CARM1 Is an Important Determinant of ERα-Dependent Breast Cancer Cell Differentiation and Proliferation in Breast Cancer Cells

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Abstract

Breast cancers with estrogen receptor α (ERα) expression are often more differentiated histologically than ERα-negative tumors, but the reasons for this difference are poorly understood. One possible explanation is that transcriptional cofactors associated with ERα determine the expression of genes which promote a more differentiated phenotype. In this study, we identify one such cofactor as coactivator-associated arginine methyltransferase 1 (CARM1), a unique coactivator of ERα that can simultaneously block cell proliferation and induce differentiation through global regulation of ERα-regulated genes. CARM1 was evidenced as an ERα coactivator in cell-based assays, gene expression microarrays, and mouse xenograft models. In human breast tumors, CARM1 expression positively correlated with ERα levels in ER-positive tumors but was inversely correlated with tumor grade. Our findings suggest that coexpression of CARM1 and ERα may provide a better biomarker of well-differentiated breast cancer. Furthermore, our findings define an important functional role of this histone arginine methyltransferase in reprogramming ERα-regulated cellular processes, implicating CARM1 as a putative epigenetic target in ER-positive breast cancers. Cancer Res; 71(6); 2118–28. ©2011 AACR.

Introduction

In normal breast tissue, estrogen receptor α (ERα) regulates growth and development of the mammary gland by regulating the balance between cell proliferation and differentiation. This balance is deregulated in cancer. Enhanced ERα proliferative action contributes to the initiation and progression of breast cancer (1) by promoting cell-cycle progression, in particular S-phase entry (2, 3). Microarray analyses using breast cancer cell lines have revealed that a majority of ERα target genes are involved in metabolism and cell-cycle regulation (4–6). ERα is expressed in nearly 70% of breast cancers. Interestingly, ERα-positive tumors are more histologically well-differentiated (7). ERα decreases in high-grade tumors (8), and its presence serves as a hallmark of differentiation and predictor of low aggressiveness and favorable disease-free survival (9, 10). The protective effect of ERα raises the possibility that ERα functions to regulate both proliferation and differentiation in breast cancer cells, albeit with the balance tilted toward proliferation. Cell proliferation and differentiation are 2 mutually exclusive processes. Forced differentiation of primary tumors with therapeutic compounds can inhibit proliferation (11). Differentiation therapy such as all-trans retinoic acid was successfully used in treating acute promyelocytic leukemia. However, this strategy is not widely applied to breast carcinomas because breast tumors are more heterogeneous. Moreover, how ERα regulates the balance of proliferation and differentiation is not well understood.

ERα regulates transcription through recruitment of multiple cofactors (12). Although ERα coactivators share the common feature of activating ERα in transcriptional assays, to date, no ERα coactivator has been reported to promote differentiation in breast cancer cells. Coactivator-associated arginine methyltransferase 1 (CARM1) was originally identified as a steroid receptor coactivator which activates transcription of ERα target genes (13, 14). Furthermore, loss of CARM1 in the mouse embryo leads to abrogation of the estrogen response and reduced expression of some ER target genes (15), highlighting the significance of CARM1 in ER-regulated processes. CARM1 is a multifunctional protein engaged in a variety of cellular processes including gene expression (16), coupling of transcription and mRNA processing (17), regulating protein stability (18), and tissue development (15). However, the function of CARM1 in regulating cell differentiation or proliferation is contradictory and seems to be context dependent. CARM1 is required for differentiation of adipocytes (19), myocytes (20),...
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pulmonary epithelial cells (21), and early thymocyte progenitor cells (22). In contrast, CARM1 was implicated in cancer cell proliferation and was shown to regulate the expression of E2F1 and cyclin E1 factors promoting cell-cycle progression (16, 23). Thus, functions of CARM1 in ERα-dependent breast cancer require further elucidation.

Here we report an extensive study of the biological function of CARM1 in ERα-regulated processes in breast cancer cells, using both gain-of-function and loss-of-function approaches.

