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Cell Cycle Progression Stimulated by Tamoxifen-Bound Estrogen Receptor-α and Promoter-Specific Effects in Breast Cancer Cells Deficient in N-CoR and SMRT

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Estrogen receptor α (ERα) mediates the effects of estrogens in breast cancer development and growth via transcriptional regulation of target genes. Tamoxifen can antagonize ERα activity and has been used in breast cancer therapy. Tamoxifen-bound ERα associates with nuclear receptor corepressor (N-CoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) at certain target genes. Here we show the effects of reducing N-CoR and SMRT levels on the actions of estrogen and tamoxifen in breast cancer cells. Silencing both corepressors led to tamoxifen-stimulated cell cycle progression without activation of the ERα target genes c-*myc*, *cyclin D1*, or stromal cell-derived factor 1, which play a role in estrogen-induced proliferation. By contrast, expression of X-box binding protein 1 was markedly elevated in tamoxifen-treated cells in which N-CoR and SMRT had been silenced. The gain in cell cycle entry seen with tamoxifen when N-CoR and SMRT were silenced was dependent on ERα and not observed upon treatment with estradiol or epidermal growth factor. These results suggest that N-CoR and SMRT play an active role in preventing tamoxifen from stimulating proliferation in breast cancer cells through repression of a subset of target genes involved in ERα function and cell proliferation. (*Molecular Endocrinology* 19: 1543–1554, 2005)

THE ESTROGEN RECEPTOR (ER) belongs to a superfamily of nuclear receptors that function as ligand-activated transcription factors (1). This receptor mediates the effects of estrogens, which include proliferation and differentiation in reproductive tissues and have been linked to the development and progression of breast cancer (2, 3). Estrogenic effects are mediated by two forms of the ER, α and β, although a role for ERβ in breast cancer has not been clearly defined (4–6). Estrogens can stimulate cell growth through ERα-mediated transcriptional regulation of target genes involved in proliferation (7–9). Tamoxifen can antagonize ERα action and has been widely used in the prevention or treatment of breast cancer (10).

The ERs can modulate transcription by binding directly to estrogen response elements (EREs) in the promoter region of target genes or indirectly through transcription factors such as AP1 (11). Transcriptional activation is mediated by two activation function (AF) domains, AF-1 and AF-2. The AF-1 domain is located in the N terminus of the receptor and has a ligand-independent function that can be enhanced by phosphorylation through the MAPK pathway (12). The AF-2 domain is located in the ligand binding domain (LBD) in the C terminus of the receptor and its function is ligand dependent. Transcriptional activation by ERα is associated with the ligand-dependent recruitment of several coactivators, including AIB1, GRIP1, SRC1, CBP/p300 and p/CAF, and subsequent histone acetylation (13).

Tamoxifen acts as an ERα antagonist by competing with estrogen for binding to the LBD of the receptor and creating an alternative structural conformation that blocks the interaction of coactivators with the AF-2 domain (14, 15). Instead, tamoxifen-bound ERα has been shown to interact with the corepressors referred to as nuclear receptor corepressor (N-CoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) and associate with these corepressors and histone deacetylases at certain ERα target promoters (13, 16–18). However, tamoxifen can also act as an agonist, presumably through coactivator interactions involving the AF-1 domain, depending on the target gene, cell, or tissue (19–22). For example, whereas tamoxifen functions as an antagonist in breast cancer cells, it acts as a partial agonist in endometrial cells.

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Abbreviations: AF, Activation function; AtRA, all-*trans* retinoic acid; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; ER, estrogen receptor; ERE, estrogen response element; LBD, ligand binding domain; N-CoR, nuclear receptor corepressor; RAR, retinoic acid receptor; RARE, retinoic acid response element; RNAi, RNA interference; SDF-1, stromal cell-derived factor 1; si, small interfering; SMRT, silencing mediator for retinoid and thyroid hormone receptors; SRC-1, steroid receptor coactivator 1; XBP-1, X-box binding protein 1.

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How tamoxifen functions as a selective ER modulator is not completely understood, but the availability of ER coregulators has been shown to influence tamoxifen action. It has been shown in various cell lines that overexpression of the coactivators SRC-1 or L7/SPA enhances or leads to tamoxifen-stimulated transcription (17, 23, 24). Conversely, overexpression of the corepressors N-CoR or SMRT or silencing SRC-1 expression by RNA interference (RNAi) represses the partial agonist activity of tamoxifen (17, 23, 24). In addition, microinjection of N-CoR antibodies into MCF-7 cells leads to activation of an ERE/lacZ reporter by tamoxifen (25). Furthermore, tamoxifen is antagonistic in wild-type mouse embryonic fibroblasts, but agonistic in N-CoR$-/-$ type mouse embryonic fibroblasts (26).

Low levels of N-CoR have also been implicated in the acquisition of tamoxifen resistance. Most ERα-positive breast cancer patients treated with tamoxifen ultimately develop resistance, which leads to tamoxifen-stimulated tumor growth (27). The mechanisms by which resistance is acquired are not known, but one possible mechanism is the emergence of tamoxifen's agonistic abilities through changes in the levels of ER coregulators. In support of this hypothesis, low N-CoR mRNA expression has been associated with shorter relapse-free survival in ERα-positive breast cancer patients treated with tamoxifen (28). Decreased N-CoR protein expression has also been correlated with acquired tamoxifen resistance in a mouse model of breast cancer (25). In an additional study, lower N-CoR mRNA expression levels were found in tumors from patients with recurrence compared with patients without recurrence (29).

The corepressors N-CoR and SMRT were originally identified as components of a complex involved in repression associated with unliganded retinoic acid receptor (RAR) and thyroid hormone receptor (30, 31). Association of these corepressors with unliganded ER has not been clearly demonstrated. Their physiological role in tamoxifen-mediated antagonism or repression of ERα transcriptional activity and the critical target genes have also not been fully defined.

