Research Article

Retinoic acid-related orphan receptor alpha 1 (RORα1) induction of AKR1C3 promotes MCF-7 breast cancer cell proliferation and tamoxifen-resistance which is suppressed by melatonin

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ABSTRACT

The retinoic acid-related orphan receptors alpha (RORα) are members of the steroid/thyroid nuclear receptor super-family and core components of the circadian timing system. In the present study, we continue to investigate the role of RORαs in human breast cancer. Assays using the RORα response element (RORE)-tk-luciferase reporter demonstrate the functionality of the RORα1 in MCF-7 breast cancer cells and that over-expression of RORα1 stimulates MCF-7 human breast cancer cell proliferation. Genomic analysis revealed that RORα1 over-expression regulated the transcription of numerous genes in MCF-7 breast cancer cells including increasing the expression of connexin 43 (*CX43*), aldo-keto reductases 1C1 (*AKR1C1*), and *AKR1C3*. Furthermore, administration of the pineal hormone melatonin represses RORα1 induction of *CX43, AKR1C1,* and *AKR1C3* in MCF-7 cells. *AKR1C3* has been reported to impact in intra-tumoral production of androgens and estrogens and thus, might promote Tamoxifen resistance in breast cancer. Over-expression of *RORα1* and subsequently *AKR1C3* does promote Tamoxifen resistance, which can be inhibited by melatonin administration.

Key Words: Retinoic acid-related orphan receptor alpha 1, breast cancer, AKR1C3, melatonin.

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1. INTRODUCTION

A number of steroid/thyroid hormone nuclear receptor superfamily including the estrogen receptor (ER), progesterone receptor (PR), and androgen receptor (AR) influence the development and progression of breast cancer (1-3). The retinoic acid-related orphan receptors (RORs) are nuclear orphan receptors that are expressed in human breast cancer (4). There are 4 RORα splice

variants [1, 2, 3 and 4] from the same gene located on chromosome 15q21 in humans and chromosome 9 in mice that differ by small sequences in their N-terminal domains (5-7). The RORαs bind to their corresponding DNA response elements primarily as monomers (5). The RORα response element (RORE) consists of a tandem repeat of the PuGGTCA motif, spaced by two nucleotides, and proceeded by a 6-bp AT-rich sequence, referred to as Rev-DR2 site. The RORα1 has also been shown to bind as a homodimer to an RORE in the promoter region of the circadian clock gene Rev-erbα (8). The RORαs appear to be widely expressed and have been detected by us and others at both the mRNA and protein level in many tissues and even human breast cancer (4, 9-11). By identification of RORE motifs in their regulatory region, several tumor-related genes, including *N-Myc*, *laminin B1*, *p21*, *BMAL1*, *REV-ERBα*, *NM23*, and Aromatase have been designated as RORα responsive genes, suggesting that RORαs may play a role in the progression of certain neoplasias (11-16). We and others have reported that RORαs cross-talk with other steroid nuclear receptors including RARα and ERα in breast cancer cells (17, 18). The RORs are also core circadian clock genes that regulate the expression of the basic helix-loop-helix gene Brain Muscle and Arnt Protein 2 (BMAL2) of the positive limb of the circadian clock that dimerizes with Circadian Locomoter Clock Kaput (CLOCK) to modulate the expression of E-box genes including Cryptochrome, Period, and even *MYC* genes (19). These and other studies demonstrate that disruption of peripheral oscillator (clock) genes in the breast, are strongly associated with cancer (20).

The RORαs were initially classified as orphan receptors with no defined ligands, however, using X-ray crystallography, Kallen *et al.* (21) identified cholesterol and its derivatives as potential ligands of RORα, while Kane and Means (22) demonstrated that calcium/calmodulin-dependent kinase IV (CaMKIV) enhanced RORα transactivation. It was initially reported by Becker-Andre *et al.* (23) that circadian hormone, melatonin, a known inhibitor of mammary tumorigenesis (24), was a ligand for the RORαs, however this report has been retracted as melatonin was not confirmed as a ligand for these receptors (25). We have reported that RORα receptors are constitutively active in MCF-7 breast tumor cells and their transcriptional activity can be inhibited/suppressed by melatonin in MCF-7 human breast cancer cells in a ligand-independent manner (4).

Melatonin (N-acetyl-5-methoxytryptamine) is the major hormonal product of the pineal gland that regulates circadian rhythms, reproduction in seasonally breeding mammals, and has been demonstrated to have anti-tumorigenic effects on breast cancer (26). Studies with the ERα-positive MCF-7 human breast cancer cell line demonstrated that physiological concentrations of melatonin significantly inhibit breast tumor cell proliferation and induce a more differentiated phenotype (24). Furthermore, we have reported that melatonin can modulate various nuclear receptors, including inhibiting ERα, GR, and RORα transactivation, while promoting RAR, RXR, and VDR transcriptional activity in breast cancer cells (26, 27).

In the present study, we demonstrate that expression of RORα1 promotes the proliferation of ERα-positive MCF-7 cells, and induces the expression of numerous genes including *CX43*, *AKR1C1*, and *AKR1C3*. Finally, we show that MCF-7 cells over-expressing RORα1 with subsequent induction of AKR1C3 protein expression show resistance to the anti-estrogen tamoxifen which can be reversed by the administration of melatonin.