Materials and Methods

Cell culture maintenance and construction

All cell lines were purchased from American Type Culture Collection (ATCC) and used within 6 months. MCF7-Tet-on-shCARM1 was generated by 2 steps: First, we synthesized shRNA-encoding oligo DNA "CGCGGAGATCCAGCGCCAC" targeting human CARM1 and cloned the sequence into pLVTHM plasmid (24). MCF7 cells (ATCC HTB-22) were sequentially infected with the lenti-KRAB and pLVTHM-CARM1 shRNA vectors, followed by selection of single clones by Western blotting. MCF7-Tet-on-CARM1 was generated by cotransfecting pTRE-tight CARM1 plasmid with pBabe-puro vector controls, MCF7-CARM1 cells have only a 2-fold increase of CARM1 in MCF7-CARM1, because compared with MCF7-vector controls, MCF7-CARM1 cells have only a 2-fold increase in CARM1 expression (Fig. 1B). Consistent with the growth phenotype, the expression of the CDK inhibitors p21cip1 and p27kip1 was elevated in MCF7-CARM1 treated with 17beta-estradiol (E2) (Fig. 1B). Also the expression of p21cip1 and p27kip1 was stimulated by E2 in a time-dependent manner in MCF7-CARM1 cells (Supplementary Fig. 1) but not in parental MCF7 cells, suggesting that ERα and CARM1 are involved in regulating their expression. The effect of CARM1 on anchorage-independent cell growth was determined using soft-agar assays. E2 stimulates colony formation of MCF7-vector cells; in contrast, no colonies were formed in soft agar with MCF7-CARM1 (Fig. 1C). This result suggests that overexpressing CARM1 in MCF7 may inhibit anchorage-independent growth. In contrast to MCF7, no growth effects were detected by overexpressing or knocking down CARM1 in MDA-MB-231, an ERα-negative breast cancer cell line (Fig. 1D and E). Consistent with it being ERα negative, the growth rate of MDA-MB-231 was E2 independent (Fig. 1D). Similarly, overexpressing CARM1 exhibits no growth effect on MDA-MB-468, another ERα-negative breast cancer cell line (Supplementary Fig. 2), supporting the notion that the growth inhibitory effect of CARM1 in MCF7 is ERα dependent. The growth inhibitory effect of CARM1 was further validated in another ERα-positive breast cancer cell line ZR-75 (Supplementary Fig. 3). p21cip1 has been reported to induce both cell cycle arrest and cell

Additional assays and methods

Cell growth assays, quantitative real-time PCR (qRT-PCR), cell-cycle profiling, CARM1 Ab characterization, mouse xenograft experiments, and microarray gene expression analyses are described in Supplementary Methods and Materials. The microarray GEO numbers are GSE26454 and GSE26259.

Statistical analysis

One-way ANOVA and ANOVA single-factor analysis was applied and a value of P < 0.05 was regarded as statistically significant.

Results

Overexpression of CARM1 in MCF7 breast cancer cell line inhibits estrogen-dependent cell proliferation and anchorage-independent growth and stimulates the expression of CDK inhibitors p21cip1 and p27kip1

