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Anti-Proliferative Actions of a Synthetic REV-ERB α / β Agonist in Breast Cancer Cells

Yongjun Wang¹, Douglas Kojetin², and Thomas P. Burris³

¹Department of Pharmacological & Physiological Science, Saint Louis University School of Medicine, St. Louis, MO 63104

²The Scripps Research Institute, Jupiter, FL 33458

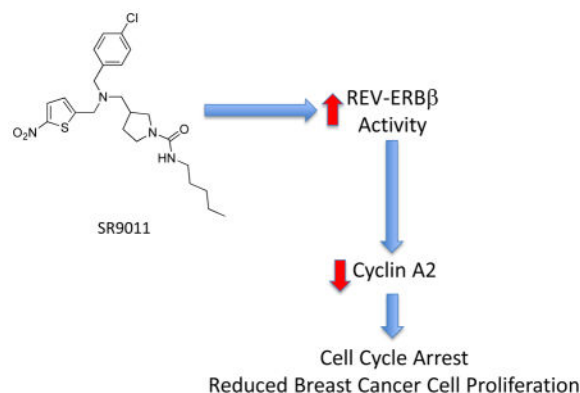
Abstract

REV-ERB α and REV-ERB β are nuclear receptors that are ligand-dependent transcriptional repressors. Heme is the natural ligand for these receptors, but several synthetic agonists and antagonists have been designed recently. The gene that encodes REV-ERB α , *NR1D1*, is closely associated with *ERBB2*, the gene that encodes the HER2 oncogene, which is amplified in HER2⁺ breast cancers. We examined the effect of a synthetic REV-ERB agonist, SR9011, on a range of estrogen receptor positive (ER⁺), ER⁻, HER2⁺, HER2⁻ and triple negative breast cancer cell lines. We found that SR9011 suppressed proliferation of the breast cancer cell lines regardless of their ER or HER2 status. SR9011 had no effect on MCF10A cell proliferation. SR9011 appears to pause the cell cycle of the breast cancer cells prior to M phase. Cyclin A (*CCNA2*) was identified as a direct target gene of REV-ERB suggesting that suppression of expression of this cyclin by SR9011 may mediate the cell cycle arrest. These data indicate that synthetic REV-ERB ligands may hold utility in treatment of diseases associated with uncontrolled cellular proliferation such as cancer.

Graphical Abstract

³To whom correspondence should be addressed: Dr. Thomas Burris, Department of Pharmacological & Physiological Science, Saint Louis University School of Medicine, 1402, South Grand Blvd, St. Louis, MO 63104, burristp@slu.edu.

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Keywords

Nuclear receptor; circadian rhythm; steroid receptor; heme; cell cycle

1. Introduction

REV-ERB α and REV-ERB β are members of the nuclear receptor superfamily and serve as receptors for heme^{1–3}. These two receptors respond to heme binding by altering the conformation of the ligand binding domain (LBD) leading to recruitment of the corepressor NCoR and subsequent repression of expression of their target genes^{1–3}. REV-ERBs play an important role in a range of physiological processes including regulation of glucose and lipid metabolism as well as regulation of the circadian rhythm^{3–5}. We and others have designed a number of synthetic ligands that target the REV-ERBs^{6–13}. In fact, we recently demonstrated that the REV-ERB agonists, SR9011 and SR9009, display a range of activity in vivo, including modulation of behavior and metabolism^{10, 12, 14}

Interestingly, the REV-ERB α gene (*NR1D1*) resides on the *ERBB2* (HER2)-containing 17q12–21 amplicon and has been suggested to play a role in the viability of HER2⁺ breast cancers. Additionally, REV-ERB α has been suggested to be a survival factor for HER2⁺ breast cancers¹⁵. Based on these reports, we sought to examine the effect of a synthetic REV-ERB agonist, SR9011, on a range of breast cancer cells including estrogen receptor positive and negative cells, HER2 positive and negative cells as well as triple negative cells.

2. Materials and Methods

2.1 Plasmids

The *CCNA2* promoter (–957 to +17) was amplified from genomic DNA of HepG2 cells and cloned into pTAL-Luc luciferase report vector (Clontech, Mountain View, CA) to make the pTAL-*CCNA2* reporter construct. The *CCNA2* promoter mutant construct was made using QuikChange II site-directed mutagenesis kit (Stratagene, Santa Clara, CA) according to the manufacturer's instructions. The REVRE (–802 to –791) was mutated from TAATAAGGTCAT to TAATAAGGCCAT. The muted primers targeting REV-ERB binding site are:

CTTCTGAAAGGAACATAATTATATCTAGGCCACTAGAACGTCATTGTG (forward)

and CACAATGACGTTCTAGTGgCCTAGATATAATTATGTTTCCTTTCAGAAG (reverse). The pGL4.73, pG5luc, pGL3-mBmal1, Gal4-REV-ERB α and Gal4-REV-ERB β were previously described. pcDNA-REV-ERB α and pcDNA-REV-ERB β were cloned in our lab.

