Estrogen Receptor α Promotes Breast Cancer by Reprogramming Choline Metabolism

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Abstract

Estrogen receptor α (ERα) is a key regulator of breast growth and breast cancer development. Here, we report how ERα impacts these processes by reprogramming metabolism in malignant breast cells. We employed an integrated approach, combining genome-wide mapping of chromatin-bound ERα with estrogen-induced transcript and metabolic profiling, to demonstrate that ERα reprograms metabolism upon estrogen stimulation, including changes in aerobic glycolysis, nucleotide and amino acid synthesis, and choline (Cho) metabolism. Cho phosphotransferase CHPT1, identified as a direct ERα-regulated gene, was required for estrogen-induced effects on Cho metabolism, including increased phosphatidylcholine synthesis. CHPT1 silencing inhibited anchorage-independent growth and cell proliferation, also suppressing early-stage metastasis of tamoxifen-resistant breast cancer cells in a zebrafish xenograft model. Our results showed that ERα promotes metabolic alterations in breast cancer cells mediated by its target CHPT1, which this study implicates as a candidate therapeutic target. Cancer Res; 76(19); 5634–46. ©2016 AACR.

Introduction

Complementary to viewing cancer as a genetic disease, cancer can be considered a metabolic disease (1, 2). Altered metabolism in cancer directs enhanced nutrient acquisition and facilitates angiogenesis and choline (Cho) metabolism. Cho phosphotransferase CHPT1, responsible for Cho metabolism, is required for cell and tissue specific (17). Following uptake of Cho, the intracellular metabolism of Cho is partitioned along two major pathways: (i) converted to PCho for the synthesis of phosphatidylcholine (PtdCho), a major constituent of cell membranes; or (ii) oxidation to produce the methyl donor betaine (16). Abnormally high synthesis of PtdCho via the cytidine diphosphate–chole (CDP-Cho) pathway, where CTP:phosphocholine cytidylyltransferase (CCT) has been identified as the rate-limiting enzyme, is generally recognized as a metabolic hallmark of cancer (18, 19). PtdCho can also be synthesized through methylation of phosphatidylethanolamine (PE) by phosphatidylethanolamine N-methyltransferase (PEMT; ref. 20). The PEMT gene has been shown to be induced by estrogen in hepatocytes (20).

Increased levels of PCho and total Cho-containing metabolites have been identified as markers for breast cancer (21). Furthermore, increased synthesis of PtdCho is one of the earliest metabolic events associated with the initial stimulation of cell growth and proliferation by tumor promoters in normal cells (22–24). PtdCho has also been found to be increased in breast cancer cells by tumor promoter (25). Consistently, human breast cancer cells have been shown to have higher levels of PtdCho than normal human mammary epithelial cells (26). Underlying mechanisms and potential drug targets in abnormal Cho phospholipid metabolism have been widely investigated in different cancers, as reviewed in ref. 19. Overall, the regulation...
of Cho phospholipid metabolism in breast cancer cells has been shown to depend on breast cancer subtype with respect to gene expression profiles and metabolic fingerprints (27).

Several studies have provided important insights into global estrogen-regulated gene networks based on profiling ERα-binding regions and estrogen-regulated expression (28–33). To extend global estrogen-regulated networks to effects on breast cancer metabolism, we report a comprehensive analysis of estrogen-regulated metabolic pathways in two breast cancer cell lines. We integrate cistrome, transcriptome, and metabolome data to identify metabolic pathways regulated by estrogen signaling via ERα. We focus on effects conserved between two ERα+ breast cancer cell lines with the aim to identify general effectors of metabolic signaling rather than cell type-specific effects.

Materials and Methods

Additional and detailed methods are included in the Supplementary Materials and Methods.

Cell culture

MCF7 cells developed at the Michigan Cancer Foundation (Detroit, MI) were kindly provided by Dr. Robert P.C. Shiu (University of Manitoba, Winnipeg, Manitoba, Canada; 2012). T47D cells were purchased from the ATCC (2004). Tamoxifen-sensitive MCF7 cells and tamoxifen-resistant LCC2 cells were kindly provided by Dr. Janne Lehtiö (Karolinska Institutet, Stockholm, Sweden; 2012). LCC2 cells originate from MCF7 cells. These cell lines were authenticated by short tandem repeat profiling (Upsala Genome Center, Upsalla, Sweden) in June 2016. MCF7 and LCC2 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/Streptomycin (Gibco). T47D cells were grown in RPMI1640 supplemented with 10% FBS and 1% penicillin/Streptomycin. DMEM and RPMI1640 culture medium contain 28.57 and 21.43 μmol/L Cho, respectively. The content of Cho and Cho phospholipid in bovine serum is not provided by the manufacturer. No additional growth factors were added to the cell culture medium.

