in T47D and UCD65 cells (**Fig. 3A**). Compared to vehicle, E2 treatment increased mitochondrial axis length whereas R5020 did not alter mitochondria compared to vehicle. In cells treated with E2+R5020, mitochondrial length shifted back towards vehicle, suggesting that R5020 blocks the elongating effect of E2 (**Fig. 3B**). To determine if this effect was consistent in vivo

in solid tumors given chronic treatment, we performed EM analysis of T47D xenografts and ER+PR+UCD4 PDX tumors, grown with either E2 alone or E2 plus P4 for 8-12 weeks. Mitochondria in tumor cells grown in mice supplemented with E2 were generally more elongated than in mice supplemented with E2+P4 (**Fig. 3C** and **Fig. 3D**). These data support that co-treatment with E2+progestins versus E2, in cell lines and tumors, shifts mitochondria to a less elongated phenotype, which is consistent with lower functional capacity (42).

Progestin-treated cells have a more aged mitochondrial network

To visualize the entirety of the mitochondrial network within cells, mitochondria in T47D cells were labeled with a baculovirus GFP construct (MitoGFP) and then treated for 24 h with vehicle, E2, P4, or E2 plus P4. (**Fig. 4A**). Confocal images show that MitoGFP appears visually enhanced with E2, but not P4, compared to vehicle, and that P4 attenuates the E2 signal. Since this is suggestive of impaired mitochondrial biogenesis, we evaluated and quantitated mitochondrial turnover in relation to hormone treatments using the inducible MitoTimer system developed by Hernandez et al (**Fig. 4B**) (40), in which newly made mitochondria fluoresce green but photoconvert to red fluorescence over approximately 48 h. Representative confocal microscopy images (**Fig. 4C**) are shown for each hormone treatment of vehicle, E2, R5020, and the combination. A general trend was observed of more green and yellow mitochondria in E2-treated cells, indicative of continual mitochondrial biogenesis, whereas mitochondria from R5020 or E2+R5020-treated cells were relatively more orange or red, indicative of older mitochondria. Quantitation of MitoTimer green:red fluorescence ratio found that, compared to vehicle, cells with E2 treatment trended towards increased green cells ($P=0.0524$) while cells R5020 treatment showed significantly decreased cells with green mitochondria and a gain in cells with predominantly red mitochondria (**Fig. 4D**). Treatment with E2+R5020 significantly reduced the green:red ratio compared to E2 alone. These results imply mitochondrial turnover is impacted by progestin treatment and can reduce baseline biogenesis in addition to blocking E2-induced biogenesis.

Progestins block the E2-induced PGC1 α mitochondrial biogenic signaling cascade

Mitochondrial biogenesis is regulated by the peroxisome proliferator activated receptor gamma coactivator (PGC) family of proteins, particularly PGC1 α . Since progestin treatment resulted in a more aged mitochondrial network, we evaluated the expression of transcripts for the mitochondrial biogenesis signaling cascade depicted in **Fig. 5A** in response to hormone treatment over 72 h. E2 increased transcript expression of Nrf2 (*NFE2L2*) (**Fig. 5B**) and PGC1 α (*PPARGC1A*) (**Fig. 5C**), and PGC1 β (*PPARGC1B*) (**Fig. 5D**), upstream regulators of mitochondrial biogenesis at 48 and 72 h. Conversely, the time-dependent induction of Nrf2, PGC1 α , and PGC1 β by E2 was entirely mitigated by co-treatment with R5020. Downstream effectors of PGC1 α include the Nrf1 (*NFE2L1*) transcription factor and mitochondrial transcription factor A (*TFAM*) which activate mitochondrial biogenesis and trigger replication of the

mitochondrial genome, respectively. E2 induction of these targets was abolished by E2+R5020 treatment (**Fig. 5E** and **Fig. 5F**, respectively). Detection of PGC1 α protein is complex, due to the presence of more than 10 isoforms with variable stability, biological activity, and tissue expression (43). However, immunoblot against Nrf2 as the inducer of the downstream signaling cascade suggested a decrease in Nrf2 protein expression with R5020 alone or E2+R5020. Collectively these data support that progestins target the PGC1 α /Nrf2 signaling cascade as a potential mechanism to disrupt mitochondrial turnover.