2. MATERIALS AND METHODS

2.1. Materials.

All chemicals and tissue culture reagents were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI-1640 medium, fetal bovine serum (FBS), and lipofectamine were purchased from Gibco BRL (Gaithersburg, MD). TRIzol, the pcDNA3.1/Zeo (+) plasmid and zeocin were purchased from Invitrogen (Carlsbad, CA). Molecular biology enzymes were purchased from Promega (Madison, WI) and RORα primers were synthesized by integrated DNA technologies, Inc. (Coralville, IA). The chemiluminescence reagents were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL) and Kodak X-Omat AR films were purchased from Eastman Kodak Co. (Rochester NY). The pCMV-β-galactosidase plasmid was provided by Dr. Jean Lokeyer (New Orleans, LA); the pCMX-RORα expression vectors and the RORE$\alpha 2_3$TKLUC reporter plasmid were kindly provided by Dr. Vincent Giguere from McGill University (Montreal, Quebec, Canada).

2.2. Cell lines and breast tumors.

MCF-7 ERα-positive breast cancer cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA), expanded and frozen in liquid nitrogen. MCF-7 cells below passage 20 were employed for these studies. Breast cancer cell lines were routinely maintained at 37° C in a humidified atmosphere of 5% CO_2 and 95% air in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 mM MEM non-essential amino acids, 1 mM sodium pyruvate and 10 mM BME.

2.3. Expression vector and stable transfection.

The plasmid pCMX-RORα1 was digested with the restriction enzymes *Kpn*I and *Bam*HI to obtain the full-length RORα1 cDNA which as then cloned into the *Kpn*I-*Bam*HI sites of the pcDNA3.1/Zeo (+) expression vector to generate the expression construct pcDNA3.1-RORα1, that was stably transfected into MCF-7 cells. The pcDNA3.1/Zeo (+) (pcDNA3.1-vt) vector was used to transfect MCF-7 cells, which were then used as controls in studies of RORα1-transfected cells. Details regarding stable transfection and RORα1 expression can be found in the supplemental materials.

2.4. Growth studies.

Vector-transfected and RORα1-transfected MCF-7 human breast cancer cells were plated onto 6-well plates at a density of 5×10^4 cells/well in RPMI-1640 medium supplemented with 10% FBS, in triplicate. On Days 1, 3, 5 and 7, cells were harvested in phosphate-buffered saline (PBS) solution containing 0.1% EDTA and passed through a 21-gauge needle to generate a single cell suspension. Cell numbers were determined by counting on a hemocytometer using the trypan blue dye exclusion method.

2.5. Tamoxifen resistance growth studies.

MCF-7 vector (control) and MCF-7 RORα1 expressing cells were seeded at the density of 10 x 10^4 cells/well into 35 mm 6-well plates. After 24 h cells were administered 4-hydroxy-tamoxifen (4-OHT) or melatonin at concentrations of 5 μM and 10 nM, respectively, for 96 h (4 days). Before staining with trypan blue to test for cell viability, cells were dislodged with trypsin and rinsed with PBS. Viable and dead cells were counted on the hemocytometer using a microscope.

2.6 Transient transfection and luciferase transcriptional reporter assays.

Parental MCF-7 cells were plated onto 35 mm 6-well plates at a density of 2×10^5 cells/well in RPMI-1640 medium supplemented with 10% FBS. After 24 h of serum-starvation, cells were transfected in serum-free RPMI-1640 medium for 6 h with 2 μg of the RORE$\alpha 2_3$-tk-LUC reporter construct or 1 μg of pCMVβ control plasmid per well, using 6 μl of lipofectamine (Life Technologies, Inc.). Luciferase was measured using a Model 2010 luminometer (Analytical Luminescence) and luminescence was normalized to both the protein concentration and the β-galactosidase activity, as previously described (28).

2.7 Western blot analysis.

The concentrations of total cellular protein in extracts from cell line lysates were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Total protein (50 μg per sample) from cell line lysates was electrophorectically separated on 10% SDS polyacrylamide gels and electro-blotted onto a Hybond membrane. After incubation with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20, the blots were probed with antibodies against RORα (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), AKR1C1, AKR1C3 (Abcam, Cambridge, MA), and β-actin (Cell Signaling Technology). The same blots were subsequently stripped and re-probed with a β-actin antibody as an internal loading control (Cell Signaling Technology Inc.). Band intensity was quantified using ImageJ v1.50e (NIH, Bethesda MD) to normalize to internal control.

2.8 cDNA microarray analysis.

Total RNA from the MCF-vt, MCF-ROR#3, and MCF-ROR#8 cell clones was isolated by RNeasy Mini kits (Qiagen, Valencia, CA) following the manufacturer's instructions. The cDNA microarray analysis was performed by the Tulane University Gene Therapy Center employing Affymetrix Gene Chip microarray (Affymetrix, Santa Clara, CA). Technical and experimental details are described in the Supplemental Materials. In brief, Twenty-five micrograms of biotinylated cRNA was fragmented and hybridized to Affymetrix HG-U133A array, which contains 22,215 oligonucleotides representing 15,003 human genes. The array was stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR) and the staining signal was amplified by biotinylated anti-streptavidin (Vector Laboratories, Burlingame, CA) and by a second staining with streptavidin-phycoerythrin, and then scanned on a Hewlett-Packard Gene Array Scanner. The expression data were analyzed using Microarray Suite 5.0 (MAS5.0).

2.9. Northern blot analysis of Cx43 mRNA expression.

Fifty micrograms of total cellular RNA isolated from RORα1-over expressing clones (#3 and #8), pcDNA3.1 vector-transfected, and parental MCF-7 cells was separated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde and transferred onto Hybond-XL membranes, as previously described (23, 28). The membranes were then hybridized with full-length α-[^{32}P]–labeled RORα1 or CX43 probes purchased from American Type Culture Collection (ATCC). The membranes were then washed and exposed to Kodak X-Omat AR film.