Chromatin immunoprecipitation followed by sequencing or qPCR

Chromatin immunoprecipitation (ChIP) was performed as described previously (34).

Gene expression microarray analysis

The gene expression data for MCF7 cells has been published previously (33). For T47D cells, Human Gene 2.1 ST Arrays were used for analysis of global gene expression profiling. The microarray data are deposited in GEO (accession number GSE36683 and GSE74034 for MCF7 and T47D cells, respectively).

Gene ontology (GO) analysis and identification of enriched pathways were performed using the WEB-based GENE Set Analysis Toolkit.

Nuclear magnetic resonance spectroscopy

Water-soluble metabolites were extracted using ethanol. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 600 MHz spectrometer. A multivariate comparison of metabolic profiles from estrogen-stimulated and control cells was performed using partial least squares discriminant analysis on Pareto-scaled NMR spectra using PLS_Toolbox v7.5.2 (Eigenvector Research Inc.). This technique identifies linear combinations of metabolic features referred to as latent variables (LV) that discriminate between classes of samples. Quantification was performed by binning spectral regions containing signals from identified metabolites. To aid in the identification of these metabolites, Chenomx NMR suite v7.7 (Chenomx Inc.) was used. In addition, various 2D NMR spectra (HSQC, HMBC, COSY, TOCSY) were recorded to assure identification.

Tissue microarray analysis

CHPT1 expression in human breast cancers was analyzed in tissue microarrays (TMA; US BioMax BR1503d) by IHC. Anti-CHPT1 antibody was from The Human Protein Atlas.

Zebrafish metastatic model

Tamoxifen-sensitive MCF7 cells and tamoxifen-resistant LCC2 cells were used. The zebrafish metastatic model was established as described previously (35).

Results

A core set of direct ERα-regulated genes in ERα+ breast cancer cells

To identify a core set of direct ERα-regulated genes, we combined genomewide ERα-binding profiles with detailed transcript profiling for the two breast cancer cell lines, MCF7 and T47D. A total of 18,040 and 12,659 ERα-binding regions were identified for MCF7 cells and T47D cells, respectively (Fig. 1A).

General properties of the identified ERα-binding regions, such as peak distribution and enriched motifs, are consistent with previously published studies (Supplementary Fig. S1A–S1C). Overlapping the MCF7 and T47D cistromes revealed 6,480 shared binding regions, corresponding to 36% and 51% of the MCF7 and T47D cistromes, respectively (Fig. 1A and B). The shared binding regions overlapped with published ERα cistromes for breast cancer cell lines (Supplementary Fig. S1D).

Global gene expression profiling revealed 2,531 and 1,800 estrogen-regulated genes in MCF7 and T47D cells, respectively (Fig. 1C). Overlapping the estrogen-regulated MCF7 and T47D transcriptomes identified 420 common estrogen-induced and 348 common estrogen-repressed genes (Fig. 1C). ERα binding was enriched in regions associated with estrogen-induced genes as compared with repressed genes for both MCF7 and T47D cells (Fig. 1D). To further identify a core set of direct ERα target genes common to MCF7 and T47D cells, we combined genome-wide ERα-binding profiles and estrogen-regulated transcript profiles focusing on the 4,739 ERα-binding regions within 25 kb up- and down-stream of TSSs of their most proximal genes. Integrating these ERα-binding regions with estrogen-regulated transcript profiling, we identified a core set of 207 direct ERα target genes (Supplementary Table S1). A number of well-established direct ERα target genes were included in the identified core set, such as trefoil factor 1 (TFF1), growth regulation by estrogen in breast cancer 1 (GREB1), and progesterone receptor (PR). A majority (71%) of the core set of direct ERα target genes were induced by E2 treatment (Fig. 1E).

GO pathway analysis for the core set of direct ERα target genes showed significant enrichment of cancer pathways. Interestingly, metabolic pathways were also enriched (Fig. 1F).