2.2 Luciferase assay

HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C under 5% CO₂. Cells were plated in 96-well plates at a density of 15 X 10³ cells/well 24 h before transfection. Eight hour post-transfection, the cells were treated with SR9011 or DMSO. Twenty-four hours post-treatment, the luciferase activity was measured using the Dual-Glo™ luciferase assay system (Promega, Madison, WI). The values indicated represent the means \pm S.E. from three independently transfected wells. The experiments were repeated three times, and representative experiments are shown.

2.3 Cell culture, compound treatment, overexpression and knockdown

MCF10A, MDA-MB-231, MCF-7, MDA-MB-361, SKBR3, BT474 are from ATCC. Cells were plated in 6-well plates one day before treatment. The cells were treated with SR9011 or DMSO for 24 hr and harvested for RNA isolation or western blot. For over expression, the cells were infected with adenovirus for 24 hours and then switched to regular growth media. Twenty-four hours later, the cells were harvested to isolate total RNA. For knockdown assay, the control siRNA, human REV-ERB β siRNA (Thermo Scientific) were transfected with Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA) by using reverse transfection following the manufacture's instruction. After 24 hours, cells were harvested to perform quantitative PCR assay.

2.4 MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assays (Invitrogen)¹⁶ were performed according to the manufacturer's manual. Briefly, 3 \times 10³ to 5 \times 10³ cells per well were plated in 96-well plates. Twenty-four hours later, cells were treated with SR9011 or DMSO. Seventy-two hours after treatment, the cells were labeled with 1.2 mM MTT and incubated for 4 hours. DMSO was then added and readings were taken on a plate reader at 540 nm.

2.5 Cell Cycle and BrdU assays

The SKBR3 cells were incubated in growth media without serum for 72 hours and re-stimulated with normal growth media to synchronize the phase of the cell cycle. Every four hours after synchronization, the cells harvested for isolation of mRNA and assessment of *CCNA2* expression by QPCR. In MDA-MD231 cells, a BrdU assay¹⁷ was used to determine their phase within the cell cycle. The cells were incubated with BrdU (Invitrogen) for 30 min and then trypsinised and fixed with 70% ice-cold ethanol on ice for at least 30 minutes. The cells were then incubated with 2 M hydrochloric acid for 20 minutes, washed and stained with an anti-BrdU antibody (Invitrogen), FITC-labeled goat anti-mouse IgG

(eBiosciences, San Diego, CA) and propidium iodide (Sigma, St. Louis, MO) subsequently. Data were collected in a LSRII flow cytometer and analyzed using FloJo software.

2.6 cDNA synthesis and quantitative PCR

Total RNA extraction and cDNA synthesis were performed as described before. The quantitative PCR was performed using ABI Prism 7900 HT detection system (Applied Biosystems, Foster City, CA). The primers for quantitative PCR are: *CYPB*, GCAAATTCATCGTGTAATCAAG (forward) and CGTAGATGCTCTTTCCTCCTG (reverse); *REV-ERB α* , TTCCGCTTCGGTGGAGCAGC (forward) and CCGTTCTTCAGCACCAGAG (reverse); *REV-ERB β* , GAACAGACAGCCTTGCCAGC (forward) and AGCTCTGGTCACCATGCCAA (reverse); *CCNA2*, CGGGACAAAGCTGGCCTGAA (forward) and GTTGTGCATGCTGTGGTGCT (reverse). The expression of target gene was normalized to housekeeping gene *CYPB*.

2.7 Western blot

Antibodies against human cyclin A were obtained from Santa Cruz (Dallas, TX). Tubulin is from Sigma. Western blot were performed according to the standard protocols. Briefly, cells were washed once with phosphate-buffered saline and then incubated for 10 min at 4 °C in 100 μ l of TNT lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 1% Triton X-100) and a complete miniprotease inhibitor mixture (Roche Applied Science, Indianapolis, IN). Samples were then scraped and harvested into 1.5-ml microcentrifuge tubes, vortexed, and then centrifuged. Protein levels in the supernatants were determined using a Coomassie protein assay kit (Bio-Rad, Hercules, CA), and 20 μ g of protein from each sample was separated by SDSPAGE (BioRad - 10%) and then transferred to a polyvinylidene difluoride membrane (Millipore, Milford, MA) and immunoblotted with primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA). Detection of the bound antibody by enhanced chemiluminescence was performed according to the manufacturer's instructions (Santa Cruz).