In this study, we wanted to know whether reducing the levels of the corepressors N-CoR and SMRT would lead to changes in tamoxifen action in breast cancer cells. This would help to better understand whether these corepressors are required for tamoxifen-mediated repression and how the relative levels of ER coregulators in a target tissue may influence the response to tamoxifen. Therefore, we silenced N-CoR and SMRT separately or together using small interfering (si) RNAs and tested whether tamoxifen could now stimulate proliferation or function as an agonist on endogenous ERα target genes that mediate estrogen-induced cell growth in breast cancer cells. The loss of N-CoR and SMRT function was validated by an observed relief of constitutive repression by the RAR on a retinoic acid response element (RARE) reporter gene. We found that silencing both N-CoR and SMRT led to tamoxifen-stimulated proliferation in MCF-7 cells, but not through activation of the c-*myc*, *cyclin D1*, or *SDF-1* genes. By contrast, expression of X-box binding protein 1 (*XBP-1*) was markedly elevated when cells in which N-CoR and SMRT had been silenced were treated with tamoxifen. The cell cycle progression stimulated by tamoxifen when N-CoR and SMRT were silenced was not observed in an ER-negative cell line and thus dependent on ERα. In addition, the gain in proliferation was specific to tamoxifen and not observed upon treatment with estradiol or epidermal growth factor (EGF). These results suggest that N-CoR and SMRT play a role in the antiproliferative action of tamoxifen in breast cancer cells through repression of a subset of target genes involved in ERα function and cell proliferation.

RESULTS

Silencing of N-CoR and SMRT by RNA Interference

To examine whether reducing N-CoR and SMRT function alters tamoxifen action in breast cancer cells, these corepressors were silenced separately or together using RNAi. MCF-7 cells were transfected with siRNA oligonucleotide duplexes targeting N-CoR (siN-CoR), SMRT (siSMRT), or luciferase (siLuc) as a nonspecific control. For cosilencing of N-CoR and SMRT both specific siRNAs were transfected simultaneously. Immunoblot analysis of whole-cell extracts prepared from cells 48 h after transfection of siRNA demonstrates that N-CoR and SMRT were efficiently silenced or cosilenced by at least 60% at the protein level when quantified relative to calnexin (Fig. 1A). To confirm equivalent and efficient silencing at the mRNA level under each silencing condition, RNA was isolated 48 h after transfection with siRNA oligonucleotides and the relative levels of N-CoR and SMRT mRNA were analyzed by quantitative real-time RT-PCR. The results of this analysis showed that N-CoR mRNA levels were consistently reduced by 75% when silenced separately or together with SMRT (Fig. 1B). The mRNA levels of SMRT were reduced by approximately 65% when SMRT was silenced separately or together with N-CoR. Surprisingly, N-CoR mRNA levels were reduced by about 25% by siSMRT, but this effect was not seen at the protein level (Fig. 1A). This was not due to significant homology in the target siRNA sequence and thus may be due to regulation at the transcriptional level.

Silencing N-CoR or SMRT Relieves Constitutive Repression by the RAR

To validate that N-CoR and SMRT were functionally silenced at the observed reduced levels of mRNA and protein expression, the effect of silencing the corepressors separately or together on constitutive repression of

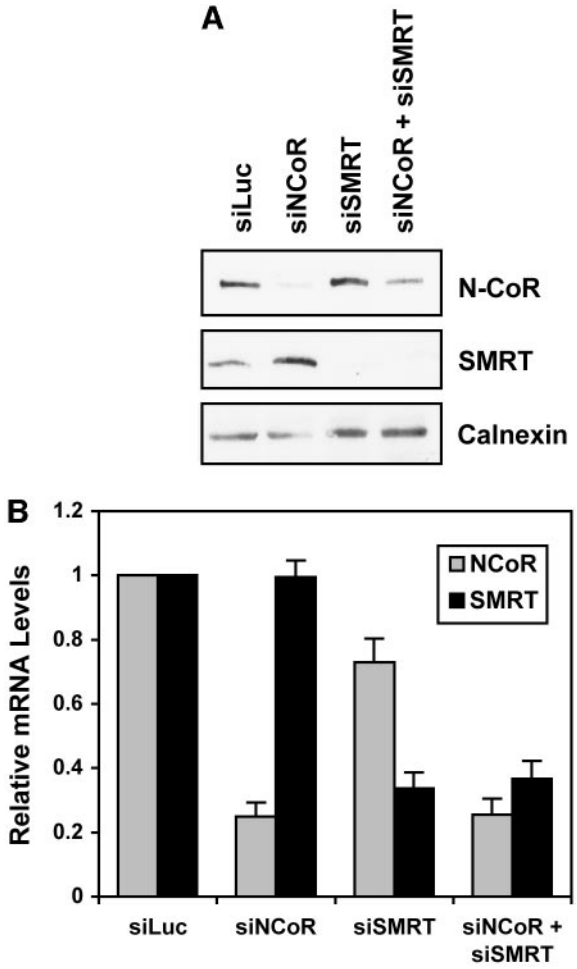

Fig. 1. Silencing of N-CoR and SMRT Separately or Together

MCF-7 cells were transfected with siRNA oligonucleotide duplexes targeting N-CoR (siNCoR), SMRT (siSMRT), both corepressors, or luciferase (siLuc) as a nonspecific control. A, Western blot analysis of proteins from whole-cell extracts prepared 48 h after transfection. B, RNA was isolated from cells 48 h after transfection and analyzed by quantitative real-time RT-PCR using primers for N-CoR or SMRT. Results shown are the average expression of N-CoR and SMRT mRNA (normalized to GAPDH) relative to in the siLuc control from four independent experiments (±SEM).

a RARE reporter gene by the RAR was investigated. For these experiments, the reporter constructs (RAREβ)$_2$tk-luciferase and pCMVβ-galactosidase, which was used as a normalization control, were cotransfected with siRNA oligonucleotides targeting N-CoR, SMRT, or both into MCF-7 cells. A scrambled N-CoR target sequence (scrNCoR) was used as a nonspecific control. Cells were treated for 24 h with vehicle [dimethylsulfoxide (DMSO)] or 100 nM all-*trans* retinoic acid (AtRA) 48 h after transfection and cellular extracts were prepared for use in the measurement of luciferase and β-galactosidase activity. The reduced levels of N-CoR and SMRT protein expression before treatment were similar to those shown in Fig. 1A. We found that silencing N-CoR, SMRT, or both corepressors relieved constitutive repression on the RARE reporter gene in the absence of hormone 3.2-, 2.4-, or 3.3-fold, respectively, as compared with the scrNCoR vehicle-treated control (Fig. 2). These levels of activation were near the average seen in the AtRA-treated scrN-CoR control, which was 4.2-fold. In addition, the activation of the RARE reporter gene upon treatment with AtRA was significantly elevated when both N-CoR and SMRT were silenced to an average of 10.7-fold as compared with the vehicle-treated scrNCoR control. The average AtRA-induced luciferase activity in cells with reduced levels of N-CoR and SMRT relative to the corresponding vehicle-treated control was 3.2-fold. Thus, the fold activation stimulated by the RAR when N-CoR and SMRT were silenced was not increased as compared with that of the scrNCoR control, but the overall activity was higher. This may be due to elevated basal levels of activation and these findings suggest that N-CoR and SMRT also play a role in modulating ligand-activated transcription by the RAR on the RARE reporter gene. Taken together, these results indicate that N-CoR and SMRT were functionally silenced.