2.10. Quantitative real time PCR (qPCR).

qPCR was performed using an iCycler iQ apparatus (Bio-Rad) associated with the iCycler Optical System Interface software (version 2.3; Bio-Rad) as we described previously (28). The sequences of primers used are shown in Supplementary Table 1. For the analysis of *AKR1C1* and *AKR1C3* mRNA expession, qPCR analysis was performed in triplicate. The differences in the expression of all the gene transcripts were normalized with respect to *GAPDH* expression. The relative level of expression was calculated with the formula $2^{-\Delta ct}$.

2.11. Statistical analysis.

Statistical difference in cell proliferation and RORα transcriptional activity was determined by two-way analysis of variance (ANOVA) followed by a Newman-Kuels post-hoc test. P-values < 0.05 were considered statistically significant.

3. RESULTS

3.1. Stable expression of RORα1 receptor in MCF-7 human breast cancer cells lines.

RORα1 was expressed at high levels in ERα-positive MCF-7 human breast cancer cells following stable transfection with the pcDNA3.1-*RORα1* construct. Nine clones of MCF-7 breast cancer cells were characterized for *RORα1* expression. Two clones, MCF-*ROR*#3 and MCF-*ROR*#8, which exhibited the greatest levels of *RORα1* mRNA (Figure 1A) and protein (Figure 1B) expression levels were chosen for further analysis. To confirm the functionality of the *RORα1* receptor in these cells, RORE luciferase assays were conducted. These assays showed that the *RORα1* over-expressing cell lines possessed significantly greater *RORα* transcriptional activity than their parental or vector transfected counterparts. As illustrated in Figure 1C, the relative luciferase activities increased 2 to 3-fold in RORα1 over-expressing MCF-7 cells in comparison with their corresponding vector-transfected control cells. These data demonstrate that expressed recombinant *RORα1* receptors are functional in MCF-7 cells.

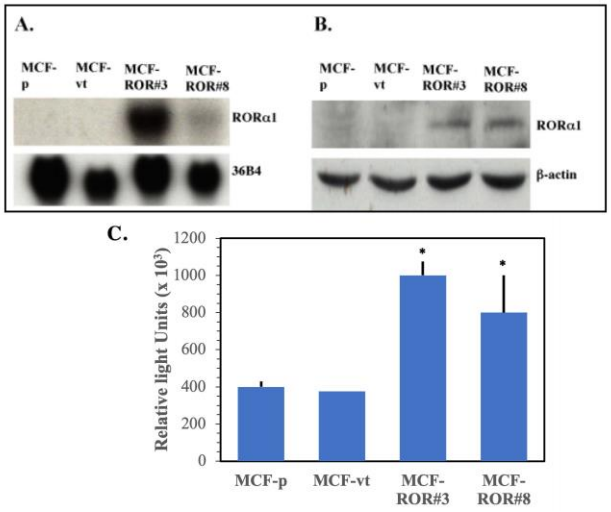

Fig. 1. Northern and Western blot analyses of RORα in MCF-7 human breast cancer cells and RORα1 transactivation in RORα1-transfected ERα-positive MCF-7 breast cancer cells.

In the Northern blot analysis (A), fifty micrograms of total cellular RNA from different RORα1-transfected cell clones (MCF-ROR#3, MCF-ROR#8), pcDNA3.1 vector-transfected (MCF-vt) or MCF-7 parental cells (MCF-p) were separated on a 1% denaturing agarose gel and transferred to Hybond-XL membranes. The RORα1 and 36B4, loading control, mRNA signals were detected on the blot by probing with the RORα1 and 36B4 cDNAs. In the Western blot analysis (B), fifty micrograms of cellular protein from each cell clone was subjected to a 12% SDS-PAGE and transferred to nitrocellulose paper. Expression of RORα and β-actin were detected by ECL reagents following a sequential incubation with a pan RORα and β-actin antibodies. (C) RORα1 transactivation RORE$\alpha 2_3$-TKLUC reporter assays in ERα-positive MCF-7 breast cancer cells. Transient transfection with the RORE$\alpha 2_3$-TKLU reporter construct was conducted on different RORα1-over-expressing MCF-7 cell clones (MCF-ROR#3, MCF-ROR#8), pcDNA3.1 vector-transfected (MCF-vt), or MCF-7 parental cells (MCF-p). Cells were harvested 24 h following transfection and subjected to luciferase assays. The transfection efficiency was normalized by β-galactosidase activities. (P< 0.05 vs. MCF-p or MCF-vt, n=3).*

3.2. Effects of RORα1 on MCF-7 human breast cancer cell proliferation.

The role of the *RORα1* receptor on human breast cancer cell proliferation was determined by comparing the growth rates between *RORα1* over-expressing cells and vector-transfected control cells. *RORα1* over-expressing MCF-7 cells grew significantly faster ($p < 0.05$) than their corresponding parental and vector-transfected cells. As illustrated in Figure 2, the cell number in the MCF-*ROR*#3 and MCF-*ROR*#8 clones increased by 150% and 166%, respectively, in comparison with vector-transfected cells after 7 days of culture.

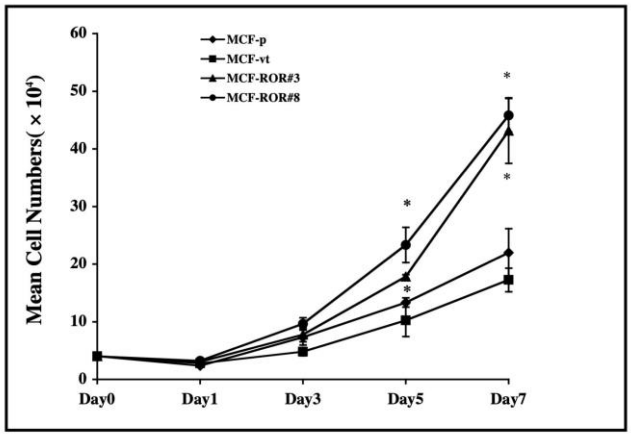

Fig. 2. Mitogenic effect of RORα1 in ERα-positive MCF-7 cells.