2.8 ChIP/Microarray and ChIP assay

The *REV-ERB α* Chromatin Immunoprecipitation (ChIP)/Microarray experiment was previously described¹⁸. Standard ChIP assays were performed according to the manufacturer's instructions (Active Motif, Carlsbad, CA) as we have previously described¹⁹. Generally, the cells were grown to 70–80% confluency, fixed with 1% formaldehyde and sheared by sonication. Sheared chromatin was immunoprecipitated with IgG (Abcam, Cambridge MA), anti-Histone H3 (Abcam) and anti-*REV-ERB α* (Cell Signalling, Danvers, MA) overnight. The eluted chromatin was reverse cross-linked at 95°C for 15 min and treated with Proteinase K at 37°C for 1hr. The primers for the PCR reaction are: TGTTAAAGGCACGTATAGTTAAGAGAGT (forward) and AGATGGCACCTTGAACACTGTTG (reverse).

3. Results

3.1 SR9011 Inhibits Breast Cancer Cell Proliferation

In order to examine the effect of SR9011 (Fig. 1a) on breast cancer cell proliferation, we assessed the ability of this compound to alter cell viability using a MTT assay. We began using two cell lines. In the non-tumorigenic breast epithelial MCF-10A cell line had no effect on cell viability; however, in the tumorigenic HER2⁺ (estrogen receptor (ER) negative and progesterone receptor (PR) negative) SKBR3 cell line there was a dose-dependent decrease in viability of the cells (Fig. 1b). MCF-10A cells are commonly used as normal controls in breast cancer studies²⁰ and it is interesting to note that the SR9011 effects were specific to the tumorigenic breast cancer cell line. When we examined the effects of SR9011 on additional breast cancer cell lines including ER⁺ PR⁺ HER2⁻ (MCF-7), ER⁺ PR⁺ HER2⁺ (BT-474 and MDA-MB361), and triple negative (MDA-MB231) cell lines we observed that the compound also caused a dose-dependent reduction in cell viability (Fig. 1c). Thus, the effect of SR9011 on the breast cancer cells appeared to be independent of ER, PR or HER2 status. As shown in Fig 2, treatment with SR9011 resulted in an increase in cells in the G₀/G₁ phase and a decrease of cells in S and G₂/M phase suggesting that activation of REV-ERB may be resulting in decreased transition from G₁ to S phase and/or from S to G₂/M phase. There was no evidence of induction of apoptosis or cell death as indicated by the absence of a sub-G₁ phase.

3.2 Cyclin A is a REV-ERB target gene

Given the specific effects we observed on the cell cycle, we initially searched for putative REVERB target genes that could be responsible for accumulation of cells in the G₀/G₁ phase. Assessment of ChIP/Microarray data revealed that the *cyclin A* gene (*CCNA2*) contained a putative REV-ERB response element within its promoter (Fig. 3a) and the function of cyclin A in regulation of progression of the cell cycle into the S and M phases via interaction with CDK2 and CDK1, respectively²¹⁻²³. A subsequent ChIP assay revealed REV-ERB α occupancy of the promoter region of *CCNA2* (Fig. 3b). Adenoviral overexpression of REV-ERB α in SKBR3 cells led to significant repression of *CCNA2* gene expression, as one would expect if this transcriptional repressor directly targeted this gene (Fig. 3c). A putative REV-ERB response element (REVRE) that displayed considerably conservation between human and mouse was identified in the promoter (Fig. 3d). We constructed luciferase reporter constructs containing the promoter of the wild type *CCNA2* REVRE or a mutant *CCNA2* REVRE (Fig. 3d bottom) and assessed the ability of either REV-ERB α or REV-ERB β to regulate this reporter in a cotransfection assay in HEK293 cells. As shown in Fig. 3e, when either REV-ERB α or REV-ERB β were cotransfected into cells along with the wild type reporter significant repression of transcription was noted. If the REV-ERB agonist SR9011 was added, additional suppression of transcription was observed. However, if the reporter contained the mutant REVRE instead of the wild type the repressive activity of either of the REV-ERBs was lost (Fig. 3e). Additionally, and as one would expect, the response to SR9011 was lost when examining the mutant reporter (Fig. 3e)

In order to perform REV-ERB loss of function studies, we first examined the expression of the REV-ERB subtypes in the various breast cancer cell lines. We found that *REV-ERB β* was the predominate form of *REV-ERB* expressed in all of the breast cancer cells lines with the exception of the MCF10A line. *REV-ERB β* was expressed to a level approximately 9- to 35-fold greater than *REV-ERB α* in these cell lines (Fig 4). In MCF-10A cells *REV-ERB α* and *REV-ERB β* expression levels were similar (Fig. 4). Given that REV-ERB β is the dominant REV-ERB in the cells, we performed loss of function studies targeting REV-ERB β expression with siRNA. Suppression of REV-ERB β expression in MDA-MB231 cells resulted in loss of the ability of SR9011 to reduce cell viability as detected in a MTT assay as shown in Figure 5a. Furthermore, overexpression of REV-ERB β in the resistant MCF-10A cells resulted in gain of ability of SR9011 to reduce cell viability in the MTT assay (Fig. 5b). These data suggest that expression of REVERB β is critical for the anti-proliferative effects of SR9011 in breast cancer cells.