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A core set of direct ERα-regulated genes in breast cancer cells. A, Venn diagram showing overlap of ERα cistromes between MCF7 and T47D cells. Cells were cultured in steroid-depleted media and treated with 10 nmol/L E2 or ethanol for 45 minutes. Genome-wide ERα-binding sites were determined by ChIP-seq. B, peak intensity heatmaps of ERα-binding regions in a ±5 kb, relative to the TSS, genomic window. C, overlap of estrogen-induced up- and downregulated genes, respectively, for MCF7 and T47D cells. Cells were cultured in steroid-depleted media and treated with 10 nmol/L E2 or ethanol for 6 hours. Estrogen-stimulated gene expression was assayed by microarray analysis (n = 4 for MCF7 cells; n = 3 for T47D cells). D, correlation of estrogen-regulated gene expression with ERα-binding intensity. Heatmaps show the expression changes of all genes that were ranked on the basis of high to low fold change values in MCF7 or T47D cells. The line graphs represent the moving average plots (window size, 100; step size, 1), which were plotted as a function of average ERα-binding of genes. These genes were arranged according to the heatmaps. E, a core set of direct ERα up- and downregulated genes in breast cancer cells. Overlay of ERα cistromes with estrogen up- and downregulated genes common to MCF7 and T47D cells. F, GO network analysis for the core set of direct ERα-regulated genes. G, direct ERα-regulated metabolic genes. ERα binding is presented as the score obtained from peak analysis using MACS. Estrogen-stimulated gene expression is illustrated as fold change (FC) of estrogen treatment versus vehicle control.
Estrogen signaling leads to global metabolic reprogramming in breast cancer cells

We determined the effect of estrogen signaling on levels of intracellular and extracellular metabolites using proton NMR (1H NMR). Figure 2A demonstrates a clear effect of estrogen signaling on the intracellular metabolic profile for both cell lines. Samples from estrogen-treated cells were more separated from the control samples along LV1 for MCF7 cells compared with T47D cells, indicating a stronger metabolic response to E2 stimulation in MCF7 cells. Notably, the metabolic profiles from MCF7 and T47D cells were clearly separated along LV2 (Fig. 2A), indicating that the metabolic characteristics of MCF7 and T47D are inherently different. Quantitative analyses of the NMR spectra normalizing to protein levels are shown in Supplementary Table S2. Notably, the area-normalized spectra were used for identification of changed metabolites (Supplementary Table S3). For the MCF7 cell line, 29 unique metabolites were quantified. Levels of 19 of these metabolites were significantly modulated (FDR-adjusted P < 0.05) upon estrogen treatment (Supplementary Table S3; Supplementary Fig. S2A). For the T47D cell line, levels of 13 of 29 metabolites were significantly modulated upon estrogen treatment (Supplementary Table S3; Supplementary Fig. S2B). All estrogen-modulated intracellular metabolites, seven of which were changed in both cell lines, were mapped to metabolic pathways (Fig. 2B). Estrogen modulated amino acid synthesis in both cell lines (Supplementary Fig. S2A and S2B), resulting in increased phenylalanine, tyrosine, and 1-methyl histidine levels (Fig. 2B). In addition, estrogen modulated the Cho metabolic pathway, with PCho being reduced in both cell lines (Fig. 2B). However, we also observed differential effects of estrogen on Cho-containing metabolites between these two cell lines, with glycerophosphocholine (GPC) levels being reduced in MCF7 cells and Cho levels increased in T47D cells (Supplementary Fig. S2A and S2B).

Analysis of extracellular metabolites revealed that upon estrogen treatment, MCF7 cells consumed more glucose and produced more lactate (Fig. 2C and D), leading to a higher lactate/glucose ratio (Fig. 2E). Surprisingly, T47D cells consumed significantly higher amounts of glucose than MCF7 cells regardless of estrogen treatment (Fig. 2C, where the extracellular concentration of glucose is much lower for T47D cells compared with MCF7 cells). Similarly as observed for MCF7 cells, estrogen stimulation enhanced glucose consumption and the lactate/glucose ratio for T47D cells (Fig. 2C and E), supporting elevated aerobic glycolysis upon activation of estrogen signaling in E2-† breast cancer cells. However, estrogen treatment did not affect lactate levels in T47D cells (Fig. 2D).