Effects of Silencing N-CoR and SMRT on Tamoxifen Action at Pro-Proliferative ERα Target Genes

We next examined whether reducing the levels of N-CoR, SMRT, or both could convert tamoxifen into an agonist on endogenous ERα target genes that can stimulate cell growth. The c-*myc*, *cyclin D1*, and *SDF-1* genes have been identified as targets of ERα action that can mediate the proliferative effects of estradiol (7–9). We investigated the effects of silencing N-CoR and SMRT separately or together on tamoxifen action on these three genes in MCF-7 cells as compared with estradiol-stimulated activation as a positive control. Cells were transfected with siRNA oligonucleotides targeting N-CoR, SMRT, both corepressors, or luciferase as a nonspecific control. Forty-eight hours after transfection, cells were treated for various times with 100 nM 17β-estradiol (estradiol) or 1 μM 4-hydroxytamoxifen (tamoxifen) before RNA isolation and analysis of target gene induction by quantitative real-time RT-PCR.

Induction of c-*myc* by estradiol was maximal at 1 h and the levels of mRNA returned to near basal levels after 7 h, even though estradiol was present (Fig. 3A, *left panel*). Tamoxifen treatment did not lead to a significant gain in activation of c-*myc* when N-CoR, SMRT, or both were silenced as compared with the siLuc control (Fig. 3A, *right panel*). *Cyclin D1* mRNA levels induced by estradiol were maximal at 3 h and this target gene was also not significantly activated upon treatment with tamoxifen up to 7 h even under cosilencing conditions (Fig. 3B, *left* and *right panels*, respectively). Activation of c-*myc* and *cyclin D1* by estradiol was also not significantly altered under any of the silencing conditions (Fig. 3, A and B, *left panels*). There was no activation of c-*myc*

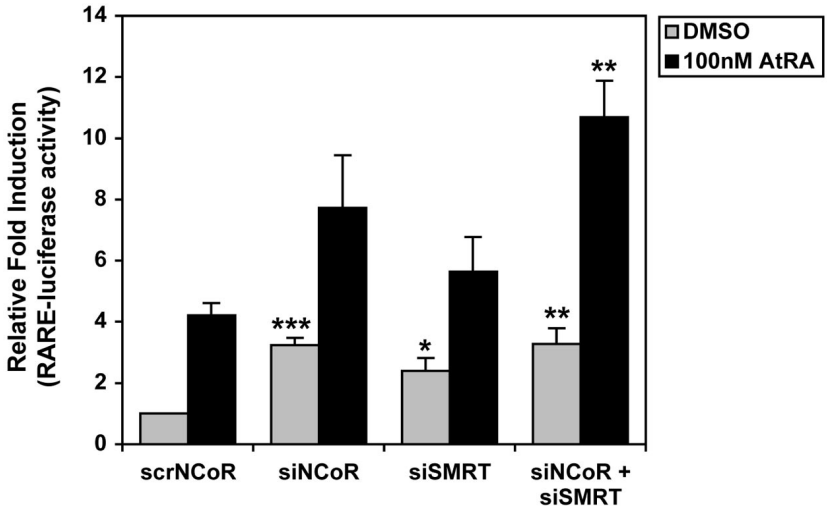

Fig. 2. Effect of Silencing N-CoR or SMRT on Constitutive Repression and Ligand-Activated Transcription by the RAR

MCF-7 cells were transfected with (RAREβ)$_2$tk-luciferase and pCMVβ-galactosidase reporter plasmids and siRNA oligonucleotide duplexes targeting N-CoR, SMRT, or a scrambled N-CoR target sequence (scrNCoR) as a nonspecific control. Forty-eight hours after transfection, cells were treated for 24 h with vehicle (DMSO) or 100 nM AtRA. Cell lysates were then prepared and used to measure reporter activity. Results shown are the average fold induction of luciferase activity normalized to β-galactosidase activity relative to the scrNCoR vehicle-treated control from three independent experiments $\pm$ SEM (***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$).

or *cyclin D1* by tamoxifen after treatment for as long as 30 h (data not shown). These results suggest that N-CoR and SMRT are not required for tamoxifen-mediated repression of ERα on the c-*myc* and *cyclin D1* genes.

The *SDF-1* gene was strongly activated by estradiol and mRNA levels increased up to 8-fold after treatment for 30 h in MCF-7 cells transfected with siLuc (Fig. 3C, *left panel*). The maximum fold estradiol-induced *SDF-1* mRNA levels were similar under each silencing condition. The levels of *SDF-1* mRNA were slightly increased to about 1.5-fold in response to treatment with tamoxifen for 12, 18, 24, or 30 h in cells transfected with siLuc (Fig. 3C, *right panel*). The tamoxifen-stimulated fold induction of *SDF-1* was similar when N-CoR was silenced, but was slightly elevated to a maximum of 2.3-fold when SMRT or both SMRT and N-CoR were silenced as compared with the corresponding untreated controls. However, the overall relative mRNA levels were not significantly elevated under these conditions. These results suggest that N-CoR and SMRT are not essential for tamoxifen-mediated repression on the *SDF-1* gene.