We also examined the effect of suppression of REV-ERB β on *CCNA2* gene expression in three cell lines, MCF7, MDA-MBA-231 and SKBR3. Transfection of these cells with *REV-ERB β* siRNA led to substantial decrease in *REV-ERB β* expression in all three cases ranging from 70–90% (Fig. 6). In all three cases, suppression of *REV-ERB β* expression led to an increase in *CCNA2* gene expression (Fig. 6). These data clearly indicate that the cyclin A gene is a direct REV-ERB target gene.

3.4 SR9011 suppresses cyclin A expression in breast cancer cells

Since we had observed a clear effect of the REV-ERB agonist SR9011 on cell cycle that led us to hypothesize that *CCNA2* was a direct REV-ERB target gene, we next examined whether SR9011 would regulate *CCNA2* expression. First, we examined this possibility using the *CCNA2* promoter luciferase reporter transfected along with either REV-ERB α or REV-ERB β in HEK293 cells. As shown in Fig. 7a, in cells transfected with the *CCNA2* reporter only SR9011 treatment resulted in suppression of transcription consistent with SR9011 enhancing the transcriptional repressor activity of endogenously expressed REVERB. Overexpression of either REV-ERB α or REV-ERB β enhanced the effect of SR9011, as one would expect (Fig. 7a). Treatment of a range of breast cancer cell lines with SR9011 for 24h led to suppression of *CCNA2* gene expression in all cases (Fig. 7b). Interestingly, the most sensitive cell line in terms of SR9011-dependent suppression of proliferation (SKBR3) was also most sensitive in terms of maximal suppression of *CCNA2* gene expression (~90% suppression) while the least sensitive cell line for proliferation response (MCF10A) was also the least sensitive to SR9011 in terms of *CCNA2* response (~15%) (Fig. 7b). We also examined *CCNA2* expression in SKBR3 cells where we synchronized the cells for the phase of cell cycle and found that 20h after synchronization *CCNA2* expression was suppressed in SR9011 treated cells vs. vehicle treated cells (Fig. 7c). This suppression was sustained for the duration of the experiment that concluded at 44h post synchronization. When we examined Cyclin A2 protein expression in three of the cell lines (MDA-MB231, MDA-MB361, and BT-474) in response to SR9011 treatment we also observed a decrease consistent with the decrease in gene expression (Fig. 7d).

4. Discussion

Two subfamilies of nuclear receptors, REV-ERBs (REV-ERB α and β) and retinoic acid receptor-related orphan receptors (ROR α , β , γ) play an important role in modulation of the circadian clock by directly regulation the expression of core circadian clock genes such as BMAL1, NPAS2, and CLOCK^{18, 24–27}. Circadian (~24h) rhythms in biological processes are essential for normal physiological function in nearly all living organisms. These 24h recurrent patterns of behavior and physiology are controlled by a feedback loop at the cellular level. Circadian rhythms play an essential role in aspects of physiology and behavior including the sleep-wake cycle, body temperature, blood pressure, and renal function, and are generated by feedback loops in gene expression where heterodimers of BMAL1 and CLOCK (the positive limb) activate the expression of the *Cryptochrome* and *Period* genes (the negative limb). Once CRY and PER have reached a critical level of expression they are able to block the stimulatory effect of the CLOCK/BMAL1 complex on their own genes completing the loop. Epidemiological data indicates that disruption of circadian rhythmicity is associated with an increased risk of development of breast cancer^{28–31}. Based on these data, the World Health Organization has classified shift-work associated with a disrupted circadian rhythm as a probable carcinogen³². The observation that disruption of the circadian rhythm leads to increased risk of cancer has been replicated in animal models where rodents experience an increased rate of tumor progression if their rhythms are disrupted^{33–36}. The molecular mechanism underlying the link between circadian rhythm disruption in humans and the increased risk of breast cancer is not clear, but several studies have demonstrated links between abnormal regulation of “clock” genes (genes that regulate the core circadian rhythm) and tumorigenesis^{37–40}.

