TOXICOLOGICAL SCIENCES **131(1)**, 71–83 (2013)
doi:10.1093/toxsci/kfs290
Advance Access publication October 10, 2012

Endocrine Disruptors Fludioxonil and Fenhexamid Stimulate miR-21 Expression in Breast Cancer Cells

Yun Teng,*[,1] Tissa T. Manavalan,*[,1] Chuan Hu,* Svjetlana Medjakovic,† Alois Jungbauer,† and Carolyn M. Klinge*[,2]

*Department of Biochemistry & Molecular Biology and Center for Genetics and Molecular Medicine, University of Louisville School of Medicine, Louisville, Kentucky 40292; and †Department of Biotechnology, University of Natural Resources and Life Sciences Vienna, Muthgasse 18, Vienna, Austria

[1]These authors contributed equally to this study.

[2]To whom correspondence should be addressed at Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Delia Baxter Building, 581 South Preston Street, Louisville, KY 40202. Fax: (502) 852-3659. E-mail: carolyn.klinge@lousiville.edu.

Received June 25, 2012; accepted September 26, 2012

Fenhexamid and fludioxonil are antifungal agents used in agricultural applications, which are present at measurable amounts in fruits and vegetables. Fenhexamid and fludioxonil showed endocrine disruptor activity as antiandrogens in an androgen receptor reporter assay in engineered human breast cancer cells. Little is known about how environmental chemicals regulate microRNA (miRNA) expression. This study examined the effect of fenhexamid and fludioxonil on the expression of the oncomiR miR-21 in MCF-7, T47D, and MDA-MB-231 human breast cancer cells and downstream targets of miR-21 in MCF-7 cells. Fenhexamid and fludioxonil stimulated miR-21 expression in a concentration-dependent manner and reduced the expression of miR-21 target Pdcd4 protein. Antisense to miR-21 blocked the increase in Pdcd4 protein by fenhexamid and fludioxonil. Fenhexamid and fludioxonil reduced miR-125b and miR-181a, demonstrating specificity of miRNA regulation. Induction of miR-21 was inhibited by the estrogen receptor antagonist fulvestrant, by androgen receptor antagonist bicalutamide, by actinomycin D and cycloheximide, and by inhibitors of the mitogen-activated protein kinases and phosphoinositide 3-kinase pathways. Fenhexamid activation was inhibited by the arylhydrocarbon receptor antagonist α-napthoflavone. Fenhexamid and fludioxonil did not affect dihydrotestosterone-induced miR-21 expression. Fludioxonil, but not fenhexamid, inhibited MCF-7 cell viability, and both inhibited estradiol-induced cell proliferation and reduced cell motility. Together these data indicate that fenhexamid and fludioxonil use similar and distinct mechanisms to increase miR-21 expression with downstream antiestrogenic activity.

Key Words: **endocrine disruptor; fungicide; breast cancer; microRNA; estrogen receptor; androgen receptor.**

Fenhexamid and fludioxonil are antifungal agents used in a variety of agricultural applications. The structures of fenhexamid and fludioxonil are provided in Supplementary figure 1. Fenhexamid is the International Organization for Standardization (ISO) approved name for *N*-(2,3-dichloro-4-hydroxypheynyl)-1-methylcyclohexanecarboxamide, a hydroxyanilide fungicide that inhibits the growth of fungal spore germ tubes and mycelia (JMPR, 2005). Fenhexamid inhibits C3-ketoreductase, which is involved in ergosterol biosynthesis (Fillinger *et al*., 2008). Fludioxonil is the ISO-approved name for 4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrile. Fludioxonil was not carcinogenic in rats or mice (www.federalregister.gov). Both fenhexamid and fludioxonil are considered to have high exposure scores based on usage and residual concentrations on fruits and vegetables (Orton *et al*., 2011). Fenhexamid and fludioxonil are sprayed on tomatoes and appear to be present on the harvested fruits at concentrations below the legal maximum residue levels (Angioni *et al*., 2012; Cabizza *et al*., 2012). Human tissue or serum levels of fenhexamid and fludioxonil have not been examined.

Endocrine disrupting chemicals (EDC) are environmental chemicals that mimic or block transcriptional activation elicited by naturally circulating steroid hormones by binding to steroid hormone receptors and acting as either agonists or antagonists of that receptor (Diamanti-Kandarakis *et al*., 2009). In addition, EDC may also act at the level of enzymes involved in steroid hormone synthesis or metabolism and may alter the expression or activities of transcriptional coregulators (Diamanti-Kandarakis *et al*., 2009). The role of EDC in breast cancer is suspected but not proven (Weyandt *et al*., 2008). Pesticides and fungicides have been reported to be tumorigenic in rodent models of breast cancer (e.g., Ross and Leavitt, 2010). Based on their widespread use, environmental persistence, and high incidence of breast cancer, the possible role of EDC in stimulating breast tumorigenesis is of clear importance (Casals-Casas and Desvergne, 2011; Diamanti-Kandarakis *et al*., 2009; Weyandt *et al*., 2008).