Estrogen signaling regulates transcripts and metabolites of the Cho metabolic pathway in breast cancer cells

The glycerophospholipid pathway, which includes the Cho metabolic pathway, was enriched for direct ERTx target genes common to MCF7 and T47D cells (Fig. 1F). Specifically, of the 119 genes involved in the KEGG Homo sapiens glycerophospholipid pathway hsa: 00654 (27), 26 and 10 were regulated by estrogen in MCF7 and T47D cells, respectively (Fig. 3A). Regulation of a subset of these genes was confirmed using qPCR (Fig. 3B).

In addition, metabolic profiling confirmed alterations in Cho metabolism in response to estrogen signaling for these two cell lines (Fig. 2B and Supplementary Fig. S2A and S2B). Figure 3C shows changes in the levels of metabolites in the Cho metabolic pathway upon estrogen stimulation. NMR metabolic profiling of polar extracts is not suitable for determination of the liposoluble metabolite PtdCho, and CDP-Cho is present in too low concentration for detection by NMR. To obtain a more complete overview of the effects of estrogen on Cho metabolism, we assayed these metabolites by alternative assays, that is, PtdCho levels by PtdCho Assay Kit and CDP-Cho levels by LC-MS. We observed increased levels of CDP-Cho in MCF7 cells after estrogen stimulation, while no difference in T47D cells was observed (Fig. 3C). Interestingly, PtdCho levels were significantly increased 24 hours after estrogen treatment in both MCF7 and T47D cells (Fig. 3D). Furthermore, CHPT1, the direct upstream enzyme to catalyze PtdCho synthesis, was identified as the only direct ERTx target gene in the Cho pathway, which was upregulated upon estrogen stimulation in both analyzed cells (Fig. 3A, E, and F).

All estrogen-regulated genes and metabolites in the Cho metabolic pathway are indicated in Fig. 3G. The expression of cell membrane Cho transporters, solute carrier family 44 members 1 and 2 (SLC44A1, SLC44A2) encoding CTL1 and CTL2, was reduced significantly after estrogen stimulation in MCF7 cells (Fig. 3B). The expression of CHKB and CHKA, which are responsible for Cho phosphorylation, was reduced by estrogen in MCF7 and T47D cells, respectively (Fig. 3B). Consistently, PCho levels were decreased in both cell lines (Supplementary Table S4). Phospholipase A2, group VI (PLA2G6) was downregulated upon estrogen stimulation in MCF7 cells (Fig. 3B), which may result in decreased levels of its downstream product GPC, which is consistent with reduced GPC levels in this cell line in response to estrogen stimulation. The expression of PLCd1 and PLCe1 was significantly decreased in estrogen-treated MCF7 cells (Fig. 3B). Another isofrom of PLC, PLCB3, was downregulated by estrogen in T47D cells (Fig. 3B). Downregulation of PLCs may result in less PCho production, consistent with what was observed for MCF7 and T47D cells (Supplementary Table S4). However, it should be noted that changes in gene expression do not necessarily translate into changes in enzyme activity and metabolite concentrations.

GPC is formed by the deacylation of PtdCho. Elevated PCho/GPC ratio has been observed in breast cancer cell lines compared with normal breast epithelial cells (36). Furthermore, it has been proposed that this ratio can predict breast cancer aggressiveness (37, 38). Our results show that estrogen stimulation increased the PCho/GPC ratio in MCF7 cells (Supplementary Table S4). On the contrary, in T47D cells, the PCho/GPC ratio was reduced (Supplementary Table S4).