These studies led us to consider that our efforts to pharmacologically target components of the clock machinery may hold utility in treatment of cancer. We were particularly intrigued by recent studies implicating REV-ERB in regulation of proliferation and cancer^{15, 41, 42}. Our data indicate that activation of REV-ERB with a synthetic agonist leads to decreased proliferation of a range of breast cancer cells independent of their ER or HER2 status. It appears that this may be due to direct targeting of cyclin A2 expression. A previous study linked REV-ERB α to breast cancer based on the localization of the *NR1D1* gene within the same chromosomal region of the *ERBB2* gene (HER2) that is amplified in many breast cancers. Interestingly, the REV-ERB α gene (*NR1D1*) resides on the *ERBB2* (HER2)-containing 17q12–21 amplicon and has been suggested to play a role in the viability of HER2⁺ breast cancers.¹⁵ REV-ERB α expression was associated with increased lipogenesis that improved BT474 cell survival¹⁵. Based on this, we might expect that a REV-ERB antagonist would be beneficial, which is in contrast to our current work indicating that activation of REV-ERB leads to decreased breast cancer cell proliferation in the BT474 cell line as well as a range of other breast cancer cells lines. In a more recent study, suppression of REV-ERB α expression did not alter BT474 cell viability⁴². However, this same study found that although a REVERB β antagonist did not alter BT474 viability alone, it did enhance the cytotoxicity of the autophagy inhibitor chloroquine⁴².

Thus, there is some controversy surrounding whether REV-ERB is a “survival” factor in HER2⁺ breast cancer cells based on REV-ERB α knock-down experiments performed in

BT474 cells, but our data clearly demonstrate that pharmacological activation of REV-ERB leads to reduced proliferation of a range of breast cancer cells independent of their ER or HER2 status. Overexpression of REV-ERB β relative to REV-ERB α appears to be critical for the efficacy of SR9011 in the breast cancer cells since suppression of REV-ERB β expression reduces that activity of SR9011 in MDA-MB231 cells while overexpression of REV-ERB β in the “resistant” MCF10A cells renders them sensitive to the drug. Overall, we have not observed cytotoxicity of SR9011 or other REV-ERB agonists in “normal” non-cancerous cells as well as in a number of in vivo studies indicating that these compounds are not overtly toxic^{10, 12, 14, 43}. These data suggest that targeting REV-ERB may be an effective method for treating breast cancers clinically.

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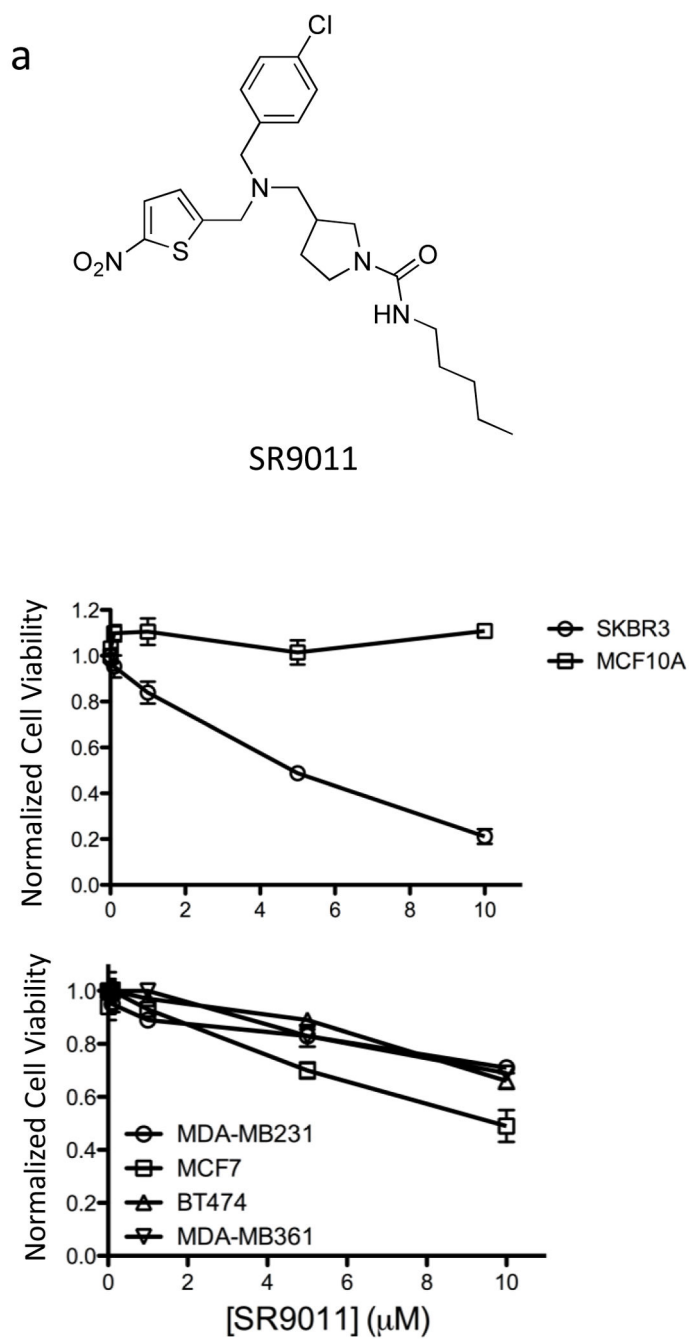


Figure 1. Activation of REV-ERB suppresses breast cancer cell viability (MTT assay). a. Chemical structure of the REV-ERB agonist SR9011. b. Effect of SR9011 on viability of SKBR3 and MCF10A cells. c. Effect of SR9011 on a range of breast cancer cell viability. See results for description of each cell line in terms of ER and HER2 expression.