MCF7 breast cancer cells stably overexpressing CARM1, MCF7-CARM1, were generated (25). MCF7-CARM1 grows at a slower rate than parental cells carrying empty vector (MCF7-vector) as measured by MTT assays (Fig. 1A). The altered growth rate was not due to the superphysiologic amounts of CARM1 in MCF7-CARM1, because compared with MCF7-vector controls, MCF7-CARM1 cells have only a 2-fold increase in CARM1 expression (Fig. 1B). Consistent with the growth phenotype, the expression of the CDK inhibitors p21cip1 and p27kip1 was elevated in MCF7-CARM1 treated with 17beta-estradiol (E2) (Fig. 1B). Also the expression of p21cip1 and p27kip1 was stimulated by E2 in a time-dependent manner in MCF7-CARM1 cells (Supplementary Fig. 1) but not in parental MCF7 cells, suggesting that ERα and CARM1 are involved in regulating their expression. The effect of CARM1 on anchorage-independent cell growth was determined using soft-agar assays. E2 stimulates colony formation of MCF7-vector cells; in contrast, no colonies were formed in soft agar with MCF7-CARM1 (Fig. 1C). This result suggests that overexpressing CARM1 in MCF7 may inhibit anchorage-independent growth. In contrast to MCF7, no growth effects were detected by overexpressing or knocking down CARM1 in MDA-MB-231, an ERα-negative breast cancer cell line (Fig. 1D and E). Consistent with it being ERα negative, the growth rate of MDA-MB-231 was E2 independent (Fig. 1D). Similarly, overexpressing CARM1 exhibits no growth effect on MDA-MB-468, another ERα-negative breast cancer cell line (Supplementary Fig. 2), supporting the notion that the growth inhibitory effect of CARM1 in MCF7 is ERα dependent. The growth inhibitory effect of CARM1 was further validated in another ERα-positive breast cancer cell line ZR-75 (Supplementary Fig. 3). p21cip1 has been reported to induce both cell cycle arrest and cell
differentiation in various carcinomas (28, 29). The findings that p21cip1 expression is increased by E2 in the presence of exogenous CARM1 (Supplementary Fig. 1) raises the possibility that CARM1 may inhibit breast cancer growth by modulating key ERα target genes involved in cell-cycle control and differentiation.

CARM1 decreases estrogen-dependent breast cancer cell growth and S-phase entry

To eliminate the possibility that the growth effects of CARM1 in MCF7-CARM1 cells could be attributed to additional changes during retroviral integration events, we generated 2 inducible MCF7 stable cell lines: one overexpresses CARM1 (MCF7-Tet-on-CARM1) and the other expresses CARM1 shRNA (MCF7-Tet-on-shCARM1) under the control of a tetracycline-inducible promoter. These stable cell lines serve as gain-of-function and loss-of-function cell culture models for studying the effects of CARM1 in estrogen-dependent breast cancer growth. Cells were preincubated with doxycycline (Dox) for 4 days to induce or knockdown CARM1 expression, followed by E2 treatment for 24 hours. With either cell line, E2 alone has no significant effect on CARM1 expression at both mRNA and protein levels (Fig. 2A and B). Dox could increase CARM1 expression in MCF7-Tet-on-CARM1 cells by 2-fold (Fig. 2A) and reduce CARM1 to greater than 90% in MCF7-Tet-on-shCARM1 cells (Fig. 2B). E2 has no additional effect on CARM1 expression compared with Dox alone when both are present. The 2 cell lines were employed to measure cell growth by MTT assays under 4 treatment conditions: vehicle, E2, Dox, or combination of Dox and E2 for 4 time points (24, 48, 72, and 96 hours). As expected, E2 treatment significantly increases MCF7 cell growth starting from day 2 (P value <0.001; Fig. 2C). Overexpression of CARM1 by Dox treatment alone decreased MCF7 cell growth (Fig. 2C). Statistical analysis of 3 independent experiments suggested that overexpression of CARM1 by Dox treatment significantly repressed E2-induced cell growth in 2 individual clones, clone 7 (Fig. 2C) and clone 13 (Supplementary Fig. 4). This is in contrast to the nonstatistically significant effect of Dox upon E2-induced cell growth in MCF7-Tet-on-shCARM1 cells (P > 0.05; Fig. 2D) and a CARM1 stable knockdown MCF7 (MCF7-shCARM1) cell line expressing shRNA targeting a different sequence of human CARM1 (Supplementary Fig. 5, P = 0.04).