XBP-1 has also been identified as an ERα target gene activated in response to estradiol treatment by microarray analysis and was found by serial analysis of gene expression to be highly expressed in cancerous mammary epithelial cells (32–35). XBP-1 is a basic region leucine zipper protein that is a member of the CREBP/ATF family of transcription factors (36). Although a role for XBP-1 in estradiol-stimulated proliferation has not been demonstrated, this protein has been shown to play a role in hepatocyte growth and plasma cell differentiation (37, 38). In addition, XBP-1 can enhance ERα-dependent transcriptional activity in a ligand-independent manner, and its expression was found to be high in ERα-positive breast tumors (39–45). Therefore, we wanted to investigate the effect of silencing N-CoR and SMRT on expression of the *XBP-1* gene in response to tamoxifen treatment in MCF-7 cells. Figure 4 (*left panel*) shows that *XBP-1* mRNA levels were increased in response to estradiol treatment for 3–30 h to a maximum of 5.8-fold in cells transfected with the siLuc control. Interestingly, silencing N-CoR and SMRT separately or together led to elevated basal expression of *XBP-1* mRNA to a maximum of 2-fold under cosilencing conditions (Fig. 4, *left* and *right panels*). This led to lower fold activation of *XBP-1* by estradiol when N-CoR and/or SMRT were silenced, although the absolute mRNA levels reached nearly the same maximum. In response to tamoxifen, the *XBP-1* mRNA levels increased to a maximum of 1.9-fold in cells transfected with siLuc (Fig. 4, *right panel*). The tamoxifen-stimulated fold induction *XBP-1* activation increased slightly when N-CoR, SMRT, or both were silenced to a maximum of 2.1-, 2.5-, or 2.6-fold, respectively, after treatment for 24 h, as compared with the corresponding untreated controls. More significantly, the absolute levels of *XBP-1* mRNA were elevated to 3-, 3.7-, or 5-fold upon tamoxifen treatment when N-CoR, SMRT, or both were silenced, respectively. Thus, the *XBP-1* gene was activated to about the estradiol-induced level by tamoxifen when N-CoR and

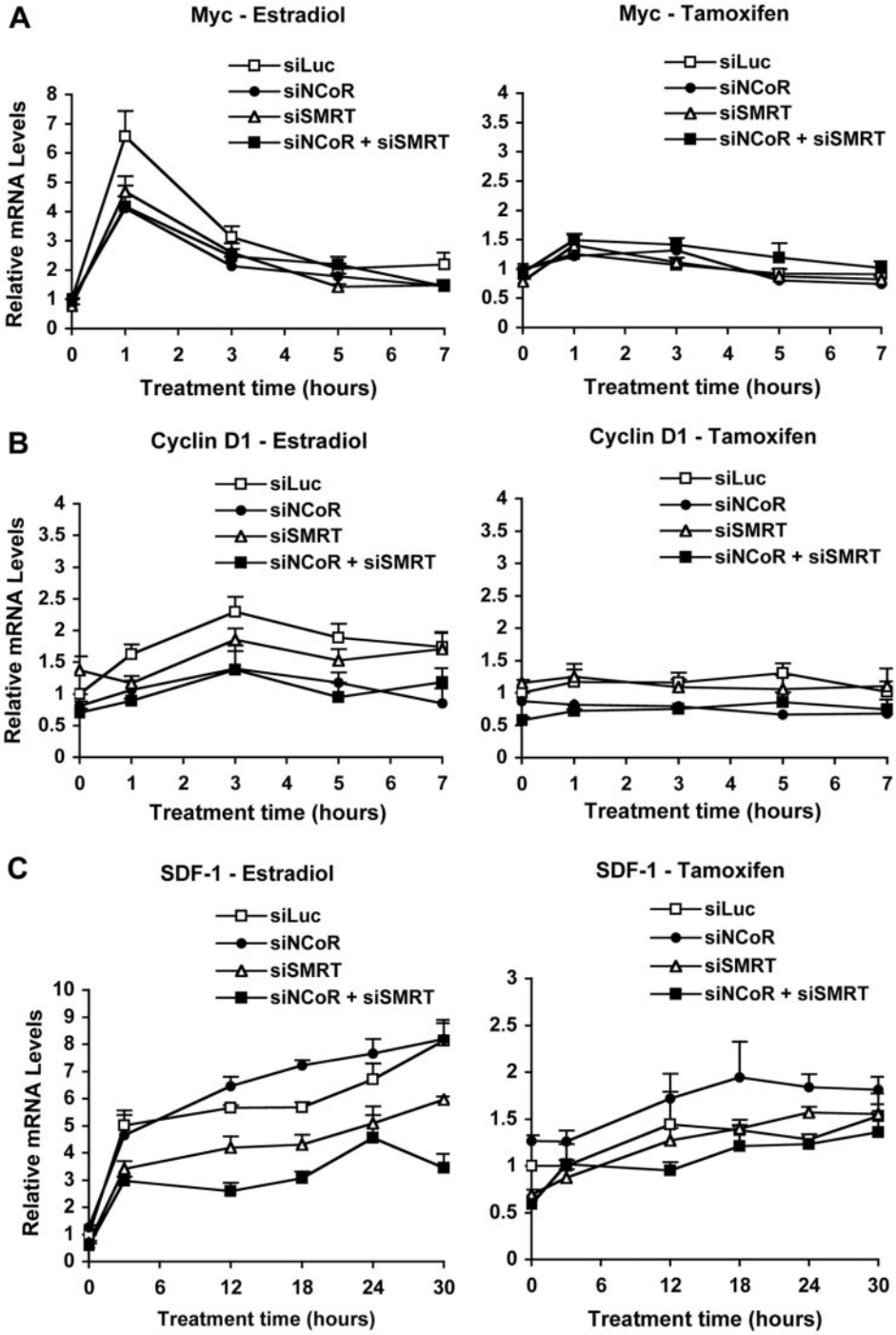

Fig. 3. Effect of Silencing N-CoR and SMRT on ERα-Mediated Transcriptional Activation of the c-*myc*, *cyclin D1*, and *SDF-1* Genes in Response to Estradiol or Tamoxifen Treatment

A-C, MCF-7 cells were transfected with siRNA oligonucleotide duplexes targeting N-CoR, SMRT, both corepressors, or luciferase as a nonspecific control. Forty-eight hours after transfection, cells were treated for the indicated times with 100 nM 17β-estradiol (estradiol), or 1 μM 4-hydroxytamoxifen (tamoxifen). RNA was isolated and analyzed by quantitative real-time RT-PCR. Data shown are the average mRNA levels (normalized to GAPDH) relative to those in the untreated siLuc control calculated from at least three independent experiments. *Error bars* represent SEM.

SMRT were cosilenced. The above findings indicate that N-CoR and SMRT are involved in constitutive repression of the *XBP-1* gene. Also, cosilencing N-CoR and SMRT, coupled with tamoxifen treatment, can lead to changes in gene expression that could contribute to increased ERα action.