Different RORα1 over-expressing MCF-7 cell clones (MCF-ROR#3, MCF-ROR#8), vector-transfected (MCF-vt) or MCF-7 parental cells (MCF-p) were plated onto 6-well plates at a density of 5 × 10^4 cells/well in RPMI-1640 medium supplemented with 10% FBS and the cell numbers were determined by trypan blue dye exclusion method on days 1, 3, 5 and 7 following plating. (P< 0.05 vs. MCF-p or MCF-vt at the same time point, n=3).*

3. Genes modulated by RORα1 overexpression in MCF-7 cells.

To explore the function of *RORα1* in human breast cancer we conducted a cDNA microarray analysis to compare the gene expression profiles of MCF-7 cells transfected with and over-expressing *RORα1* vs. the vector-transfected cells. In this study, Human U133A genomic gene chips, which contain 22, 215 oligonucleotides representing 15,003 human genes, were used. To increase the reliability of the results, we examined two *RORα1* over-expressing MCF-7 clones (#3 and #8) in this study. As shown in Table 1, the cDNA microarray results revealed 38 genes, whose expression was altered (either up- or down-regulated) significantly and consistently between two *RORα1* over-expressing MCF-7 cell clones versus the control cell RNA samples. Twenty-three genes were elevated and 15 genes were down regulated in the *RORα1* over expressing cell clones compared to the control cells. Genes regulated by *RORα1* were grouped into seven functional categories: 1. cell proliferation and cell cycle, 2. apoptosis, 3. invasion and metastasis, 4. cell adhesion, 5. signaling transduction molecules and transcriptional factors, 6. calcium signaling, and 7. lipid metabolism as shown in Table 1. Our results suggest that *RORα1* is involved in a variety of cellular activities in breast cancer cells. Of the genes induced by *RORα1* some are also reported to be estrogen regulated, including; insulin-like growth factor binding protein 3, transforming growth factor alpha and beta, *CX43*, and bone morphogenetic protein 7 genes.

Table 1.
Microarray results: Genes modulated by RORα1 overexpression in MCF-7 cells

Gene name	Gb accessory #	Fold-change (#3 vs. Vt)*	Fold-change (#8 vs. Vt)*
Cell proliferation and cell cycles			
Insulin-like growth factor binding protein 3 (IGFBP3)	M31159	4.00	6.06
Transforming Growth factor alpha (TGFα)	NM 003236	3.48	2.30
Transforming Growth factor beta (TGFβ)	M60316	-2.46	-2.14
Adrenomedullin (ADM)	NM_001124	4.00	3.25
human protein tyrosine phosphatase(TEP1)	U96180	3.48	2.64
v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 3 (ERBB3)	NM_001982	2.46	2.83
Fibroblast growth factor receptor 2	NM_022969	-2.46	-2.30
arachidonate-5-lipoxygenase (ALOX5)	NM_000698	4.29	2.14
Janus kinase 2 (JAK2)	NM_004972	-2.00	-2.14
Cyclin-dependent kinase 5, regulatory subunit (p35)	NM_003885	-8.00	-2.14
Connexin43 (Cx43)	NM_000165	13.93	3.03
Type XVIII collagen (COL18A1), alpha 1	AF018081	-2.64	-2.46
Fibrinogen, gamma polypeptide (FGG)	NM_000509	3.03	21.10
Apoptosis			
BCL2-like 1 (BCL2L1, Bcl-xL)	NM_001191	2.14	2.00
Prostate differentiation factor	AF003934	6.50	3.73
Bone morphogenetic protein 7 (BMP7)	NM_001719	-2.83	-2.83
ADM	NM_001124	4.00	3.25
TGF-β	M60316	-2.46	-2.14
Invasion and metastasis			
Cx43	NM_000165	13.93	3.03
Bone marrow stromal cell antigen 2 (BST2)	NM_004335	39.40	131.79
S100 calcium-binding protein A7 (S100A7)	NM_002963	3.73	2.00
N-myc downstream regulated (NDRG1)	NM_006096	3.25	2.83
PAPS synthetase-2 (PAPSS2)	AF074331	3.73	2.30
Cell adhesion			
Protocadherin beta 12	NM_018932	-2.64	-4.29

*#3 and #8 represent two RORα1-overexpressing MCF-7 cell clones. Vt represents pcDNA3.1 vector-transfected MCF-7 cells. Fold-change > 2.0 indicates significant increase compared to Vt. Fold-change < -2.0 indicates significant decrease compare to Vt.

Table 1. (continued).

Microarray results: Genes modulated by RORa1 overexpression in MCF-7 cells

Gene name	Gb accessory #	Fold-change (#3 vs. Vt)*	Fold-change (#8 vs. Vt)*
Stem cell growth factor, lymphocyte secreted C-type lectin (SCGF)	BC005810	-3.03	-3.25
Keratin 13	NM_002274	-5.66	-8.00
Keratin, hair, basic 1(KRTHB1)	NM_002281	-5.66	-4.92
Neural cell adhesion molecules 2 (NCAM2)	NM_004540	-2.00	-2.14
COL18A1	AF018081	-2.64	-2.46
Calcium signaling			
Bradykinin receptor B2 (BDKRB2)	NM_000623	5.66	2.30
S100A7	NM_002963	3.73	2.00
S100A14	NM_020672	2.83	3.25
Inositol 1,4,5-triphosphate receptor type I (IP3R1)	L38019	4.92	4.92
Signal transduction molecules and transcriptional factors			
CBP/p300-interacting transactivator, with GluAsp-rich carboxy-terminal domain 2 (MRG1)	AF109161	2.64	2.83
BDKRB2	NM_000623	5.66	2.30
JAK2	NM_004972	-2.00	-2.14
G protein coupled receptor 65 (GPR65)	NM_003608	-6.06	-8.00
Opsin 3 (OPN3)	NM_014322	-3.03	-3.25
SH3 domain binding glutamic acid-rich protein like (SH3BGRL)	NM_003022	-8.00	-4.00
Protein tyrosine phosphatase D1 (PTPD1)	X79510	-8.57	-8.00
Endothelial PAS domain protein 1(EPAS-1)	NM_001430	2.46	4.29
Lipid metabolism			
ALOX5	NM_000698	4.29	2.14
Dihydrodiol dehydrogenase 1	NM_001353	19.70	19.70
Dihydrodiol dehydrogenase 3	U05598	25.99	7.26
PPAR gamma angiopoietin related protein	NM_016109	2.14	3.73
PDE1A	NM_005019	-3.03	-4.59