The main proliferative action of E2 in breast cancer is to promote cell-cycle progression during G1/S transition (3). Since CARM1 can induce expression of p21cip1 and p27kip1, which are negative regulators of the cell cycle, and inhibit E2-dependent growth, we determined whether CARM1 would interfere with E2-induced cell cycle progression. MCF7-Tet-on-CARM1 cells were preincubated with Dox for 4 days, followed by E2 treatment for 24 hours. Fluorescence-activated cell-sorting analysis of MCF7-Tet-on-CARM1, using propidium iodide labeling, showed that E2-induced S-phase entry was inhibited by overexpressing CARM1 (Fig. 2E). This result was
validated by bromodeoxyuridine (BrdU) labeling (Fig. 2F). Although E2 and E2 + Dox both increased S-phase entry as compared with that of the vehicle (P value <0.001 and 0.0015, respectively), results from 3 independent experiments showed that the percentage of S-phase entry induced by Dox + E2 was significantly decreased compared with E2 treatment alone (P = 0.0013), indicating that overexpression of CARM1 decreased E2 induction of S-phase entry. In contrast, in MCF7-Tet-on-shCARM1, Dox + E2 treatment displayed no difference in S-phase entry compared with E2 alone and both treatment groups induced S-phase entry compared with the vehicle treatment (P = 0.0014). In either MCF7-Tet-on-CARM1 or MCF7-Tet-on-shCARM1 cells, Dox alone had no significant effect on S-phase entry (Fig. 2F). These data suggest that overexpression of CARM1 can inhibit E2-stimulated cell growth through modulating cell cycle, while loss of CARM1 could not further accelerate E2-stimulated growth within 4 days of treatment.

Changes of cell morphology and differentiation marker expression by increasing CARM1 level in MCF7 cells

In addition to the growth inhibitory effects of CARM1, we noticed that MCF7 cells stably overexpressing CARM1 displayed a distinct cell morphology from that of MCF7-vector cells (Fig. 3A) and exhibited increased cell adhesion (requires longer trypsin treatment time). Next we investigated desmoplakin 1 (DSP1) expression, a known differentiation marker of epithelial cells that plays an essential role in maintaining cell adhesion and differentiation (30, 31), and a CARM1 target gene identified in this study. Three independent experiments showed that E2 significantly decreased DSP1 mRNA, which was reversed by overexpressing CARM1 in MCF7-Tet-on-CARM1 cells (Fig. 3B). In addition, induction of 2 additional differentiation markers, GATA-3 and E-cadherin, by overexpressing CARM1 was observed in MCF7-Tet-on-CARM1 (Fig. 3C) by Western blotting. These data suggested that growth inhibitory
function of CARM1 may be accompanied by the induction of cell differentiation.

**CARM1 levels in MCF7 cells modulate the ERα gene signature**

Since CARM1 inhibits E2-dependent growth of MCF7 cells and induces a morphology change, we determined the global effect of CARM1 on E2-dependent ERα gene signature by microarray analyses of CARM1 gain-of-function and loss-of-function cell lines treated with vehicle or E2. MCF7-inducible cells were treated under 4 conditions: DMSO, Dox, E2, and E2 plus Dox. The gene signature as calculated by fold change was normalized to vehicle control (Fig. 4A). Microarray analysis of MCF7-Tet-on-CARM1 reveals that E2 upregulated expression of 313 genes and downregulated 157 genes (P < 0.05, fold change ≥1.6). Overexpression of CARM1 drastically altered E2-regulated gene signatures. Approximately 16% of E2-induced genes including cell-cycle regulators (e.g., c-Myc; Fig. 4B) were inhibited. The most profound effect of CARM1 overexpression on E2-dependent signature was to relieve the repression of approximately 56% of E2-repressed genes (Fig. 4C; P < 0.05, fold change ≤0.6 compared with vehicle).

To our knowledge, CARM1 is the only coactivator which affects global expression of E2-repressed genes. Interestingly, gene ontology (GO) of the affected genes suggested that most E2-repressed, CARM1-activated genes are involved in cell differentiation and development (Fig. 4C). The ability of CARM1 both to inhibit E2-dependent growth and S-phase entry and to modulate E2-dependent genes involved in cell-cycle progression, cell differentiation, and development supports a role of CARM1 in modulating the programming of E2-dependent cellular processes (i.e., regulating the balance between cell differentiation and proliferation).