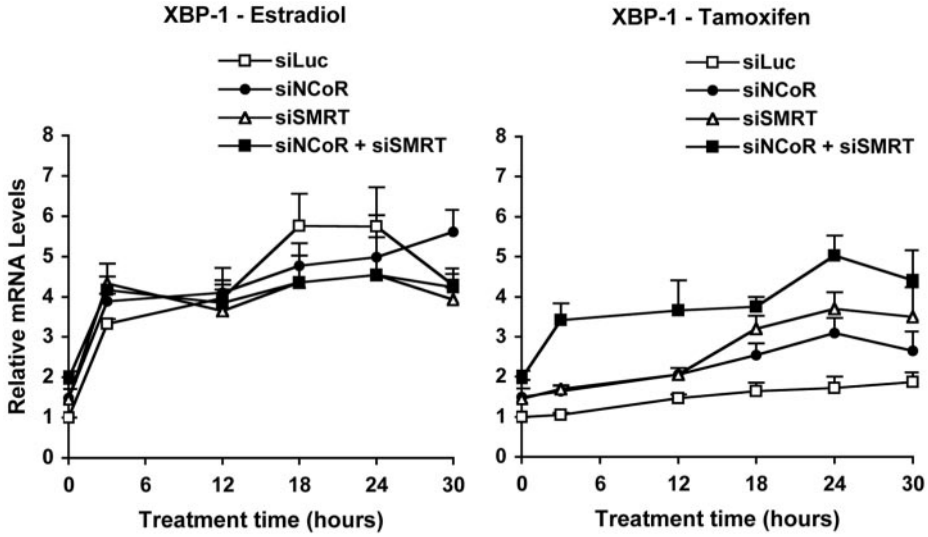

Fig. 4. Effect of Silencing N-CoR and SMRT on ERα-Mediated Transcriptional Activation of the *XBP-1* Gene in Response to Estradiol or Tamoxifen Treatment

MCF-7 cells were transfected with siRNA oligonucleotide duplexes targeting N-CoR, SMRT, both corepressors, or luciferase as a nonspecific control. Forty-eight hours after transfection, cells were treated for the indicated times with 100 nM 17β-estradiol or 1 μM 4-hydroxytamoxifen. RNA was isolated and analyzed by quantitative real-time RT-PCR. Data shown are the average mRNA levels (normalized to GAPDH) relative to those in the untreated siLuc control calculated from at least three independent experiments. *Error bars* represent SEM.

Silencing of Both N-CoR and SMRT Leads to Tamoxifen-Stimulated Cell Cycle Progression

We did not observe a change in tamoxifen-bound ERα action on the c-*myc*, *cyclin D1*, and *SDF-1* genes when the levels of N-CoR, SMRT, or both were reduced, but that did not rule out the possibility that N-CoR and SMRT are required for the antiproliferative action of tamoxifen in breast cancer cells. Therefore, we measured the change in entry of MCF-7 cells into the cell cycle upon treatment with tamoxifen when N-CoR and SMRT were silenced separately or together. Forty-eight hours after transfection, cells were treated with vehicle, 100 nM 17β-estradiol, or 1 μM 4-hydroxytamoxifen for 24 h. Cells were then harvested and fixed before staining with propidium iodide for DNA content. The results in Fig. 5A show that cosilencing N-CoR and SMRT led to proliferation upon treatment with tamoxifen, indicated by an average 5% increase in cells entering G2, S, and M phases. This finding suggests that N-CoR and SMRT play a role in preventing tamoxifen from stimulating proliferation in breast cancer cells. Interestingly, there was no significant increase in cell cycle entry upon treatment with tamoxifen when N-CoR and SMRT were silenced individually. Also, the tamoxifen-stimulated increase in cell cycle entry did not reach that stimulated by estradiol, which was an average of 17.3% in the control siLuc-transfected cells. These results indicate that in cells with reduced levels of N-CoR and SMRT that tamoxifen is a partial agonist for the stimulation of cell cycle progression.

To determine whether the pro-proliferative effects of tamoxifen when N-CoR and SMRT were silenced was dependent on ERα, we repeated the cell cycle entry experiments in the ER-negative cell line MDA-MB231. The results of immunoblot analysis using MDA-MB231 whole-cell extracts isolated after transfection with siRNA oligonucleotide duplexes demonstrate that N-CoR and SMRT were effectively cosilenced in this cell line (Fig. 6A). These cells were treated as above or with 10 nM EGF as a positive control for growth stimulation. As expected, we observed an increase in cell cycle entry only upon treatment with EGF and not with estradiol or tamoxifen in these ER-negative cells. This was also the case when N-CoR and SMRT were silenced. These findings support the conclusion that tamoxifen-stimulated proliferation in the setting of N-CoR and SMRT silencing is strictly dependent on ERα and in addition that N-CoR and SMRT silencing does not have a general growth promoting effect (Fig. 6B).

To examine whether silencing N-CoR and SMRT allowed MCF-7 cells to become more sensitive to mitogens other than tamoxifen, we investigated the effect of silencing the corepressors on cell cycle entry in response to increasing doses of estradiol. Cells in which N-CoR and SMRT were silenced were not more sensitive to estradiol. The EC_{50} values were similar for estradiol under control and cosilencing conditions, and the cell cycle entry in response to different doses of estradiol was actually reduced overall when N-CoR and SMRT were silenced (Fig. 6C). Thus, cells in which N-CoR and SMRT are silenced demonstrate an increase in proliferation specifically upon treatment with tamoxifen and not a more generalized increased sensitivity to growth stimuli.