*#3 and #8 represent two RORa1-overexpressing MCF-7 cell clones. Vt represents pcDNA3.1 vector-transfected MCF-7 cells. Fold-change > 2.0 indicates significant increase compared to Vt. Fold-change < -2.0 indicates significant decrease compare to Vt.

To confirm that RORα1 regulates the expression of these genes, we chose to examine connexin 43 which were increased by 13.9-fold, as well as *AKR1C1* and *AKR1C3* genes, also known as dihydrodiol dehydrogenases 1 and 3, which *RORα1* induced by 19- and 25-fold, respectively. Interestingly, *AKR1C3* has been implicated in the progression of prostate and breast cancers and is reported to be involved in promoting drug resistance and in intra-tumoral endocrine production in these cancers (29).

3.3. Over-expression of RORα1 up-regulates the expression of CX43 mRNA.

It is reported that elevated expression of *CX43* is involved in tumorigenesis, tumor cell migration, and metastasis in breast cancer (30). Recent studies support the therapeutic potential of targeting *CX43* as a treatment in breast cancer (31, 32). Thus, we decided to determine if RORα1 over-expression could induce *CX43* expression in MCF-7 breast cancer cells. As shown in Figure 3, Northern blot analysis of *CX43* shows *CX43* mRNA expression is increased by approximately 37-fold in MCF-7 cells stably over-expressing RORα1.

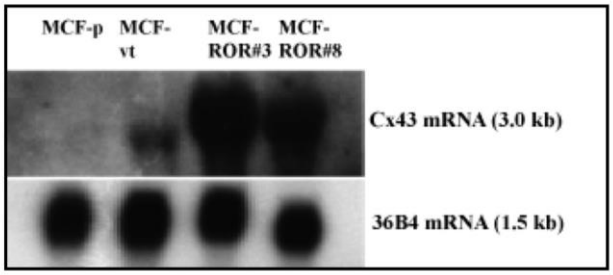

Fig. 3. RORα1 over-expression up regulates the expression of Cx43 mRNA.

Cx43 mRNA expression measured by Northern blot. Fifty micrograms of total RNA isolated from parental (MCF-p), pCDNA3.1 vector-transfected (MCF-vt), and RORα1-overexpressing MCF-7 cell clones (MCF-ROR#3 and MCF-ROR#8) were subjected to 1% MOPS/formaldehyde agarose gel and transferred to Hybond-XL membranes. Full-length human Cx43 cDNA was radiolabeled and used as probe to hybridize with membranes. The blots were stripped and re-probed with 36B4 cDNA, which is a loading control.

3.4. Over-expression of RORα1 up-regulates AKR1C1 and AKR1C3 mRNA and protein expression in MCF-7 breast cancer cells which can be suppressed by treatment with melatonin.

To confirm the effect of RORα1 on regulating *AKR1C1* and *AKR1C3* mRNA and protein expression in MCF-7 cells and to define if melatonin inhibition of RORα1 transcriptional activity might repress *AKR1C1* and *AKR1C3* expression, we employed both real time PCR (qPCR) and Western blot analysis. As shown in figure 4 over-expression of *RORα1* in MCF-7 cells increase the expression of *AKR1C1* mRNA (Figure 4A) and protein (Figure 4B/C), which was significantly inhibited by 72% and 54%, respectively, in response to administration of physiologic levels (1 nM) of melatonin. Figure 5 shows that RORα1 over-expression greatly up regulated the expression *AKR1C3* mRNA (Figure 5A) and protein (Figure 5B/C) in MCF-7 cells and the administration of physiologic levels of melatonin (1 nM) significantly suppressed *AKR1C3* mRNA and protein by 78% and 91%, respectively.

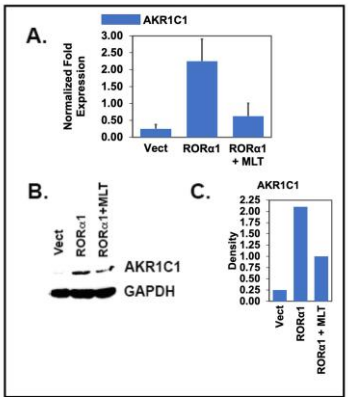

Fig. 4. Effects of melatonin on RORα1 over-expression induction of AKR1C1 mRNA and protein expression in MCF-7 cells.

(A) AKR1C1 mRNA expression was measured by qPCR in parental MCF-7 cells, vector transfected (Vect.) MCF-7 cells, MCF-7 cells transiently transfected with pcDNA3.1-RORα1, and RORα1 transfected MCF-7 cells treated with 10-9 M melatonin (RORα1+MLT) for 48 hours. Cells were collected and lysed and 5 µg total mRNA was used for qPCR analysis of AKR1C1 mRNA expression using primers and described in mateirals in methods. AKR1C1 protein expression (B) in the same groups above after total protein was extracted and determined by Western blot analyisis using a rabbit anti-AKR1C1 antibody at 1: 1000 and anti-GAPDH antibody at 1:750, as described in "Materials and Methods". Graphical data (C) represents the mean of 3 independent experiments.