Since CARM1 has putative effects on ERα-dependent proliferation and differentiation, we applied qRT-PCR to validate the effect of CARM1 overexpression on 6 differentially expressed genes identified by microarray. p21cip1 and p27kip1 are known to inhibit breast cancer growth (32). Cyclin G2 is an ERα target gene and a negative regulator of cell cycle (33). Among genes involved in cell differentiation, GATA-3 is an ERα target gene and prodifferentiation marker of breast cancer (8, 34). MAZ is a transcriptional factor (35), and KRTAP10.12 is a potential prodifferentiation marker. As shown in Figure 4D, E2 alone significantly decreased cyclin G2 and KRTAP10.12 mRNA but not p21cip1, p27kip1, MAZ, and GATA-3 mRNA after 4-hour treatment. However, overexpression of CARM1 relieved E2 repression of cyclin G2 and KRTAP10.12 mRNA but not p21cip1, p27kip1, MAZ, and GATA-3 mRNA after 4-hour treatment. Consistently, the protein levels of GATA-3, E-cadherin (Fig. 3C), and p21cip1 and p27kip1 (Fig. 1B) were also increased by CARM1 overexpression and E2 treatment. These results validate our microarray data and reinforce the hypothesis that CARM1 may antagonize the proliferative action of estrogen in breast cancer cells by activating multiple cell-cycle--negative regulators and prodifferentiation genes. It is worth noting that p21cip1 induction requires both CARM1 overexpression and E2 treatment. In contrast, overexpressing CARM1 alone is sufficient to induce genes such as p27kip1.
suggesting that CARM1 may regulate some genes in hormone-deprived conditions.

The global effects of CARM1 on ER target genes were next examined in the loss-of-function model, MCF7-Tet-on-shCARM1 under aforementioned conditions. The heat map of gene expression calculated as fold changes compared with vehicle indicated that CARM1-induced genes (+Dox) are largely nonoverlapping with E2-activated genes. Among CARM1-repressed genes, many are activated by E2 (see blowup of the heat map), indicating that overexpressing CARM1 can inhibit some E2-activated genes. B, pie graph shows that among all E2-upregulated genes, 16% of them are downregulated by CARM1 overexpression. The bottom of chart shows, among all CARM1-downregulated, E2-upregulated genes, the percentage of genes in each molecular function category. GO of the affected genes was determined by GOSTat tool online (http://gostat.wehi.edu.au/cgi-bin/gostat.pl), “goa_human” database, where % represent the percentage of the affected genes that belong to each represented category. C, pie graph shows that among all E2-downregulated genes, 56% of them are upregulated by CARM1 overexpression. The bottom chart shows, among all CARM1-upregulated, E2-downregulated genes, the percentage of genes in each molecular function category. D, Q-RT PCR analyses of p21\(^{cip1}\), p27\(^{kip1}\), cyclin G2, MAZ, KRTAP10.12, and GATA-3 expression in MCF7-Tet-on-CARM1. Error bars, SD from 3 independent experiments.

Figure 4. Overexpression of CARM1 modulates E2-dependent gene signature. A, the ratios of normalized intensities for Dox, E2, or Dox + E2-treated samples versus that of samples treated with control vehicle (Dox vs. DMSO, E2 vs. DMSO, and Dox + E2 vs. DMSO) were used to show the activation or repression. Heat map of gene expression calculated as fold changes compared with vehicle indicated that CARM1-induced genes (+Dox) are largely nonoverlapping with E2-activated genes. Among CARM1-repressed genes, many are activated by E2 (see blowup of the heat map), indicating that overexpressing CARM1 can inhibit some E2-activated genes. Among CARM1-downregulated, E2-upregulated genes, the percentage of genes in each molecular function category. GO of the affected genes was determined by GOSTat tool online (http://gostat.wehi.edu.au/cgi-bin/gostat.pl), “goa_human” database, where % represent the percentage of the affected genes that belong to each represented category. C, pie graph shows that among all E2-upregulated genes, 16% of them are downregulated by CARM1 overexpression. The bottom of chart shows, among all CARM1-downregulated, E2-upregulated genes, the percentage of genes in each molecular function category. D, Q-RT PCR analyses of p21\(^{cip1}\), p27\(^{kip1}\), cyclin G2, MAZ, KRTAP10.12, and GATA-3 expression in MCF7-Tet-on-CARM1. Error bars, SD from 3 independent experiments.