Estrogen stimulation increases the activity of CCTx

CCTx is a key enzyme in the CDP-choline pathway for de novo PtdCho biosynthesis. This enzyme is inactivated when it is phosphorylated and activated by a phosphatase, which...
ERα reprograms cell metabolism. Cells were cultured in steroid-depleted media and treated with 10 nmol/L E2 or ethanol for 24 hours. Intra- and extracellular metabolites were extracted and analyzed by 1H NMR. A, partial least squares discriminant analysis of NMR identified intracellular metabolites in MCF7 and T47D cells. Data are plotted using two latent variables (LV1 and LV2; n = 5). B, metabolic profile of MCF7 and T47D cells in response to estrogen stimulation. Metabolites in red, metabolic changes observed only in MCF7 cells; metabolites in green, metabolic changes observed only in T47D cells; metabolites in blue, metabolic changes observed in both MCF7 and T47D cells. Full names of the metabolites are shown in Supplementary Data. TCA, the citric acid cycle. C, decreased glucose levels in the culture medium in response to estrogen treatment for MCF7 and T47D cells. D, lactate levels in the culture medium in response to estrogen. Increased lactate levels are observed in MCF7 cells but not in T47D cells. E, increased lactate/glucose ratio in the culture medium in response to estrogen treatment for MCF7 and T47D cells. C–E, data, mean ± SD (n = 5). Student t test was used for calculation of statistical significance.
Figure 3.

ERα regulates the Cho metabolic pathway. **A**, heatmap of estrogen-regulated genes in the Cho metabolic pathway for MCF7 and T47D cells, respectively. ETOH, ethanol. **B**, confirmation of a subset of estrogen-regulated genes in the Cho metabolic pathway using qRT-PCR for MCF7 and T47D cells, respectively. TFF1 was used as positive control for estrogen-stimulated gene expression. The assay was performed in triplicates. **C**, change of CDP-Cho level in response to estrogen treatment. **D**, increased PtdCho levels in response to estrogen treatment for MCF7 and T47D cells. **E**, ERα-binding site within the CHPT1 gene locus derived from ChIP-seq. **F**, ChIP-qPCR confirms recruitment of ERα to the CHPT1 gene. Data, fold enrichment relative to IgG. **G**, changes in gene expression levels and metabolite levels in the Cho metabolic pathway upon estrogen treatment for MCF7 and T47D cells, respectively. Gene names in red, upregulated genes; gene names in green, downregulated genes; arrow, changes in metabolite levels; red arrows, upregulated metabolites; green arrows, downregulated metabolites. **C, D, and F**, data, means ± SD (n = 5 for C and n = 3 for D and F). Student t test was used for calculation of statistical significance.
allows it to translocate to membranes (39). To understand whether E2 regulates CCT activity, we investigated the cellular distribution of CCTα. Notably, CCTα expression was detected in cytosol, membrane, and nuclear fractions (Fig. 4A). Interestingly, the level of CCTα was significantly decreased in the cytosol fraction, while it was increased in the membrane upon E2 stimulation in MCF7 and T47D cells (Fig. 4A), suggesting that CCTα was recruited to the membrane and activated in response to E2 treatment. However, no change of CCTα was observed in nuclear fraction upon E2 treatment (Fig. 4A). The separation of cytosol, membrane, and nuclear proteins was confirmed by assaying the cytosolic proteins GAPDH and β-tubulin, the membrane-associated proteins cadherin and TIM 23, and nuclear-associated protein lamin (Fig. 4A).

CHPT1 is critical for estrogen-induced PtdCho synthesis

E2 stimulation led to upregulation of both CHPT1 and its direct downstream metabolite PtdCho (Fig. 3B and D). To confirm the role of CHPT1 in estrogen regulation of PtdCho, we assayed PtdCho levels upon CHPT1 depletion with and without E2 treatment in MCF7 and T47D cells. Efficient knockdown of CHPT1 was confirmed by qRT-PCR and Western blot analysis (Fig. 4B). Importantly, PtdCho levels decreased significantly after CHPT1 knockdown, supporting a critical role of CHPT1 in regulating PtdCho synthesis (Fig. 4C). Furthermore, the effect of estrogen in promoting PtdCho synthesis was significantly reduced after CHPT1 knockdown (Fig. 4C), suggesting that estrogen-induced PtdCho synthesis is dependent on CHPT1 expression. To further explore how metabolites of the Cho metabolic pathway are affected upon CHPT1 depletion, we determined levels of Cho-containing metabolites by 1H-NMR in MCF7 cells upon CHPT1 depletion compared with control in the presence and absence of E2. As shown in Fig. 4D, CHPT1 depletion increased the levels of Cho, suggesting that CHPT1 contributes significantly to metabolic turnover in the Cho pathway in MCF7 cells. Interestingly, a significant reduction of the PCho/GPC ratio was observed upon CHPT1 depletion, and additionally, increase of the PCho/GPC ratio by E2 was abolished by CHPT1 depletion (Fig. 4E). This indicates that CHPT1 is a critical regulator of the PCho/GPC ratio, which previously has been suggested as a potential prognostic biomarker in breast cancer (40). To understand whether the PEMT pathway contributes to the increase in PtdCho levels, we assayed PEMT mRNA levels in response to E2 stimulation. However, the expression of PEMT was not induced by estrogen in the assayed breast cancer cell lines (Supplementary Table S5).