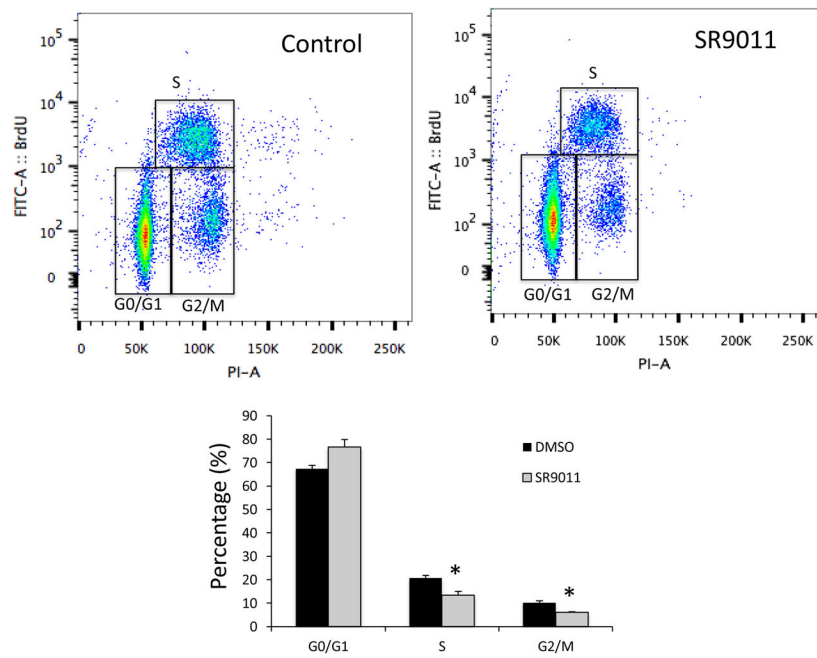


Figure 2. SR9011 treatment of breast cancer cells (MDA-MD231) results in cell cycle arrest. Results from flow cytometry analysis of MDA-MD231 cells stained with BrdU and propidium iodide (vehicle or SR9011 treatment (10 μ M)) are shown. The percentage of cells identified in various stages of the cell cycle are illustrated. *, indicates $p < 0.05$ using Student's t -test.

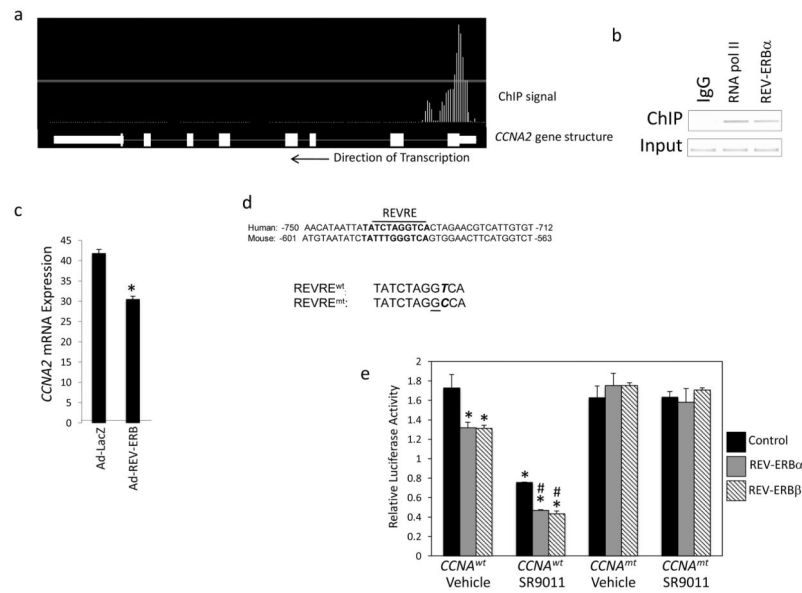


Figure 3. The *CCNA2* promoter contains a functional RORE site and is responsive to REV-ERB. a. Illustration of REV-ERB α occupancy of the *CCNA2* gene. b. Results from a chromatin immunoprecipitation assay examining RNA polymerase II occupancy (positive control) or REV-ERB α occupancy of the *CCNA2* promoter. c. Overexpression of REV-ERB α with an adenovirus suppresses *CCNA2* expression. d. Illustration of the putative REV-ERB response element (REVRE) in the *CCNA2* promoter. The human sequence is compared with the mouse and the mutation is indicated at bottom. e. Mutation of the REVRE (*CCNA2^{mt}:luc*) reduces *CCNA2* expression in the presence of REV-ERB α or REV-ERB β . Cells were cotransfected with wt or a mutant (REVRE mutated) *CCNA2* promoter-reporter with or without REV-ERB α or REV-ERB β expression vectors. SR9011 treatment at 10 μ M. *, indicates $p < 0.05$ vs. untreated control and # indicates $p < 0.05$ vs. SR9011 treated control.