Together these data suggest that progestins, alone or in combination with E2, shift the energetic potential of ER+PR+ breast cancer cells towards a more glycolytic phenotype with reduced mitochondrial activity. A schematic of our proposed effects of progestins on breast cancer cell metabolism is depicted in **Fig. 6**. Progestins promote fatty acid biogenesis which requires ample acetyl-CoA availability (28). Citrate may be drawn from the TCA cycle to satisfy acetyl-CoA demands. Glutamine can be converted to glutamate and then alpha-ketoglutarate in a process called anaplerosis which restores TCA cycle flux. Other glutamine dependent pathways such as glutathione synthesis and amino acid biosynthesis have decreased activity with progestins. While the overall effect of progestins on cellular metabolism supports a glycolytic shift, the consequences on reduced OXPHOS and TCA cycle activity are shown in multiple metabolic pathways. The significance and permanence of the progestin-induced metabolic latency are important questions relevant to their use in breast cancer treatments.

Discussion

Metabolic changes are an essential hallmark of all cancer cells. However, there is considerable variability in the specific changes each tumor adopts. Breast cancers are unique in that the majority depend on estrogens for growth and multiple metabolic processes are cited as estrogen targets. A growing body of data support that progestins frequently counteract estrogenic activity, while having suppressive, neutral, or occasional stimulatory effects on breast cancer cell growth. In this study, we investigated the impact of progestins on breast cancer cell metabolism. We demonstrate that progestins move the metabolic phenotype of breast cancer cells towards glycolysis while reducing estrogen stimulated ATP production, mitochondrial biogenesis, and amino acid biosynthesis.

Oxidative phosphorylation is the most efficient bioenergetic mechanism to produce ATP. However, discovery of the Warburg effect revealed that tumor cells frequently depend on aerobic glycolysis despite its lower ATP yield (44). There are other advantages to glycolysis, for example, providing metabolic intermediates essential for biosynthetic pathways such as the Pentose Phosphate Pathway (45). Two of our experiments support that, in estrogen driven breast cancers, progestins induce a shift towards glycolysis. In our metabolomics analysis, lactate, a byproduct of glycolysis, was one of the few significantly increased metabolites with E2 plus progestin (R5020) vs E2 alone (**Supplemental Tables S1-3**). Moreover, the only pathway that increased between these two treatment groups in two cell lines (T47D,

UCD65) was the Pentose Phosphate Pathway which utilizes glycolysis byproducts for NADPH production (45). Seahorse metabolic analysis confirmed an increase in extracellular acidification rate, an indicator of glycolysis, with both R5020 and E2+R5020 treatments compared to vehicle or E2, respectively (**Fig. 2**). Interestingly, progestin-induced glycolysis does not displace OXPHOS, which was only marginally reduced. We therefore speculate that pyruvate is still entering the mitochondria to feed the TCA cycle, potentially at reduced levels suggested by our metabolomics pathway analysis. Acetyl-CoA is a necessary substrate for fatty acid biosynthesis which can be resourced from citrate in the TCA cycle. We speculate that citrate may be the source of acetyl-CoA for progestin-mediated fatty acid biosynthesis (28) resulting in anaplerosis, or replenishment of TCA cycle intermediates (**Fig. 6**). Glutamine conversion to α -ketoglutarate can restore TCA cycle movement; however, glutamine is essential to biosynthetic pathways such as amino acid synthesis, glutathione synthesis, and nucleotide synthesis (46, 47), all processes downregulated by progestins (**Fig. 1**), supporting a loss of glutamine availability. Collectively, we propose that progestins shift metabolic priorities to energy-storage with reduced ATP production that can result in phenotypic antagonism of estrogen-induced growth. While this seems favorable, a recent meta-analysis found that tumors expressing high levels of glycolytic proteins corresponded to shorter overall survival of breast cancer patients (48).