CHPT1 increases anchorage-independent growth and proliferation of breast cancer cells

To further uncover the role of CHPT1 in ERα breast cancer cells, we examined anchorage-independent growth and proliferation after CHPT1 knockdown. As shown in Fig. 5A and B, CHPT1 knockdown reduced the number of colonies of MCF7 and T47D cells in soft agar compared with the control. Furthermore, we observed that knockdown of CHPT1 decreased cell proliferation in both cell lines (Fig. 5C).

CHPT1 is overexpressed in breast cancer

To confirm CHPT1 dysregulation in breast cancer, we determined CHPT1 protein levels in tumor tissue and adjacent normal breast tissue by IHC, using TMAs for which data were provided regarding tumor nodes and metastasis (TNM), clinical stage and pathology grade, and IHC staining for HER-2, ER, and PR. Consistent with published data by The Human Protein Atlas (http://www.proteinatlas.org/ENSG0000011666-CHPT1/tissue), we observed that almost all CHPT1 staining was localized to the cytoplasm (Fig. 5D). Although cytoplasmic staining was observed in both normal and cancerous tissue, staining was stronger for tumor tissues (Fig. 5D and E). Interestingly, higher CHPT1 expression was observed in ER+ breast cancer compared with ER− breast cancer (Supplementary Table S6), consistent with CHPT1 being an ERα target gene. There was no significant correlation between CHPT1 expression and HER-2, TNM, clinical stage, and pathology grade (Supplementary Table S6).

Knockdown of CHPT1 inhibits early stage of metastasis of tamoxifen-resistant breast cancer cells in vivo

To increase knowledge about the role of CHPT1 in invasion of tamoxifen-resistant breast cancer cells, we performed Transwell cell invasion assays for both tamoxifen-sensitive MCF7 cells and tamoxifen-resistant LCC2 cells upon CHPT1 knockdown (Fig. 6A). The invasion assay showed that LCC2 cells were more invasive than MCF7 cells (Fig. 6B). Knockdown of CHPT1 markedly inhibited invasion of both MCF7 and LCC2 cells (Fig. 6B). To further study the role of CHPT1 in regulating early stage of metastasis of tamoxifen-resistant breast cancer cells in vivo, we used a zebrafish tumor model (36). Tumor-implanted fish embryos were scored for the dissemination of tumor cells at day 4 after injection. Control siRNA–treated LCC2 cells disseminated more widespread in the fish body as compared with control siRNA–treated MCF7 cells. Reduced dissemination of tumor cells was observed for both MCF7 and LCC2 cells after CHPT1 knockdown (Fig. 6C). Notably, a stronger suppression of invasion and metastasis following CHPT1 depletion was found in LCC2 cells compared with MCF7 cells (Fig. 6D).

Discussion

Here, we combine global determination of ERα-binding regions with global determination of estrogen-induced gene expression for two breast cancer cell lines to define a set of 207 core direct ERα target genes. The two ERα breast cancer cell lines investigated in this study, MCF7 and T47D, represent distinct molecular backgrounds for ERα activity in breast cancer (32, 41). Notably and consistent with previous findings, the number of ERα-binding regions and fold induction in expression of estrogen-induced genes in MCF7 cells exhibit greater sensitivity to estrogen treatment as compared with T47D cells (32, 41). This may be due to differential impact of chromatin configuration (32) and/or different ERα expression levels between the cell lines (41).