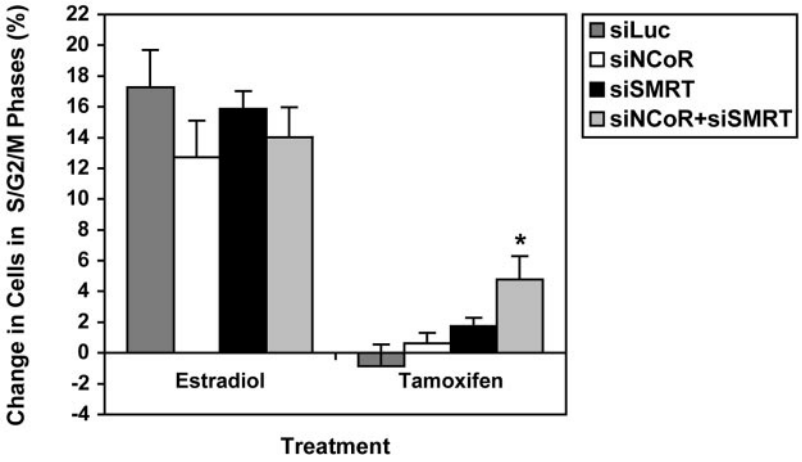

Fig. 5. Effect of Silencing N-CoR and SMRT on Cell Cycle Entry and Growth in Response to Estradiol or Tamoxifen Treatment

MCF-7 cells were transfected with siRNA oligonucleotide duplexes targeting N-CoR, SMRT, both corepressors, or luciferase as a nonspecific control. Forty-eight hours after transfection, cells were treated with vehicle, 100 nM 17β-estradiol, or 1 μM 4-hydroxytamoxifen. After treatment for 24 h, cells were harvested by trypsinization and fixed before staining with propidium iodide to analyze DNA content by flow cytometry. Results shown represent the average change in cells in G2, S, and M phases stimulated by estradiol or tamoxifen relative to the vehicle-treated control under each silencing condition. Data are from three independent experiments $\pm$ SEM (*, $P \leq 0.05$).

DISCUSSION

In this study, we examined whether silencing N-CoR, SMRT, or both corepressors together using RNAi could alter the antagonistic profile of tamoxifen in breast cancer cells. We tested whether reducing the expression of N-CoR and/or SMRT would allow endogenous levels of ER coactivators to mediate tamoxifen agonism and whether N-CoR and SMRT are essential for tamoxifen-mediated repression. Most previous studies used simple ERE reporter genes to measure changes in tamoxifen action (23–26). We wanted to examine breast cancer cell proliferation and endogenous ERα target genes involved in estradiol-stimulated growth to better understand how tamoxifen may stimulate growth in certain tissues or in tamoxifen-resistant cells.

We have shown that the expression of N-CoR and SMRT was reduced by more than 60% at the level of mRNA and protein by siRNA oligonucleotide duplexes transfected into MCF-7 cells. These reduced levels of N-CoR and/or SMRT were sufficient to relieve constitutive repression by the RAR on a RARE reporter gene and led to a significant increase in retinoic acid-stimulated activation when both N-CoR and SMRT were silenced, suggesting that the corepressors were functionally silenced. A role for N-CoR in modulating both basal and ligand-activated transcription by the RAR has been previously demonstrated (46). We found that silencing N-CoR or SMRT individually did not lead to tamoxifen-stimulated proliferation in MCF-7 cells, but silencing both corepressors together led to a significant increase in cell cycle entry upon tamoxifen treatment. These findings suggest that N-CoR and SMRT play a role in tamoxifen-mediated repression of cell growth in breast cancer cells. The finding that silencing both N-CoR and SMRT is required to observe tamoxifen-stimulated proliferation suggests that these corepressors have redundant or overlapping roles at the relevant target gene(s) involved and compensate for one another when each is silenced separately. These results also suggest the possibility that overexpression of N-CoR and SMRT in a tamoxifen-sensitive cell line, such as endometrial cells, may partially inhibit the agonistic action of tamoxifen.

The increase in cell cycle entry when N-CoR and SMRT were silenced was specific to tamoxifen because we did not observe an increase in the sensitivity of MCF-7 cells to estradiol under cosilencing conditions. These findings suggest that N-CoR and SMRT play a more important role in tamoxifen-mediated repression than in basal repression or prevention of estradiol stimulation of ERα and that reducing the expression of these proteins does not have a general growth promoting effect. Interestingly, it appears that N-CoR and SMRT may be needed for the optimal response to estradiol.

Our analysis of ERα target genes that mediate estradiol-stimulated proliferation indicated that tamoxifen action on the c-*myc*, *cyclin D1*, and *SDF-1* genes was not significantly altered when N-CoR and/or SMRT were silenced. Therefore, changes in the expression of these genes are not responsible for the increased cell cycle progression, we observed in response to tamoxifen. In addition, these results indicate that N-CoR and SMRT are not required for tamoxifen-mediated repression of these target genes and imply that other ER coregulators likely play a more important role. This suggests that although the corepressors can be recruited to some of these target genes upon treatment with tamoxifen, presumably as part of a complex

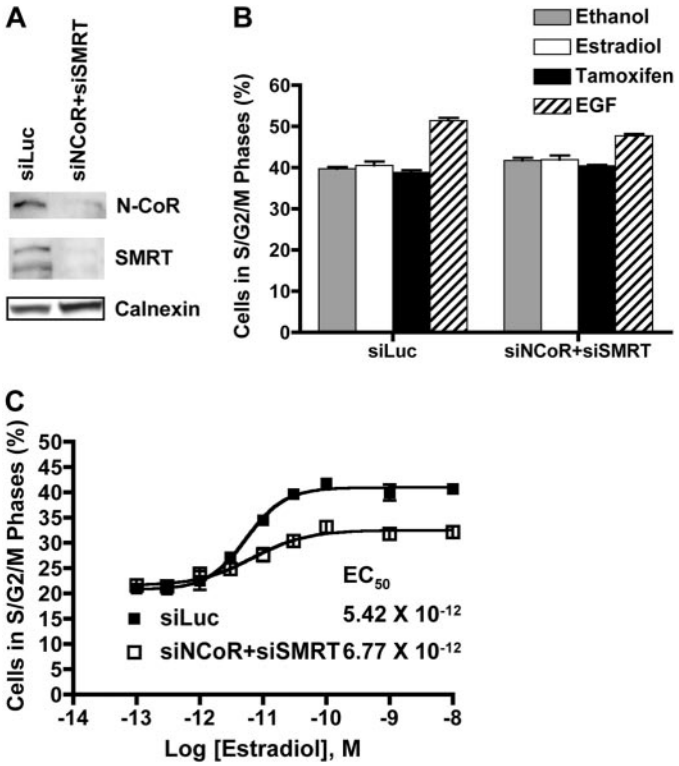

Fig. 6. Effect of Silencing N-CoR and SMRT on Cell Cycle Entry in Response to Estradiol, Tamoxifen, or EGF Treatment in ER-Negative MDA-MB231 Cells or MCF-7 Cells