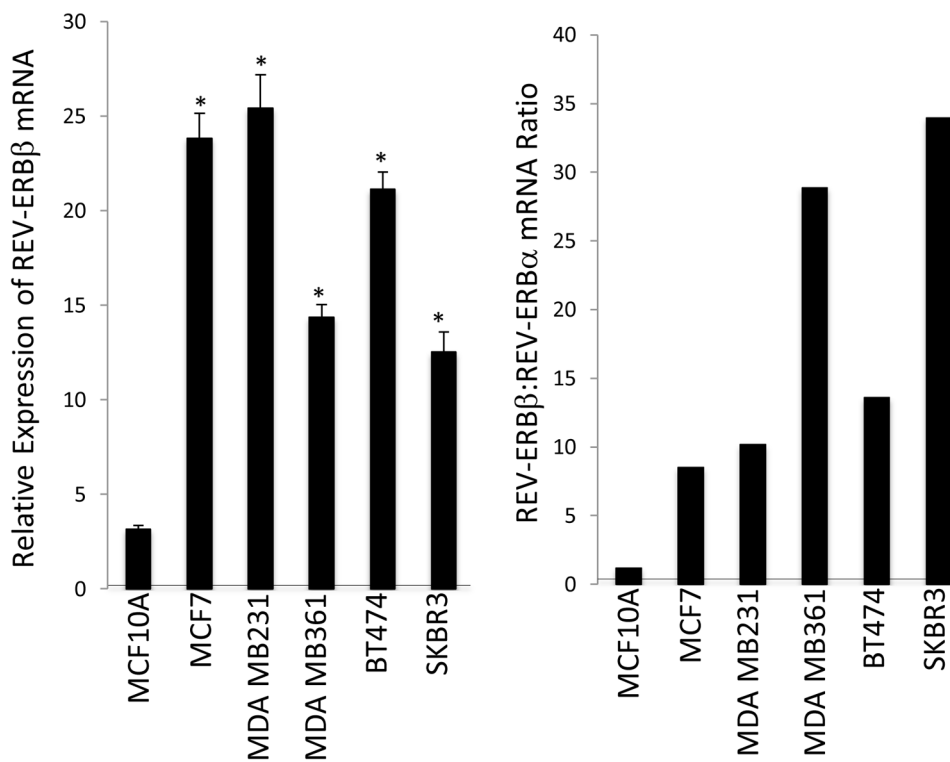


Figure 4.

REV-ERB β mRNA is highly expressed in breast cancer cell lines. REV-ERB mRNA expression was measured by QPCR and normalized to cyclophilin. REV-ERB β is overexpressed relative to REV-ERB α in breast cancer cells. Most tissues we have examined with the exception of the breast cancer cell lines display a ratio near 1 (data not shown). *, indicates $p < 0.05$ relative to MCF10A cell expression using Student's t -test.