Mitochondria are pivotal in determining the energetic state and overall physiology of cancer cells (49). In breast cancer, estrogens drive mitochondrial biogenesis and activity (22). Our studies describe that progestins have a profound but different impact on mitochondrial morphology and function. First, as described above, progestins boost the glycolytic capacity of cells (**Fig. 2**), suggesting that progestins reduce reliance on mitochondrial activity. Second, progestins block estrogen-induced changes in mitochondrial morphology, favoring smaller, rounder rather than elongated mitochondria (**Fig. 3**). Mitochondrial morphology is tightly linked to energy metabolism: a highly inter-connected mitochondrial network and enlarged cristae are associated with enhanced respiration, whereas low oxphos and high glycolysis correlates with smaller mitochondria displaying reduced intracristae space (50). Third, progestins block estrogen-induced mitochondrial biogenesis (**Fig. 4**). Progestins potently decreased basal and E2-induced expression of PGC1 α and its downstream targets in breast cancer cells, suggesting a potential mechanism for disrupting biogenesis (**Fig. 5**). Although progestins clearly alter mitochondrial morphology, our data suggests mitochondria maintain partial functionality. OXPHOS, even at reduced capacity, is necessary for cell function and survival. We propose that progestins prime breast cancer cells for a metabolic shift in energetic dependency towards glycolysis.

A lower energy state and reduced mitochondrial function may benefit more dormant tumor cell populations and CSC maintenance. In normal tissues, embryonic and induced pluripotent stem cells generally fulfill energy needs through glycolysis while differentiation reduces glycolytic rate with increased oxphos (51). Furthermore, disrupting or modulating mitochondrial dynamics also impacts stem

cell behavior (52). For example, stimulation of mitochondrial biogenesis promotes differentiation of embryonic stem cells and induced pluripotent stem cells (52). Energetic processes in CSCs appear to be tumor type dependent; in many cases, CSCs preferentially utilize glycolysis but show considerable metabolic plasticity and increase oxphos and fatty acid oxidation, for example, under stressed conditions (reviewed in (53)). Progestins increase stem cell populations in the normal and malignant breast (reviewed in 54, 55). Our studies demonstrate that progestins increase glycolysis, disrupt mitochondrial biogenesis, and reduce amino acid metabolism in the total breast cancer cell population. How and whether these processes differ between progestin-induced CSCs and non-CSCs will require further study, but we speculate this could even more exacerbated within the CSC population. Furthermore, the progestin-induced metabolic state may even facilitate the expansion of CSCs.

Endocrine and other breast cancer therapies are thought to keep occult tumor cells dormant by inducing long term cytostasis, and this is supported by an increase in recurrences upon cessation of tamoxifen after 5-10 years of use (56). The metabolic phenotype of cancer cells that are forced into cytostasis, nor the factors that trigger exit from dormancy are not well understood. Studies by Havas et al (57), using primary murine mammary organoid cultures with inducible oncogene expression noted metabolic shifts in residual cells following oncogene withdrawal that were important for recurrence. Notably, several of the pathways identified as distinct between induced (oncogenes on) vs. regressed (oncogenes off) organoids overlapped with metabolic pathways we identified as counter-regulated by E2+R5020 vs. E2 in breast cancer cells including reduced aminoacyl-tRNA biosynthesis, TCA cycle, and Ala/Asp/Glu metabolism (**Fig. 1C**) (57). Interestingly, the PR antagonist mifepristone reduced organoid recurrence upon re-induction of oncogenes (57). It is conceivable that PR provides favorable energetics for dormancy, although this has not been tested. It will also be interesting to determine how PR antagonists affect breast cancer cell metabolism which, to our knowledge, has also not been studied. These types of studies are relevant to ongoing clinical trials of selective PR modulators including both agonists (micronized progesterone and megestrol acetate) and antagonists (onapristone) for treatment of ER+PR+ breast cancer, and which types of ligands are able to both reduce growth and prevent recurrence.