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In agreement with the mRNA results, cyclin G2, GATA-3, and E-cadherin (Supplementary Fig. 6B) were decreased at protein levels with the loss of CARM1. Since both cyclin G2 (33) and GATA-3 (36) are ERα target genes, CARM1 may antagonize E2 action via ERα during reprogramming of ERα-dependent differentiation and proliferation processes. Fold changes of key cell-cycle regulators and genes involved in cell differentiation in MCF7-Tet-on-shCARM1 are listed in Supplementary Table S1. Overall, our data suggest that loss of CARM1 induces gene signatures resembling those affected by E2 and CARM1 is a regulator of E2-dependent, key cell cycle progression, and differentiation genes. Collectively, the microarray analyses using CARM1 gain-of-function and loss-of-function cell models reveal that CARM1 is a unique ER coactivator that profoundly affects the balance of genes involved in cellular differentiation and proliferation (i.e., inhibit cell growth and promote cell differentiation).

Knocking down CARM1 increased E2-dependent tumor growth in an MCF7 xenograft mouse model

To examine the effects of CARM1 in vivo, we transplanted MCF7-Tet-on-CARM1 shRNA cells in nude mice. The design of the xenograft experiment is shown in Figure 6A, representing one of triplicate experiments. We first validated that the growth of xenografted tumors was E2-dependent because no growth or only tiny tumors developed in the negative control group not receiving estrogen. Tumors collected from mice engrafted with MCF7-Tet-on-shCARM1 cells and receiving Dox showed a reduction of CARM1 expression at the mRNA and protein levels (Fig. 6B). Knocking down CARM1 increased the size of E2-induced tumors (Fig. 6C) and was associated with a modest increase in BrdU labeling. The differential rate of BrdU labeling for xenografted tumors was further increased in mice receiving a higher dose of E2 and that was associated with higher mitotic index (Fig. 6D and E). All the data suggest that knocking down CARM1 enhances...
E2-dependent proliferation of breast cancer cells in vivo. Since CARM1 inhibits E2-dependent growth by modulating negative cell-cycle regulators p21cip1, p27kip1, and cyclin G2 and pro-differentiation genes, we examined the relationship between p21cip1 and E-cadherin, a differentiation marker, in E2-induced xenografted tumors. A direct correlation was observed between p21cip1 and E-cadherin expression in tumors derived from xenografts (Fig. 6F), suggesting inhibition of cell growth and induction of differentiation are coherent processes in ERα-positive tumors.

CARM1 expression in human breast tumor biopsy samples

Our rabbit polyclonal CARM1 Ab was determined to be specific because it detects both nuclear and cytoplasmic CARM1 in normal breast tissues and breast tumors while exhibiting no activity toward mouse embryonic fibroblasts derived from CARM1 knockout mice (MEF Δ/C0; Supplementary Fig. 7). CARM1 expression was determined by IHC in ERα breast tumor TMAs available in the MBTB (26, 37). Statistically significant correlations between ERα expression as determined by IHC (n = 310, Spearman r = 0.324, P < 0.0001) and tumor grade (n = 328, Spearman r = −0.159, P = 0.004) were found. Significantly higher CARM1 expression as determined by IHC score was found in tumors with higher ERα expression than in those with lower ERα expression (Fig. 7A). Significantly higher CARM1 expression was found in lower-grade (3, 4) tumors as well (Fig. 7B and C). In addition, CARM1 expression was positively correlated with ERα levels in ERα-positive, node-negative human breast tumors (P < 0.0001; Figure 6B).
Supplementary Fig. 8A). We also found an inverse correlation between CARM1 expression and tumor grade in ER-positive, node-negative human breast tumors (P < 0.0398; Supplementary Fig. 8B). Collectively, the findings from clinical samples support a role of CARM1 in regulating ERα-dependent differentiation in ERα-positive tumors.