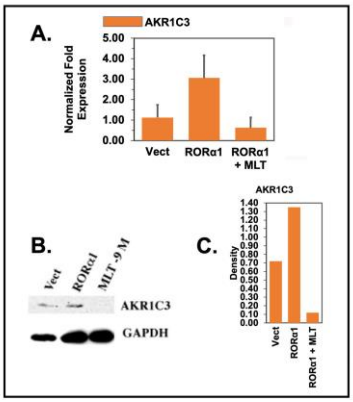

Fig. 5. Effects of melatonin on RORα1 over-expression induction of AKR131 mRNA and protein expression in MCF-7 cells.

(A) AKR1C3 mRNA expression was measured by qPCR in parental MCF-7 cells, Vector transfected (Vect.) MCF-7 cells, MCF-7 cells transiently transfected with pcDNA3.1-RORα1, and RORα1 transfected MCF-7 cells treated with 10-9 M melatonin (RORα1+MLT) for 48 hours. Cells were collected and lysed and 5 ug total mRNA was used for qPCR analysis of AKR1C3 mRNA expression using primers and described in mateirals in methods. AKR1C3 protein expression (B) was determined in the same groups above after total protein was extracted was determined by Western blot analyisis using a rabbit anti-AKR1C3 antibody at 1: 1000 and anti-GAPDH antibody at 1:750, as described in "Materials and Methods". Graphical data (C) represents the mean of 3 independent experiments.

3.5. Melatonin suppresses RORα1 transcriptional activity in MCF-7 breast cancer cells.

We previously reported that physiologic concentrations of melatonin inhibited RORα1 transcriptional activity to suppress the proliferation of MCF-7 human breast cancer cells (4, 28). In the present study, RORα1 over-expression significantly stimulated MCF-7 cell growth. To test whether the inhibitory effect of melatonin on MCF-7 proliferation is due to its repression of RORα1 transcriptional activity, we used transcriptional reporter assays to evaluate the effect of melatonin on the transactivation of RORα1. As shown in Figure 6, physiologic concentrations of melatonin (10^{-9} M and 10^{-8} M) significantly repressed the transactivation of RORα1 as measured in a RORE-Luc reporter assay.

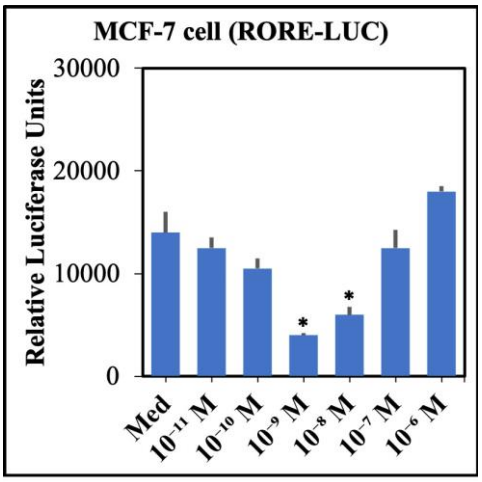

Fig. 6. Melatonin represses the transactivation of RORα1.

Two μg of the ROREα2$_3$-TKLUC reporter construct and 1 μg of pCMVβ control plasmid per well were transfected into MCF-7 cells by 6 μg of lipofectamine for 6 h. Following transfection, cells were treated with melatonin (10-11 M to 10-6 M) or diluent ethanol control (Med) for 24 h. The RORα transcriptional activities were determined by luciferase assays and the transfection efficiency was normalized by β-galactasidase assay. (P< 0.05 vs. ethanol control group, n=3).*

3.6. RORα1 expression drive tamoxifen resistance in MCF-7 breast cancer cells.

To determine if elevated expression of RORα1 contributes to tamoxifen resistance we examined the response of MCF-7RORα1 cells (clone #3) to 4-OH TAM in the presence or absence of 17-β estradiol (E2) and melatonin. As shown in Figure 7A, administration of E2 to MCF-7RORα1 cells induced as significant 41% increase in cell number, while treatment with 4-OH TAM did not significantly decrease cell proliferation after 4 days of treatment. However, treatment of RORα1 expressing cells with physiologic levels of melatonin significantly diminished cell proliferation by 27% and treatment of RORα1 expressing cells with the combination of 4-OH TAM and melatonin blocked cell proliferation and promoted a decrease in cell number. As shown in Figure 7B, Western blot analysis showed strong AKR1C3 protein expression in RORα1 expressing cells that was further increased when cells were treated with E2. Conversely, AKR1C3 levels were diminished in cells treated with melatonin and almost completely abolished in the presence of 4-OH TAM and melatonin.

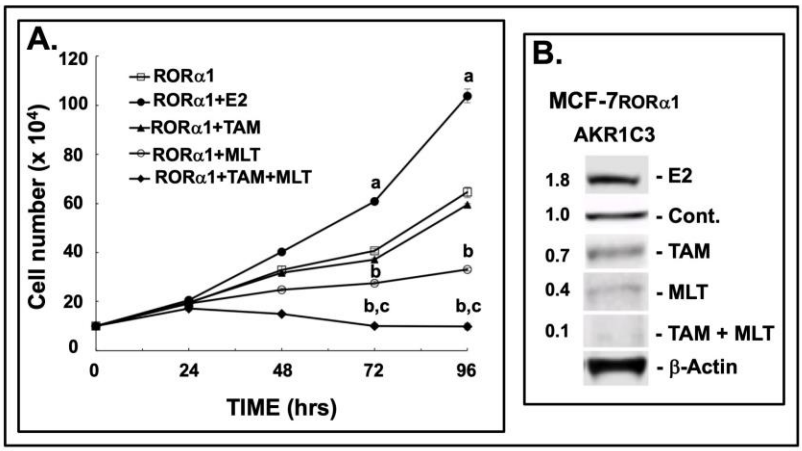

Fig. 7. Effects of melatonin administration on RORα1-induced AKR1C3 expression and RORα1/ARK1C3-mediated tamoxifen resistance in MCF-7 breast cancer cells.