In conclusion, our studies support that progestins induce a metabolic transition in ER+PR+ breast cancer cells from a high-energy mitochondria-powered phenotype to a low-energy glycolysis-powered phenotype with reduced anabolic molecules such as amino acids. We speculate that this shift is important for the dual actions of progestins in subduing estrogen-driven growth while promoting expansion of CSCs. Further studies into progestin and antiprogestin regulated metabolic phenotypes may help design treatment strategies that include relatively non-toxic PR targeted therapies. Our work has implications for selective PR ligands that are being tested in clinical trials, suggesting that immediate benefits may be counteracted by long term adeptness of cytostatic cells, and furthermore, that some progestin-induced metabolic features could be potential vulnerabilities.

Declarations

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Data Availability

Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in Materials and Methods.

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Abbreviations

ANOVA

Analysis of variance

ATP

Adenosine triphosphate

BCA

Bicinchoninic acid

BSO

Buthionine sulfoximine

cDNA

Complementary DNA

CK5

Cytokeratin 5

COI

Cytochrome oxidase I

COII

Cytochrome oxidase II
CSC
Cancer stem cell
DAPI
4',6-Diamidino-2-phenylindole
DMEM
Dulbecco's modified Eagle's medium
DNA
Deoxyribonucleic acid
Dox
Doxycycline
Drp-1
Dynamin-related protein, DNM1L
E2
17- β -estradiol
ECAR
Extracellular acidification rate
EM
Electron microscopy
ER/ ER α
Estrogen receptor alpha, ESR1
ER β
Estrogen receptor beta, ESR2
FBS
Fetal bovine serum
GCLC
Glutamate-cysteine ligase, catalytic subunit, GCLC
GFP
Green fluorescence protein
GLUT
Glucose transporter protein family
GSH
Reduced glutathione
GSSG
Oxidized glutathione
HER2
Human epidermal growth factor receptor 2, ERBB2
HT
Hormone therapy
ICC

Immunocytochemistry
KEGG
Kyoto Encyclopedia of Genes and Genomes
MetPA
MetaboAnalyst Pathways Analysis
mPR
Putative mitochondrial PR
NADH
Nicotinamide adenine dinucleotide
ND1
NADH dehydrogenase subunit 1
Nrf1
Nuclear factor erythroid 2-like 1, NFE2L1
Nrf2
Nuclear factor erythroid 2-like 2, NFE2L2
NSG
NOD/SCID/IL1rg^{-/-} mice
OCR
Oxygen consumption rate
Oxphos
Oxidative phosphorylation
P
Synthetic progestin R5020
P4
Natural progesterone
PBS
Phosphate-buffered saline
PDX
Patient-derived xenograft
PGC1 α
PPAR-gamma coactivator alpha, PPARGC1A
PGC1 β
PPAR-gamma coactivator beta, PPARGC1B
PLS-DA
Partial least squares discriminant analysis
PPAR
Peroxisome proliferator activated receptor
PR
Progesterone receptor, PGR
qPCR

Quantitative real-time polymerase chain reaction
RNA
Ribonucleic acid
STR
Short tandem repeat
TCA
Tricarboxylic acid
tRNA
Transfer RNA
UCD
University of Colorado Denver; prefix for PDX-derived tumors and cell lines
UHPLC-MS
ultra-high performance liquid chromatography coupled with mass spectrometry
Veh
Vehicle for hormone treatment, ethanol
VIP
Variable importance in projections

Supplementary Files Legends

Figure S1. Progestins do not increase expression of key fission machinery. T47D cells were treated with vehicle (0.2% EtOH), E2 (10^{-8} nM), R5020 (10^{-8} nM), or E2+R5020 (10^{-8} nM each) for the indicated timepoints and analyzed for fission machinery. A-C) Transcripts corresponding to key fission machinery were evaluated through qPCR. Primer sets are reported in **Table S3**. Values are mean $\pm$ SEM of $\Delta\Delta\text{Ct}$ and represent at least 2 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for the indicated comparisons using one-way ANOVA. *n.s.* = not significant. D-E) T47D cells were treated with vehicle (0.2% EtOH), E2 (10^{-8} nM), R5020 (10^{-8} nM), or E2+R5020 (10^{-8} nM each) for 24, 48, or 72 hrs. Lysates were collected and analyzed by immunoblot with 100 mg protein loaded per lane. Incubation with rabbit polyclonal antibody against phospho-Ser⁶¹⁶ Drp-1 (1:1000) was followed by goat anti-rabbit fluorescent secondary. E) Incubation with mouse monoclonal antibody against Drp-1 (1:1000) was followed by goat anti-mouse fluorescent secondary. b-actin (1:2000) was used as a loading control for both antibodies. F) Protein levels for phospho-Ser⁶¹⁶ Drp-1 and total Drp-1 were normalized to loading control then reported as the ratio of phospho:total Drp-1.