Discussion

In most cases, proliferation and differentiation are inversely coupled: repression of proliferation is a prerequisite for initiation of differentiation (11). In many cell types, however, cell-cycle arrest is necessary but not sufficient for differentiation. CARM1 seems to be a unique ER coactivator regulating both processes. Overexpression of CARM1 in MCF7 cells results in inhibition of E2-dependent growth through inhibition of the G0/G1 transition to S phase. This is in part due to upregulation of key negative cell-cycle regulators such as p21<sup>cip1</sup>, p27<sup>kip1</sup>, and cyclin G2. Inhibition of E2-dependent cell growth by CARM1 is accompanied by morphologic changes characteristic of a more differentiated phenotype and induction of multiple differentiation markers such as GATA-3 and MAZ. This finding is supported by previous reports that CARM1 can promote cell differentiation in other systems (19–21). Nonetheless, regulation of cell differentiation by CARM1 seems to be cell-type and context dependent. In mouse embryo and embryonic stem cells, CARM1 was shown to elevate expression of key pluripotency genes and delay their response to differentiation signals (38).

In contrast to growth inhibition by CARM1 overexpression, knocking down CARM1 in MCF7 did not alter E2-dependent cell growth in cell culture, nor did it affect E2-induced S-phase entry. This observation contradicts the conclusion by Frietze and colleagues that CARM1 increases growth of MCF7 cells. The discrepancies may be due to the transient transfection of CARM1 siRNA throughout the cell-cycle study by Frietze and colleagues (16). Moreover, the authors measured the percentage of cells in S + G2 + M phase without distinguishing the percentage of cells in S phase. Also, in consistent with the observation of O’Brien and colleagues (21), we did not observe change of E2F1 with CARM1 knockdown in contrast to Frietze and colleagues (16). Interestingly, and in contrast to cells grown in culture, knocking down CARM1 enhanced E2-induced xenograft tumors. This may be due to increased breast cancer cell interaction with the microenvironment which plays essential roles in promoting tumor growth in animals.

The growth inhibitory effect of CARM1 is unique from that of SRCs. Knocking down SRC2 and SRC3 but not SRC1 inhibits growth of MCF7 cells and decreases cyclin D1 expression (39). Overexpression of SRC3 also increases breast cancer cell proliferation and invasiveness. Similarly, SRC-1 promotes breast tumor metastasis and inhibits tumor cell differentiation (40). Thus, the ERα-dependent, growth inhibitory effect of CARM1 is unlikely to be mediated through SRC1, 2, and 3.

Cell-cycle genes that are regulated by E2 or loss of CARM1 include cyclin D1, c-Myc, cyclin G2, cyclin L1, cyclin T2, p21<sup>cip1</sup>, p27<sup>kip1</sup>, p130, and Rb (Supplementary Table S1). E2 treatment alone significantly represses cyclin G2 (33), which is reversed by overexpressing CARM1. Cyclin D1 is a well-known, E2-induced ERα target gene; however, its expression is not affected by overexpression of CARM1 in the presence of E2, yet knocking down CARM1 upregulates cyclin D1 in MCF7 cells (Supplementary Table S1). c-Myc is upregulated by E2 alone or loss of CARM1 (Supplementary Table S1) but is not affected by depletion of any of the p160 coactivators in MCF7 cells (39). Thus, the mechanism of CARM1 regulation of cell-cycle regulators is complex and only partially depends on the p160 coactivators.