*(**A**) MCF-7 breast cancer cells were plated at a density of 10 x 10^4 cells per ml in six-well plates and 5 hours after seeding, cells were treated with diluent (0.01% ethanolic cell culture media), E2 (10 nM), 4-OH TAM (4.5 μM), MLT (10 nM), or TAM + MLT for 96 h, (4 days). On specific days total and viable cells counted on a haemocytometer. n = 3 independent studies, and **a** = p<0.05 difference vs. RORα1, **b** = p<0.05 vs. RORα1 and RORα1 + TAM, and **c** = p< 0.005 vs. RORα1 and p<0.05 vs. RORα1 + TAM. (**B**) Expression of AKR1C3 was determined by Western blot analysis as described in materials and methods from cells harvested after 4 days in treatments described above. Densitometric levels of AKR1C3 protein expressed in each treatment group is noted, with the control RORα1 group normalized to 1.0.*

4. DISCUSSION

We and others have reported that the RORαs are constitutively active and their transcriptional activity in human breast cancer cells can be induced by cholesterol but repressed by melatonin (4, 13-15). In addition, Kane and Means (22) demonstrated that CaMKIV enhances RORα1 transactivation via phosphorylation of co-activators, such as CBP/p300 (33).

To determine the role of RORαs in human breast cancer cell proliferation, we developed clones of ERα-positive (MCF-7) breast cancer cells that are stably transfected with and constitutively over-express the RORα1. Comparison of the growth rates of RORα1-transfected MCF-7 cells with vector-transfected and parental controls, demonstrates significantly increased proliferation (Figure 2) in *RORα1* expressing MCF-7 breast cancer cells. The *RORαs* have been reported to cross-talk with other steroid hormone receptors, such as TR and RARα (16-17) and we have reported that they cross-talk with the ERα (28), all of which play important roles in promoting breast cancer cell proliferation. The observation that response elements for RORα are present in the promoter region of various tumor-related genes, such as *N-MYC*, *P21$^{WAF1/CIP1}$*, *NM23*, and aromatase (*CYP19A*), support RORα's role in breast tumor growth and metastasis (11-14, 16). Based on these studies and our earlier reports, we conclude that RORαs are expressed in primary breast tumor and that RORα1 play an important role in stimulating the proliferation of ERα-positive breast cancer.

Our cDNA microarray results (Table 2) revealed that the expression of 38 genes was significantly altered in two MCF-7 RORα1 over-expressing cell clones, compared to vector-transfected controls. Five of these genes are E2-regulated, including insulin-like growth factor binding protein 3 *(IGFBP3*) (34), transforming growth factor alpha (*TGFα)* (35), transforming growth factor beta (*TGFβ)* (36), *CX43*(32), and bone morphogenetic protein 7 **(**BMP7) (37). These genes are up regulated by E2 and by *ROR*α1 over-expression and these data are in agreement with our earlier reports that RORα cross-talks and augments ligand-mediated ERα transcriptional activity in MCF-7 cells (28). At the present time however, it is still not clear which genes are regulated directly by RORα1 over-expression and which are regulated via RORα1/ERα cross talk.

Table 2: Microarray results: Genes regulated by both ERa and RORa

Gene name	Gb accessory #	Breast cancer cell proliferation*	E2**	RORa***	References
Insulin-like growth factor binding protein 3 (IGFBP3)	M31159	↓	↑	↑	222, 219
Transforming growth factor alpha (TGFa)	NM_003236	↑	↑	↑	217
Transforming growth factor beta (TGFb)	M60316	↓	↓	↓	218
Connexin43 (Cx43)	NM_000165	No effect	↑	↑	245, 220
Bone morphogenetic protein 7 (BMP7)	NM_001719	No reports	↓	↓	221

* In this column, ↓ represents that breast cancer cell proliferation is inhibited by this gene; ↑ represents that breast cancer cell proliferation is stimulated by this gene.
** In this column, ↑ represents that the expression of this gene is up-regulated by E2; ↓ represents that the expression of this gene is down-regulated by E2.
*** In this column, ↑ represents that the expression of this gene is up-regulated by RORa1 overexpression; ↓ represents that the expression of this gene is down-regulated by RORa1 overexpression.

Review of the literature shows that RORα1 regulated genes impact variety of cellular signaling pathways to regulate various cellular functions including cell proliferation and cancer. We were able to group RORα1 regulated genes into seven functional categories, including, cell proliferation

and cell cycle, apoptosis, invasion and metastasis, cell adhesion, signal transduction molecules and transcriptional factors, calcium signaling, and lipid metabolism (Table 1). For example, *TGFα* and *TGFβ* are well-known growth factors that play important roles in regulation of breast cancer cell proliferation, with TGFα promoting cell proliferation, but TGFβ in many cases decreasing proliferation (38). As shown in Table 1, RORα1 over-expression up regulated TGFα but down-regulated *TGFβ* expression in MCF-7 cells, both of which play important roles in mediating E2's mitogenic effect on human breast cancer. As RORα/ERα cross-talk (4, 28), it is possible that the mitogenic effect of RORα1 is mediated via its regulation of *TGFα* and *TGFβ* alone or in concert with ERα. The expression of IGFBP3, an known inhibitor of breast cancer proliferation (34), was also induced by RORα1. Conversely, fibroblast growth factor 2 receptor (*FGFR-2*), which is over-expressed in many breast cancers and mediates cell proliferation (39, 40), was down-regulated in response to RORα1 expression. Furthermore, RORα1 over-expression also induced the expression of v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 epidermal growth factor tyrosine kinase receptor 3 (*ERBB3*) that modulates breast cancer activities by heterodimerizing with ErbB2 to activate survival and mitogenic pathways in breast cancer cells (41).