Although lifetime estrogen exposure clearly increases breast cancer risk (Wiseman, 2004), the role of androgens is still undefined (Kotsopoulos and Narod, 2012). Androgen receptor (AR) is expressed in most breast tumors and is associated with endocrine sensitivity in estrogen receptor α (ERα)+ tumors (Lundin

© The Author 2012. Published by Oxford University Press on behalf of the Society of Toxicology. All rights reserved.
For permissions, please email: journals.permissions@oup.com

et al., 2011); however, AR is overexpressed in ERα−/HER2+ and triple negative breast cancers, indicating that androgens may play a role in the pathogenesis of these tumors (Ni *et al*., 2011). Fenhexamid (IC_{50} ~8μM) and fludioxonil (IC_{50} ~2μM) had antiandrogenic activity in an engineered MDA-MB-453 breast cancer cell line stably expressing an mouse mammary tumor virus (MMTV) promoter luciferase reporter (called MDA-kb2) (Orton *et al*., 2011). Fludioxonil and fenhexamid were ERα agonists (EC_{50} values of 3.7 and 9.0μM, respectively) in a yeast-based reporter assay (Jungbauer, unpublished data). The effect of fenhexamid and fludioxonil on endogenous microRNA (miRNA) gene expression in breast cancer cells is unknown.

miRNA silence gene expression by basepairing to the 3′-UTR of their target mRNAs resulting in translational repression and mRNA degradation (Klinge, 2012). The current miRBase release (19.0) contains 1527 human miRNAs (August 2012, http://www.mirbase.org/). Each miRNA may repress hundreds of mRNAs. Changes in miRNA expression are implicated in human cancer (Iorio and Croce, 2012). Roles for miRNAs in environmental toxicology and as possible biomarkers of exposure have been reviewed (Koturbash *et al*., 2012).

Recent reports have identified miR-21 as an oncomiR overexpressed in different types of tumors, including breast cancer (reviewed in Fu *et al.* [2011]). Inhibition of miR-21 by chemically modified antisense oligonucleotides reduced proliferation and tumor growth of MCF-7 cells (Li *et al*., 2009). miR-21 regulates invasion and metastasis by inhibiting tumor repressors, e.g., programmed cell death 4 (*PDCD4*), PTEN, and Bcl-2, an antiapoptotic protein (Wickramasinghe *et al*., 2009). miR-21 potentiates Ras signaling through inhibition of multiple negative regulators of the Ras/MEK/ERK pathway, e.g., SPRY1, SPRY2, BTG2, and PDCD4 (Hatley *et al*., 2010). There is only one report examining how EDC affect miRNA expression (Tilghman *et al*., 2012). That study showed that, like E_2 (Wickramasinghe *et al*., 2009), *o*,*p*-dichlorodiphenyltrichloroethane and bisphenol A (BPA) activate ERα in MCF-7 cells and downregulate miR-21 (Tilghman *et al*., 2012). There are no published studies on how fungicides regulate miRNAs in normal breast or in breast carcinoma and/or other neoplasias. Here, we examined how fenhexamid and fludioxonil affect the expression of the oncomiR miR-21 in MCF-7, T47D, and MDA-MB-231 breast cancer cells.

MATERIALS AND METHODS

Chemicals and antibodies. Fludioxonil (no. 46102), fenhexamid (no. 31713), 17α-estradiol (E_2, no. E8875), wortmannin (phosphoinositide 3-kinase [PI3K] inhibitor, no. W1628), and α-naphthoflavone (α-NF, an arylhydrocarbon receptor [AHR] antagonist, no. N5757) were purchased from Sigma-Aldrich (St Louis, MO). ICI 182, 780 (fulvestrant, no. 1047) was purchased from Tocris (Ellisville, MO). PD98059 (MEK1/2 inhibitor, no. 1213) was purchased from Cell Signaling (Beverly, MA). Dihydrotestosterone (DHT, no. 521-18-6) was purchased from Steraloids (Wilton, NH). The antiandrogen bicalutamide (Casodex) was generously provided by Astra Zeneca (Macclesfield, UK). The antibodies against Pdcd4 (sc-130545), Bcl-2 (sc-509), PARP (Poly(ADP-ribose) polymerase-1, (sc-7150), and ERα (HC-20, sc-543) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Pten (138G6), ERK1/2 (9102), and phosphor-ERK1/2 (9101S) were from Cell Signaling Technology (Beverly, MA); α-tubulin (MS-581-P1) was from Thermo Scientific (Kalamazoo, MI); β-actin was from Sigma-Aldrich.