Microarray gene expression analyses reveal that approximately 16% of E2-activated genes were repressed by CARM1, consistent with the repressive effects of CARM1 on some ER target genes (41). The mechanism of CARM1-mediated repression is unclear. The major effect of CARM1 overexpression was
to relieve E2-repressed genes. CARM1 methyltransferase activity may be responsible for the activation because we observed increased H3R17Me2 mark on p21\(^{Ip1}\) promoter upon CARM1 induction in MCF7-Tet-on-CARM1 (data not shown), consistent with a recent publication that CARM1 is recruited to p21\(^{Ip1}\) promoter (42). Whether CARM1 regulates ER\(\alpha\) target genes via an epigenetic mechanism remains to be determined. Nonetheless, global ER\(\alpha\) transcriptional regulation by CARM1 leads to the induction of many E2-repressed genes associated with differentiation.

Consistent with this finding, knocking down CARM1 shares more than 65% of the E2 gene signature. Most CARM1- and E2-regulated genes are involved in gene expression, metabolism, cell cycle, and differentiation. Knocking down CARM1 leads to upregulation of positive cell-cycle regulators (e.g., c-Myc) and downregulation of negative cell-cycle regulators (e.g., cyclin G2). This result suggests that loss of CARM1 function may lead to the acquisition of a proliferative phenotype resembling estrogen stimulation of breast cancer. Furthermore, knocking down CARM1 also modulates genes involved in cell differentiation. For example, combination of CARM1 shRNA and E2 treatment significantly reduced the level of PPAR\(\gamma\), which induces terminal differentiation of breast cancer (43). Loss of CARM1 also significantly decreases KRTAP10.12, an E2-repressed gene involved in keratin filament formation and potentially in cell differentiation processes (44, 45). Collectively, either loss of CARM1 or E2 treatment significantly inhibits expression of various differentiation markers (Supplementary Table S1). Overall, the gene expression data from CARM1 gain-of-function and loss-of-function models suggest that CARM1 plays an important role in regulating ER\(\alpha\) target genes in differentiation and proliferation.

Evidence for a functional interplay of ER\(\alpha\) and CARM1 was explored in human breast cancer specimens. A direct correlation was observed between CARM1 and ER\(\alpha\) in ER-positive tumors. Higher ER\(\alpha\) expression is associated with less aggressive and more differentiated tumors, and ER status is known to inversely correlate with histologic grade (46). Our observation contradicts an earlier report that CARM1 is overexpressed in grade III breast tumors (23). The difference could result from analysis of RNA versus protein and the sample size. In the study by El Messaoudi and colleagues, CARM1 was analyzed only at the RNA level in 81 human breast tumors whereas we analyzed CARM1 protein level in more than 300 human breast tumors.

Histologic grade using the Nottingham method integrates scores from glandular differentiation, nuclear morphology, and mitotic counts (47, 48), and higher grade is significantly associated with poor outcome and survival. The inverse correlation of CARM1 expression and tumor grade found in ER-positive breast cancer cases, together with enhanced tumor volume in CARM1 knockdown breast cancer xenografts in animal models, supports an association of low levels of CARM1 with less well-differentiated, high-grade breast cancers and is consistent with the hypothesis that CARM1 inhibits breast cancer progression in ER\(\alpha\)-positive tumors. Our results suggest that coexpression of ER\(\alpha\) and CARM1 together may serve as a better biomarker of well-differentiated breast cancers.

ER\(\alpha\) is believed to regulate growth and differentiation through balanced interaction with cofactors. This study reports an unexpected biological function of the ER coactivator CARM1 in breast cancer. The hallmark of CARM1 action might be due to global modulation of E2-regulated genes, leading to reprogramming of cell proliferation and differentiation. To our knowledge, CARM1 is the only ER coactivator that is able to simultaneously block cell proliferation and induce differentiation. Since CARM1 has histone modification activity, inducing differentiation of breast cancer cells by upregulating CARM1 activity may be therapeutically effective in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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