The enriched pathways in the reported core set of direct ERα target genes included cancer pathways and metabolic pathways (Fig. 1F) and combined with the metabolic profiling reveal details of estrogen-induced metabolic reprogramming in breast cancer cells. Metabolic profiling revealed metabolites with conserved modulation by estrogen between the analyzed cell lines, including higher uptake of glucose and elevated levels of PtdCho, tyrosine, phenylalanine, and 1-methylhistidine and decreased levels of PCho and ATP (Fig. 2B), which
Figure 4.
CHPT1 is critical for estrogen-induced PtdCho synthesis. A, decreased levels of CCTα in the cytosol and increased levels of CCTα in the membrane in response to estrogen treatment in MCF7 and T47D cells. GAPDH is a marker for cytosol (C) proteins, cadherin is a marker for membrane (M) proteins, and lamin is a marker for nuclear (N) proteins. ETOH, ethanol. TIM23 was used as a loading control for membrane proteins. α-Tubulin was used as a loading control for cytosol proteins, and lamin was a loading control for nuclear proteins. B, reduced mRNA and protein levels of CHPT1 72 hours after siRNAs knockdown. 36B4 was used for mRNA normalization, and β-actin was used as a loading control for Western blot analysis. C, estrogen treatment increases PtdCho levels dependent on CHPT1. D, Cho levels after CHPT1 depletion with and without E2 treatment in MCF7 cells. E, PCho/GPC ratio after CHPT1 depletion with and without E2 treatment in MCF7 cells. B–E, cells were transfected with control or CHPT1 siRNA. Transfected cells were cultured in steroid-depleted media and treated with 10 nmol/L E2 or ethanol for 24 hours, after which the lipid metabolites were extracted and quantified using a Phosphatidylcholine Assay Kit (Abcam), and the water-soluble metabolites were extracted and quantified by 1H-NMR. Data, means ± SD (n = 3). Student t test was used for calculation of statistical significance.
accounts for only 1/3 and 1/2 of E2-regulated metabolites in MCF7 and T47D cells, respectively. This complex response could be related to that the compositions of culture media, inherent metabolic characteristics (42), and E2-induced changes in gene expression differ between the two cell lines. A previous study showed that cell culture conditions, confluence, serum deprivation, and acidic extracellular pH could all affect metabolite levels (43). Furthermore, we have previously demonstrated that distinct metabolic profiles are associated with differences in gene expression for different subtypes of breast cancer (27). However, importantly, estrogen-regulated metabolites for the two breast cancer cell lines were related to four metabolic pathways, aerobic glycolysis, nucleotide and amino acid synthesis, and glycerophospholipid metabolism, suggesting that ERα activation may increase the production of metabolic intermediates for the synthesis of proteins, nucleic acids, and lipids to support the rapid proliferation of cancer cells.

Alterations in membrane phospholipids are associated with malignant transformation (36), tumorigenicity (19), and metastasis (44). Interestingly, glycerophospholipid metabolism was one of the enriched pathways of direct ERα target genes, and additionally, our metabolic profiling showed alterations in Cho-metabolite levels in response to estrogen signaling. We report that E2 decreased PCho levels and increased PtdCho levels in both MCF7 and T47D cells (Supplementary Table S4; Fig. 3D). E2 suppression of PCho in T47D cells is consistent with a previous report (12). Metabolite levels are regulated by rate of synthesis and consumption of the metabolite. Reduced PCho could be attributed to decreased CHK...
Knockdown of CHPT1 inhibits early stage of metastasis of tamoxifen-resistant breast cancer cells in vivo. A, reduced mRNA and protein levels of CHPT1 96 hours after siRNA transfection. 36B4 was used for mRNA normalization, and β-actin was used as a loading control for Western blot analysis. B, CHPT1 depletion reduces MCF7 and LCC2 cell invasiveness. A and B, data, means ± SD (n = 3). Student t test was used for calculation of statistical significance. C, CHPT1 knockdown results in reduced dissemination of MCF7 and LCC2 cells in the zebrafish metastasis assay. Left, 48 hours after fertilization (hpf), zebrafish embryos of the Tg(flit1:EGFP)y1 strain in which blood vessel endothelial cells express EGFP (green) were injected in the perivitelline space with approximately 300 DiI-labeled cells (red) transfected either with control siRNA or with siRNA complementary to CHPT1 in and imaged at 120 hpf. Regions indicated by white boxes in the upper full-embryo image were enlarged in the lower images. White arrowheads, tumor cells. Inj., injection; mets, metastases. Scale bars, 500 μm (top) and 100 μm (bottom). Right, quantification of the number of the cells in the region anterior to the intestine from the experiments. Student t test was used for calculation of statistical significance (n = 30–35 embryos). D, inhibition of invasion or metastasis after CHPT1 knockdown. Data, means ± SD. Student t test was used for calculation of statistical significance.
expression (Fig. 3B; Supplementary Table S4), but also to increased CCTα activity leading to higher consumption of PCho for CDP-Cho synthesis (Figs. 3C and 4A). Moreover, downregulation of PLC could also result in less PCho production (Fig. 3B). Furthermore, we could not exclude the possibility that PCho is dephosphorylated by phosphatases. However, it should be noted that the relationship between transcript levels and metabolite concentrations in the Cho pathway is highly complex and depends on several collateral biochemical reactions. Furthermore, it is difficult to correlate changes in metabolic flux through the pathway to steady-state metabolite levels. The metabolic effects of estrogen stimulation can therefore not be conclusively determined from changes in gene expression.