Figure S2. Progestin co-treatment with estrogen decreases mitochondrial biogenesis factors. Quantitative real-time PCR analysis of mitochondrial biogenesis machinery, including PGC family members, Nrf1, and mitochondrial transcription factor A (TFAM), on UCD65 cells treated with vehicle (0.2% EtOH), E2 (10^{-8} nM), R5020 (10^{-8} nM), or E2+R5020 (10^{-8} nM each). Values are mean $\pm$ SEM of $\Delta\Delta\text{Ct}$ and represent at least 3 independent experiments. A-D) Statistical outcomes of comparisons between Veh vs. E2 and

between E2 vs. E2+P are reported. For C), these comparisons were not significantly different. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for the indicated comparisons using one-way ANOVA. *n.s.* = not significant.

Figure S3. Progestin co-treatment with estrogen mitigates E2-induced Nrf2 expression. T47D cells were treated with vehicle (0.2% EtOH), E2 (10^{-8} nM), R5020 (10^{-8} nM), or E2+R5020 (10^{-8} nM each) for 24 hrs. Lysates were collected and analyzed by immunoblot with 50 mg protein loaded per lane. Incubation with rabbit polyclonal antibody against Nrf2 (1:1000) was followed by goat anti-rabbit fluorescent secondary. α -tubulin (1:30000) was used as a loading control.

Figures

Figure 1

Progestins mitigate E2-driven metabolite changes. A) Partial least squares discriminant analysis (PLS-DA) scores plots of metabolomics data for four biological replicates of T47D, UCD65, and UCD4 cells treated with vehicle (0.2% EtOH), E2 (10-8 M), R5020 (R5, 10-8 M), or E2+R5020 (E2+R5, 10-8 M each) for 24 h. B) Top 50 variables for each cell line as indicated arranged in heatmaps (Pearson distance, average linkage, using PLS-DA variable importance scores). Pair-wise comparisons were performed for E2 vs.

E2+R5020-treated cells using random forest analysis to identify important variables and analyzed via MetPA (P-value cutoff for enrichment, $P < .05$). C) Pathways in each cell line as indicated that are down or up in comparison of E2+R5020 vs. E2 with corresponding $-\log(10)$ P-values representing enrichment.

Figure 2

Progestins shift cells towards glycolysis. A) Energetic phenotype from Seahorse XF Metabolic Analyzer Mito Stress Test following 24 h treatment of T47D, UCD65, and UCD4 cells with vehicle (0.2% EtOH), E2

(10-8 M), R5020 (R5, 10-8 M), or E2+R5020 (E2+R5, 10-8 M each). Phenotype for both cell lines is shown under basal conditions (top) or following induction of mitochondrial stress with oligomycin (ATP synthase inhibitor) + FCCP (membrane uncoupler) (bottom). OCR = oxygen consumption rate; ECAR = extracellular acidification rate. B) OCR and ECAR profiles of T47D, UCD65, and UCD4 cells under the different hormone treatments. Experiments were performed 3 times with one representative experiment shown.