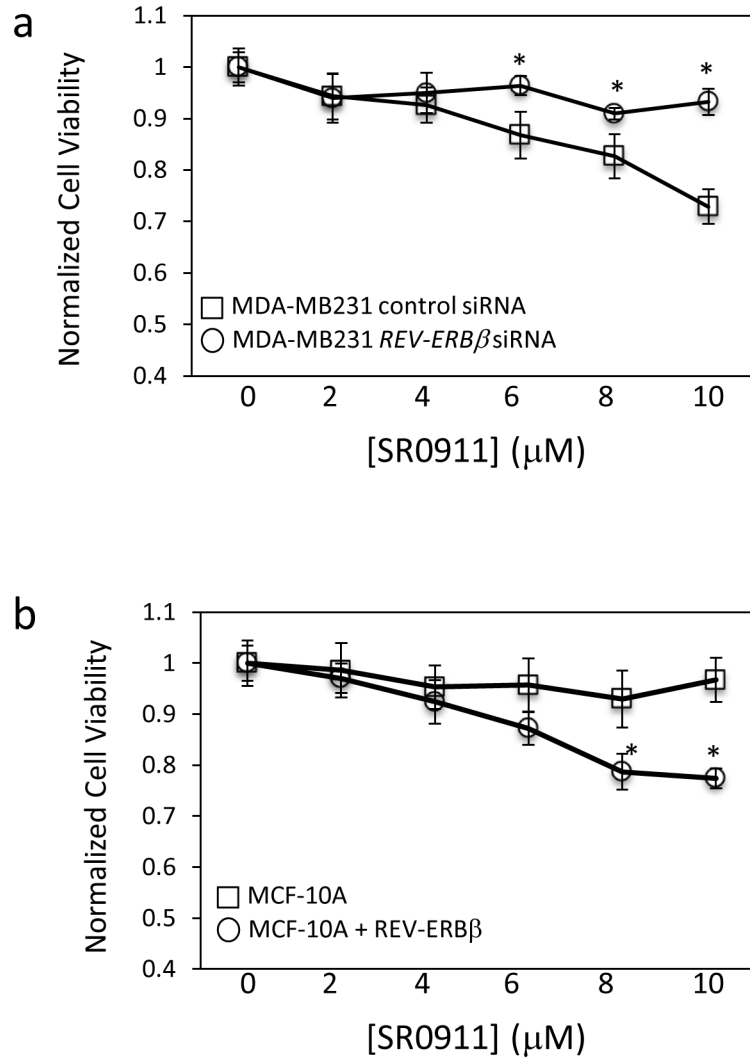


Figure 5. REV-ERB β is critical for the effects of SR9011 on breast cancer cells. A) Suppression of REV-ERB β expression in MDA-MBA231 cells renders the cells less sensitive to the actions of SR9011 as observed in a MTT cell viability assay. B) Overexpression of REV-ERB β in MCF10A cells renders these cells sensitive to the action of SR9011 in terms of suppression of cell viability. *, indicates $p < 0.05$ relative to control cells using Student's t -test.

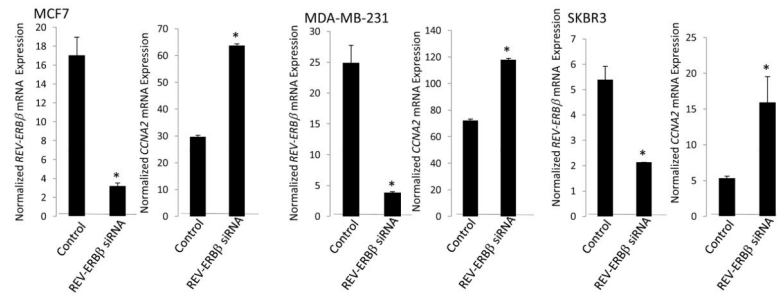


Figure 6.

CCNA2 expression is tonically suppressed by REV-ERB β . When REV-ERB β expression is suppressed by siRNA treatment, *CCNA2* mRNA expression increases in the cell lines examined above. Expression was normalized to cyclophilin and control cells received scrambled siRNA. *, indicates $p < 0.05$ using Student's *t*-test.

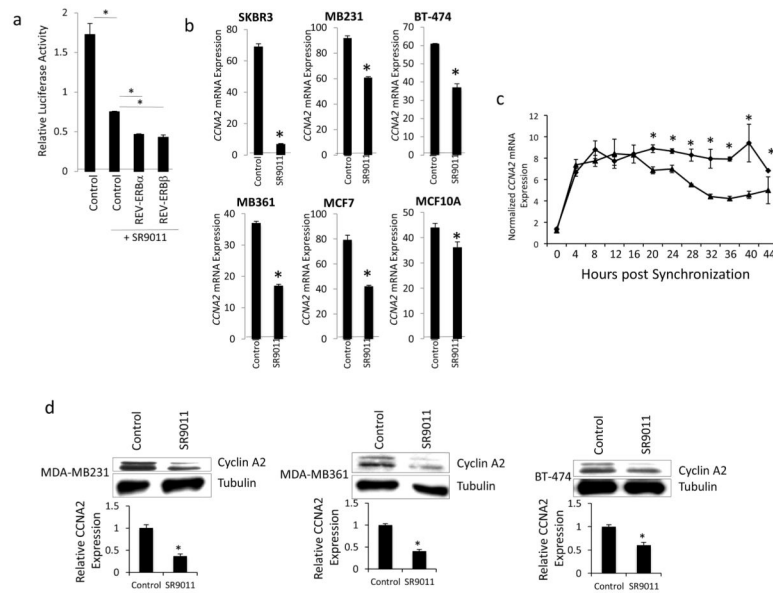


Figure 7.

The REV-ERB agonist, SR9011, suppresses the expression of *CCNA2*. **a**. SR9011 suppresses luciferase expression driven by the *CCNA2* promoter. In HEK293 cells, treatment with SR9011 (10 μ M) suppresses *CCNA2* expression and coexpression of either REV-ERB α or REV-ERB β enhances the SR9011-mediated effect. **b**. SR9011 treatment suppresses the expression of *CCNA2* in multiple breast cancer cell lines. Expression was normalized to cyclophilin. **c**. SR9011 treatment suppresses *CCNA2* expression in synchronized SKBR3 cells. **d**. SR9011 treatment suppresses the expression of CYCLIN A protein in multiple cancer cell lines. *, indicates $p < 0.05$ using Student's *t*-test.