Although it is clear from the literature and our cDNA microarray data above that RORα1 expression can induce the expression of numerous genes known to be involved in breast cancer such as *IGFBP, TGFβ, CX43* and *BMP7,* we chose to focus our efforts in understanding if *RORα1* regulated *AKRC1* and *AKR1C3* mRNA expression in MCF-7 breast cancer cells and if AKR1C3 elevated expression and its' impact on aromatase activity might be tied to the development of Tamoxifen resistance in these cells.

It has been reported that RORα1 can regulate the expression of genes encoding several phase I and phase II metabolic enzymes (42), such as cytochrome P450 enzymes and AKR1Cs (43, 44). The ARK1Cs, also termed dihydrodiol dehydrogenases, are phase 1 drug-metabolizing enzymes involved in the steroid biosynthesis and prostaglandin metabolism (45). As shown in Table 1, RORα1 over-expression induced the expression of *AKR1C1* and *AKR1C3* mRNA by 19- and 25-fold respectively, (clone #3) in MCF-7 breast cancer cells. Furthermore, RORα1 over-expression also increased AKR1C1 and AKR1C3 protein expression in MCF-7 breast cancer cells and that administration of the circadian hormone melatonin (1nM) significantly repressed AKR1C1 and AKR1C3 protein levels. The up regulation of AKR1C3 expression in response to RORα1 expression suggests that RORα1 may significantly impact the progression of luminal A breast cancer as AKR1C3 has been shown to play a central role in intratumoral endocrine production of testosterone and E2 in both prostate and breast tumor cells, driving the conversion androstenedione to testosterone and esterone to E2 (44). Elevated testosterone is a key precursor for the generation of E2 by Aromatase (CYP19A1). Given the report by Odawara *et al.* (16) that RORα can induce the expression of Aromatase, it would appear that RORαs may play a critical role in driving E2-mediated breast cancer progression. Furthermore, as luminal A breast cancer cells typically express both androgen receptor (AR) and ERα (46) the elevated intratumoral levels of testosterone and E2 in response to increased AKR1C3 expression/activity could promote breast tumor growth via activation of not only ERα but also AR.

Melatonin, synthesized and secreted at night by the pineal gland in response to darkness, has been reported to inhibit the proliferation of ERα-positive MCF-7 human breast cancer cells (24). Melatonin has been shown to modulate the transcriptional activity of various nuclear receptors, inhibiting the transcriptional activity of the ERα, glucocorticoid receptor (GR), while enhancing

the transcriptional activation of the retinoic acid receptor alpha (RARα) and Vitamin D receptor in human breast cancer cells (26, 27). RORαs were originally reported by Wiesenberg and Carlberg to be nuclear melatonin receptors, but the direct binding of melatonin to these receptors could not be repeated and thus, the report has been retracted (47). Therefore, ROR receptors, including the RORα1 receptor, are not melatonin receptors, as melatonin does not bind to them to initiate or inhibit their transcriptional activity (26). Although, our previous studies demonstrate that melatonin, when administered at physiologic (nM) concentrations decreased RORα DNA binding activity (4, 28) and transcriptional activity suppressing clock gene *BMAL1* expression in MCF-7 breast cancer cells (20), we believe this is most likely mediated by phosphor-inhibition of RORa1, similar to what we have reported with the ERα in breast cancer. The current study further confirms that RORα1 transcriptional activity can be suppressed by melatonin to significantly suppress RORα1's inductive effect on *AKR1C1* and *AKR1C3* mRNA and protein expression.

AKR1C3 also converts prostaglandin (PG) H_2 to $PGF_{2\alpha}$ and PGD_2 to 9α,11β-PGF_2 (48). The PGF_2 isomers bind to the F prostanoid receptor and induce MAPK signaling cascades that promote cell proliferation (49). Furthermore, by removing PGD_2, AKR1C3 inhibits the formation of the anti-proliferative and anti-inflammatory prostaglandin 15-deoxy-Δ (12, 14) -PGJ_2 ($15dPGJ_2$) that promotes anti-proliferative effects on breast cancer cells (50). Thus, the mitogenic actions of RORα1 we observed may not only be through enhancing AR and ERα signaling but possibly by its regulation of the prostaglandin pathway to induce expression mitogenic 9α,11β-PGF_2, while inhibiting the production of the anti-proliferative $15dPGJ_2$.

These effects of melatonin on RORα1 transcriptional activity are likely mediated via post-translational modification of RORα1 to repress its DNA-binding activity, but possibly through other mechanisms including modulation of key co-regulators to profoundly impact breast cancer progression. Melatonin, by inhibiting RORα expression and/or transcription activity and the suppression of AKR1C1 and ARK1C3 expression may impact the regulation of aromatase and intra-tumoral endocrinology in breast cancer, and thus, could have promising clinical applications for adjunctive treatment of breast cancer patients which display resistance to endocrine therapies.

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AUTHORSHIP

The following authors contributed to concept/design (SMH, DEB, SX), acquisition of data (SX, CD, RTD, Ly), data analysis/interpretation, drafting of the manuscript (SX, SMH, DEB, RTD, SMH), critical revision of the manuscript (SX, SMH RTD, DEB, TF), and approval of the article (SX, CD,LY, RD, DEB, TF, SMH).

CONFLICT OF INTEREST

The authors disclose no potential conflicts of interest.

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