Figure 3

Progestins reduce mitochondrial length. A) Cultured T47D and UCD65 cells treated with vehicle (0.2% EtOH), E2 (10⁻⁸ M), R5020 (R5, 10⁻⁸ M), or E2+R5020 (E2+R5, 10⁻⁸ M each) for 24 h were fixed, sectioned, and imaged using transmission electron microscopy. A representative image set is presented for each cell line and treatment. B) Quantitation of mitochondrial length in T47D and UCD65 cells was measured along the longest axis in Fiji in >200 mitochondria across 10-14 fields per treatment. Histograms represent mitochondrial length corresponding to 100-nm bins. T47D cells, mode: E2+R5020 = 400 nm; Veh, R5020 = 500 nm, E2 = 700 nm; UCD65 cells, mode: Veh, E2+R5020, R5020 = 300 nm; E2 = 400 nm. C) T47D and UCD4 xenograft tumors (2 each) from mice implanted hormone pellets containing E2 alone or E2+P4. A representative image set is presented for each tumor and treatment. D) Quantitation of mitochondrial length in T47D and UCD4 tumors (individual tumors plotted separately) was measured and plotted as in B. T47D tumors, mode: E2+P4 tumors 1,2 = 300; E2 tumors 1,2 = 500 nm; UCD4 tumors, mode: E2+P4 tumor 1, 300; E2+P4 tumor 2, E2 tumor 1 = 400 nm; E2 tumor 2 = 500 nm. *P <0.05, **P <0.01, ***P <0.001 for the indicated comparisons using Kolmogorov-Smirnov test for frequency distributions comparing hormone treatments.

Figure 4

Progestins reduce mitochondrial turnover. A) MitoGFP: Representative fields from laser scanning confocal images at 40X magnification on T47D cells treated with vehicle (0.2% EtOH), or P4 (10⁻⁸ M) for 48 h then labeled overnight with CellLights BacMam 2.0 MitoGFP. Cells were fixed in paraformaldehyde, counterstained with DAPI, and mounted on coverslips. Images were collected using confocal laser scanning microscopy (Zeiss LSM 780) with 40X objective. B) MitoTimer: Representative fields from laser

scanning confocal images taken at 10X magnification on T47D cells stably transduced with the doxycycline-inducible MitoTimer construct. Cells were pulsed with doxycycline for 1 h, washed, treated with hormone for 48 h, pulsed with Dox again, washout, and a final 24 h hormone treatment. Cells were fixed in 4% paraformaldehyde and imaged on an Olympus BX40 microscope at 10X magnification. C) Ratios of green to red fluorescent signal are shown and represent mean $\pm$ SEM of across 10 fields per treatment. Number of cells analyzed were vehicle (243), E2 (305), R5020 (240), and E2 plus R5020 (239). Data were log transformed to control for unequal variance, then analyzed by one-way ANOVA.

Figure 5

Progestins decrease expression of mitochondrial biogenesis factors. A) Cartoon of mitochondrial biogenesis schematic using BioRender (Toronto, Ontario). B-F) Quantitative real-time PCR analysis of mitochondrial biogenesis machinery, including Nrf2, PGC family members, Nrf1, and mitochondrial transcription factor A (TFAM), in T47D cells treated with vehicle (0.2% EtOH), E2 (10⁻⁸ M), R5020 (10⁻⁸ M), or E2+R5020 (10⁻⁸ M each). Values are mean $\pm$ SEM of $\Delta\Delta C_t$ and represent at least 4 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 for the indicated comparisons using one-way ANOVA at each time point.

Figure 6

Schematic of proposed progestin influence on breast cancer cellular metabolism. Schematic of progestin influence on cellular metabolism (made with BioRender, Toronto, Ontario). Progestins increased lactate production which indicates increased glycolysis activity. Pathway analysis suggests progestins increase Pentose Phosphate Pathway (PPP) activity which utilizes glycolysis intermediate, glucose-6-phosphate. Progestins promote Fatty Acid Biosynthesis which utilizes Acetyl-Co. Citrate can be pulled from the

Tricarboxylic Acid Cycle (TCA) cycle to satisfy Acetyl-CoA demands for Fatty Acid Biosynthesis. To prevent the TCA cycle from stalling, cells can undergo anaplerosis which converts glutamine and glutamate to alpha-ketoglutarate (alpha-KG) and restores TCA cycle flux (shown in blue). Our data suggests progestins decrease glutathione synthesis and amino acid synthesis further supporting glutamine use for anaplerosis during progestin treatment. GLUT1 = Glucose transporter 1, SLC7A5 = Solute Carrier Family 7 Member 5 (neutral amino acid transporter), SLC1A5 = Solute Carrier Family 1 Member 5 (neutral amino acid transporter), OAA = Oxaloacetate. Pathway activity increase indicated by green and decrease indicated by red.

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