Cells were transfected with siRNA oligonucleotides targeting N-CoR and SMRT or luciferase as a nonspecific control. Forty-eight hours after transfection, whole-cell extracts were prepared or cells were treated for 24 h with ethanol, 17β-estradiol, 4-hydroxytamoxifen, or EGF as shown. Cells were harvested by trypsinization and fixed before staining with propidium iodide to analyze DNA content by flow cytometry. A, Western blot analysis of proteins in whole-cell extracts from transfected MDA-MB231 cells. B, The average percent of cells in G2, S, and M phases stimulated by ethanol, estradiol, tamoxifen, or EGF under each silencing condition in MDA-MB231 cells. C, Cell cycle entry in MCF-7 cells treated with increasing concentrations of 17β-estradiol as shown. Curves and EC_{50} values were generated using GraphPad Prism software. Data shown are representative of three independent experiments done in duplicate $\pm$ SD.

of other accessory proteins, that they are not functionally required for repression. In addition, although we previously demonstrated that c-*myc* became partially responsive to tamoxifen upon overexpression of SRC-1 in breast cancer cells (17), we have shown in this study that the actions of ER coregulators are promoter specific. Thus, the balance between coactivator and corepressor expression levels and alterations in tamoxifen action may not exclusively affect the same promoters. Although the expression of known pro-proliferative ERα target genes was not increased by tamoxifen when N-CoR and SMRT were silenced, the effect of tamoxifen was dependent on the ER because the same effect was not seen in the ER-negative cell line MDA-MB231.

We found that *XBP-1* mRNA levels were elevated in cells in which N-CoR and SMRT were silenced and observed a further increase in gene expression when these cells were treated with tamoxifen. Thus, N-CoR and SMRT play a role in basal repression of this and likely other ERα target genes. Interestingly, *XBP-1* expression has been shown to be elevated 3-fold in MCF-7-derived tamoxifen-resistant cells as compared with the parent cell line (47). It is intriguing to speculate that higher *XBP-1* levels may be correlated with lower N-CoR or SMRT levels in tamoxifen-resistant cells or tumors. A role for XBP-1 in estradiol- or tamoxifen-mediated cell growth has not been demonstrated, however, so additional target genes are likely to play an important role in mediating the tamoxifen-stimulated growth that we observed. Further studies are needed to elucidate the role of XBP-1 in estradiol or tamoxifen action and breast cancer. Nonetheless, XBP-1 represents an endogenous gene involved in ERα function with increased expression when levels of N-CoR and/or SMRT are reduced and cells are treated with tamoxifen.

Our findings support those of previous studies suggesting that N-CoR and SMRT play a role in tamoxifen-bound ERα action and that the relative level of ER coregulators can influence the cellular response to tamoxifen. Overexpression of N-CoR or SMRT in various cell lines was shown to repress the partial agonist activity of tamoxifen, whereas loss or reduction of N-CoR expression led to tamoxifen-stimulated transcription or was associated with tamoxifen resistance

(23–26, 28, 29). Our results further these findings by showing that the functions of N-CoR and SMRT are promoter specific and influence tamoxifen action on an endogenous gene.

Our results also supplement previous findings by demonstrating that N-CoR and SMRT play a role in preventing tamoxifen from stimulating proliferation in breast cancer cells. These results are consistent with those of a study showing that tamoxifen enhances cell growth in MCF-7 cells stably expressing dominant-negative N-CoR (48). However, both of these findings are in contrast to those demonstrating that constitutively expressing a dominant-negative N-CoR in MCF-7 cells does not lead to tamoxifen-stimulated cell cycle entry or proliferation (49). Although we found that it was necessary to silence both N-CoR and SMRT to observe tamoxifen-stimulated cell cycle entry, the N-CoR dominant-negative consisting of a receptor interaction domain is expected to block SMRT interaction with tamoxifen-bound ERα as well due to the similarity between these domains in both corepressors. The results of these studies may vary because the dominant-negative N-CoR retains the ability to bind to ERα, which may still be able to influence the binding of other ER coregulators to the AF-1 and AF-2 domains of the receptor. Recent findings suggest that other coregulators may bind to tamoxifen-bound ERα in the AF-2 domain. A peptide was identified that recognizes a novel tamoxifen-induced binding surface of ERα within the LBD and replacement of T1F2 LXXLL receptor-interacting motifs with the peptide resulted in a tamoxifen-responsive coactivator (50).

Tamoxifen was not converted into a full agonist in the cell cycle entry assay when N-CoR and SMRT were both silenced in this study. This may be due to incomplete silencing of N-CoR and SMRT. However, it is likely that other ER coregulators and the target genes they regulate also play a role in tamoxifen-mediated repression of proliferation. Other ER corepressors such as REA, Smad4, or scaffold attachment factor B1 may influence tamoxifen action on the target genes involved (51–53). A role for additional ER corepressors in tamoxifen action has been suggested based on the finding that peptides containing a receptor interacting CoRNR box motif that differs from that found in N-CoR and SMRT interact with tamoxifen-bound ERα (54). In addition, the fact that tamoxifen does not induce recruitment of ER coactivators in breast cancer cells also plays a role in its partial agonism (14, 15, 17).

It is unclear how the tamoxifen-stimulated growth observed when N-CoR and SMRT were silenced is mediated. Microarray analysis of genes that become activated when N-CoR and SMRT are silenced and cells are treated with tamoxifen may help to identify potential target genes. It is possible that tamoxifen-stimulated proliferation is mediated via a pathway different from that stimulated by estradiol and that tamoxifen-specific target genes are involved. Defining the mechanism(s) by which tamoxifen-stimulated breast cancer cell growth occurs will help to characterize the action of tamoxifen and perhaps other selective ER modulators, understand tamoxifen resistance, and identify additional therapeutic targets.

MATERIALS AND METHODS

RNAi

siRNA oligonucleotide duplexes (Dharmacon, Inc., Lafayette, CO) were used to silence N-CoR and SMRT. The target sequence for N-CoR, located in the 5′ end of the protein, was 5′-AAGAAGGAUCCAGCAUUCGGA-3′. The target sequence for SMRT, located in the 3′UTR, was 5′-AAAGUCUAAACUGAGCUCGCA-3′. The sequence targeting luciferase as a nonspecific control was 5′-AACACUUACGCUGAGUACUUCGA-3′. The scrambled N-CoR target sequence was 5′-AAGAAGGAUCGCGCAUUCGGA-3′.