The activity of CHPT1 is regulated by thyroid hormone (45) and by arginosuccinate. Here, we demonstrate that E2 stimulation led to upregulation of CHPT1 and increased PtdCho levels in breast cancer cells (Fig. 3B and D). Notably, CHPT1 depletion not only decreased PtdCho levels, but also increased Cho levels in MCF7 cells (Fig. 4C and D). Accumulation of Cho may be the result of reduced flux through the pathway due to reduced CHPT1 activity. Our results suggest that CHPT1 contributes to regulation of PtdCho synthesis in the context of ER signaling in breast cancer. CCT, the rate-limiting enzyme for PtdCho synthesis, has been reported to display increased expression in cancer (46, 47). Interestingly, no induction of PCYT, which encodes CCT, was found upon E2 stimulation (Fig. 3G). However, E2 stimulation increased the activity of CCTα (Fig. 4A), which could result in sufficient substrate production for CHPT1 (CDP-Cho) to synthesize PtdCho (Fig. 3C). Hence, our study indicates that increased CHPT1 expression and increased CCTα activity was involved in the estrogen-induced increase in PtdCho synthesis (Supplementary Table S5).

Previous studies have shown upregulation of CHPT1 mRNA levels and activity in human breast cancer cells compared with normal mammary epithelial cells (48, 49). Furthermore, the PCho/GPC ratio has been associated with malignant transformation (36, 50). In agreement with this, CHPT1 knockdown reduced the PCho/GPC ratio in MCF7 cells (Fig. 4E) and decreased anchorage-independent growth (Fig. 5A and B) and proliferation of breast cancer cells (Fig. 5C).

In conclusion, our study has uncovered that ERα activation reprograms metabolism in breast cancer cells. We identify ERα direct target genes by integrating global ERα chromatin binding with global estrogen-regulated gene profiling. Metabolic profiling confirms functional consequences of these estrogen-mediated transcriptional changes. We show, for the first time, that the ERα target gene CHPT1 plays an essential role in estrogen-induced increases in PtdCho levels. Furthermore, knockdown of CHPT1 reduces malignant phenotype and proliferation of breast cancer cells. Importantly, CHPT1 depletion greatly suppresses early stage of metastasis of tamoxifen-resistant breast cancer cells in vivo. Mechanistically, estrogen-stimulated CHPT1 upregulation leading to increased PtdCho synthesis could contribute cell membrane synthesis (Fig. 7). Finally, as CHPT1 is overexpressed in breast cancer supports it is a potential drug target to be further investigated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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Conception and design: M. Jia, T.F. Bathen, H. Gao, C. Zhao, Y. Cao, S.A. Moestue, K. Dahlman-Wright
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References
Editor’s Note: Estrogen Receptor α Promotes Breast Cancer by Reprogramming Choline Metabolism

Min Jia, Trygve Andreassen, Lasse Jensen, Tone Frost Bathen, Indranil Sinha, Hui Gao, Chunyan Zhao, Lars-Arne Haldosen, Yihai Cao, Leonard Girnita, Siver Andreas Moestue, and Karin Dahlman-Wright

The editors are publishing this note to alert readers to concerns about this article (1). The editors were made aware of duplicated Western blot bands in Fig. 4A. Specifically, two sets of Western blot bands are duplicates: (i) T47D CCTα in the top right panel and T47D cadherin in the bottom right panel; and (ii) MCF7 CCTα band in the top right panel and MCF7 N in the bottom right panel. Because satisfactorily corrected figures could not be provided, the editors are publishing this note to alert readers to these concerns.

Reference

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