Cell Culture and Transfection

MCF-7 and MDA-MB231 cells were maintained in DMEM (Cellgro, Herndon, VA) with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 5 μg/ml insulin (Sigma, St. Louis, MO), and 100 U/ml penicillin-streptomycin. The day before transfection, 3.3×10^5 or 1.6×10^5 cells/well, respectively, were grown in six-well plates in phenol red-free DMEM (Invitrogen, Carlsbad, CA) supplemented with 5% charcoal/dextran-treated fetal bovine serum (Hyclone) and 2 mM L-glutamine. Cells were transfected with 60 nM each siRNA duplex using 4.5 μl LipofectAMINE 2000 (Invitrogen) in 2.5 ml Opti-MEM (Invitrogen) for 4 h before resuspension in fresh seeding medium. Final siRNA concentrations were brought to 120 nM with nonspecific control siRNA to equal the cosilencing condition. Forty-eight hours after transfection, cells were treated as indicated and used in subsequent analyses.

Immunoblot Analysis

Whole-cell extracts were prepared from MCF-7 or MDA-MB231 cells 48 h after transfection with siRNA duplexes. Pelleted cells were resuspended in a buffer containing 10 mM HEPES (pH 7.9), 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 1× complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN), 1.5 mM $MgCl_2$, 10 mM KCl and 0.1% Nonidet P-40, after which NaCl was added to a final concentration of 400 mM. Lysates were incubated on ice 25 min before centrifugation. Protein concentration was determined by Bradford assay using a Bio-Rad (Hercules, CA) protein assay reagent.

Proteins (70 μg) were separated by SDS-PAGE on 4–15% Tris-HCl gradient gels (Bio-Rad) and transferred to polyvinylidine difluoride for 2.5 h in 25 mM Tris, 192 mM glycine buffer with 10% methanol at 400 mA. Immunoblotting was done with polyclonal antibodies against N-CoR (1:4000) and Calnexin (1:10,000) (Stressgen Biotechnologies, Victoria, British Columbia, Canada) or a monoclonal SMRT antibody (1:3000) or polyclonal SMRTe antibody (1:250) (Upstate, Lake Placid, NY) in Tris-buffered saline containing 2% nonfat dry milk. Incubation with primary antibody was followed by incubation with horseradish peroxidase-conjugated donkey antirabbit (Pierce, Rockford, IL) at 1:5000 or goat antimouse (Bio-Rad) at 1:2000. Detection was carried out using the Pierce Super-Signal West Pico chemiluminescent substrate followed by scanning and quantification using a Fluorchem 5500 chemiluminescence imager (Alpha Innotech Corp., San Leandro, CA).

Luciferase and β-Galactosidase Assays

MCF-7 cells were transfected with siRNA duplexes as described above except that 8×10^5 cells were plated in 6-cm dishes and 200 ng pCMVβ-galactosidase (CLONTECH, Palo Alto, CA) and 400 ng $(RARE\beta)_2$tk-luciferase were included in the transfection with 9 μl LipofectAMINE 2000. Forty-eight hours after transfection, cells were treated with vehicle (DMSO) or 100 nM all-*trans* retinoic acid for 24 h. Cell extracts were prepared using 400 μl 1× Reporter Lysis Buffer (Promega, Madison, WI). Assays were performed with 25 μl extract and the Promega luciferase assay system or Tropix Galacto-Star β-galactosidase reporter gene assay system (Applied Biosystems, Foster City, CA). Luciferase and β-galactosidase activity were detected using a Monolight 2010 luminometer. Luciferase activity was normalized to β-galactosidase activity.

Real-Time RT-PCR

Total RNA was isolated from transfected MCF-7 cells treated with 100 nM 17β-estradiol (Sigma) or 1 μM 4-hydroxytamoxifen (Sigma) using the RNeasy mini kit (QIAGEN, Valencia, CA), with on-column deoxyribonuclease treatment to remove contaminating genomic DNA. Real-time RT-PCR was performed using 25–200 ng RNA and 100–200 nM of the primers listed below with SYBR Green PCR Master Mix and MultiScribe reverse transcriptase (Applied Biosystems) according to the manufacturer's protocol. Sequence detection was performed using the ABI PRISM 7700. The following primer pairs were used: N-CoR, 5′-AGCATTCCATCCCTACGGG-3′, sense, and 5′-TGGACCCCTTCACCAAAGC-3′, antisense; SMRT, 5′-CGCAACTGGTCGGCCA-3′, sense, and 5′-GCTGCAAGATCTCATCGAGGT-3′, antisense; myc, 5′-GCCACGTCTCCACACATCAG-3′, sense, and 5′-TCTTGGCAGCAGGATAGTCCTT-3′, antisense; cyclin D1, 5′-TGGAGGTCTGCGAGGAACAGAA-3′, sense, and 5′-TGCAGGCGGCTCTTTTTCA-3′, antisense; SDF-1, 5′-CCTGAGCTACAGATGCCCATG-3′, sense, and 5′-TGAGATGCTTGACGTTGGCT-3′, antisense; and XBP-1, 5-GCGCCTCACGCACCTG-3′, sense, and 5′-GCTGCTACTCTGTTTTTCAGTTTCC-3′, antisense. The primers used to amplify GAPDH as a normalization control were: 5′-TCCACCCATGGCAAATTCC-3′, sense, and 5′-TCGCCCCACTTGATTTTGG-3′, antisense. Amplification of specific targets was verified by agarose gel electrophoresis and dissociation curve analysis.

Cell Cycle Entry Assay

For cell cycle entry analysis transfected MCF-7 or MDA-MB231 cells were treated with ethanol, 100 nM 17β-estradiol, 1 μM 4-hydroxytamoxifen, or 10 nM human recombinant EGF (Sigma) for 24 h before trypsinization, washing in PBS and fixation in 40% methanol. Fixed cells were treated with 200 μl ribonuclease A (0.5 mg/ml) for 30 min at 37 C. Cells were then stained with an equal volume of propidium iodide (69 μM in 38 mM sodium citrate, pH 7.4) for at least 1 h and analyzed for DNA content by flow cytometry by a core facility.

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