Cell lines and maintenance. The human breast cancer cell lines MCF-7, T47D, and MDA-MB-231 and the normal MCF-10A breast epithelial cell line were purchased from the American Type Culture Collection (Rockville, MD). MCF-7 and MDA-MB-231 cells were cultured in IMEM (Invitrogen, Carlsbad, CA). T47D cells were maintained in RPMI-1640 media containing insulin (5 μg/ml, Invitrogen), and MCF-10A was maintained in MEGM (Lonza, Walkersville, MD; cat no. CC-3150) containing 100 ng/ml cholera toxin. Cells were cultured in their respective growth medium supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen) at 37°C under an atmosphere of 5% CO_2.

Treatments. Prior to treatment, the cells were "serum starved" in phenol red–free IMEM supplemented with 5% dextran charcoal–stripped fetal bovine serum (DCC-FBS) and 1% penicillin/streptomycin for 72 h to remove endogenous hormones from the serum and allow hormone-dependent responses to be detected in the cells (Biswas and Vonderhaar, 1987; Gill *et al*., 1991). Cells were then treated with dimethylsulfoxid (DMSO, the vehicle control), 10nM E_2, fludioxonil, or fenhexamid (at concentrations indicated in Figures) for 6 or 24 h, as indicated in Figures. Stock solutions for each compound were prepared in DMSO and frozen in aliquots of 100 μl. The final concentration of DMSO in the culture medium was <0.1%.

RNA extraction and quantitative real-time reverse transcription-PCR. Total RNA was isolated with the miRCURY RNA isolation Kit (Exiqon, Copenhagen, DEN) according to the manufacturer's instructions. For analysis of miRNA expression, cDNA was prepared with the mercury LNA Universal RT microRNA PCR Kit (Exiqon).

Quantitative real-time reverse transcription-PCR (qPCR) was performed using the miRCURY LNA SYBR Green Master Mix (Exiqon) and miRNA primer sets for miR-21, miR-22, miR-125b, miR-181a, and miR-200a (Exiqon). RNU48 and RNU38 were used for normalization of miRNA expression. For analysis of primary miR-21 and gene expression, cDNA was prepared with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions, and individual mRNAs were monitored with the following inventoried TaqMan assays (Applied Biosystems): human primary-miR-21, *TMEM49 (VMP1)*, *PDCD4, PTEN*, and *BCL2*. 18S rRNA was used for normalization of mRNA expression. The ABI 7900HT Fast Real-Time PCR System was used for all PCR (Applied Biosystems), done in triplicate. Thermal cycling parameters comprised stage 1: a 50ºC Uracil-DNA glycosylase activation step for 2 min; stage 2: 95°C for 10 min; stage 3: 40 cycles of 95°C for 15 s and 60°C for 1 min; stage 4: 95°C for 15 s; 60°C for 15 s; 95°C for 15 s. Changes in expression were calculated by the change in threshold cycle (Ct) method with endogenous control and were normalized to results obtained with untreated cells.

Transfection of anti-miR miRNA inhibitor and Pre-miR miRNA. For transfection of pre-miRNA and anti-miRNA, 2×10^5 MCF-7 cells were transfected with 90 pmol of pre-miR miR-21 precursors and anti-miR miR-21 inhibitors (Ambion) with 7 μl of Lipofectamine RNAiMAX (Invitrogen) in antibiotics-free medium. Cells were harvested for RNA isolation 48 h after transfection.

Western blot. Cells were treated as indicated in individual Figures. Whole cell extracts were prepared in modified RIPA buffer (10mM sodium phosphate, pH 7.2; 1% NP-40; 1% Na-deoxycholate; 150mM NaCl; 2mM ethylenediaminetetraacetic acid; 0.2mM Na_3VO_4; 50mM NaF; and 1 mg/ml each of aprotinin, leupeptin, and pepstatin; complete protease inhibitor (Roche); and 1mM PMSF). Protein concentrations were determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Lysates were immunoblotted according to standard methods (Klinge *et al*., 2010). Ten micrograms of protein (Figs. 3B and 4B) and 30 μg of protein (Fig. 5) were separated by 10% SDS-PAGE, transferred to polyvinylene difluoride membranes (Millipore,

Billerica, MA), and incubated with primary antibodies. Horseradish peroxidase–conjugated secondary antibodies were from Santa Cruz Biotechnology. Immunoreactive bands were visualized by chemiluminescence with the Pierce ECL Western blotting substrate (Thermo Scientific, Rockford, IL). Immunoblots were quantified by Carestream Image Station 4000R PRO using Carestream Molecular Imaging Software Version 5.0.2.30 (New Haven, CT). Regions of interest (ROI) for protein bands of interest were normalized by the ROI of the loading control (α-tubulin or α-actin), and the ratio for DMSO (vehicle) was set to one for comparison.

Transient transfection and luciferase reporter assay. MCF-7 cells were plated in 24-well plates at a density of 2.5×10^4 cells/well in phenol red–free IMEM medium supplemented with 5% FBS. Transfection of anti-miR-21 inhibitor was performed when the cells attached, as described above. Twenty-four-hour anti-miR-21 transfection, transient transfection of the same cells with 100 ng of pGL3-pro-luciferase reporter (Promega) as a control and 10 ng of pRL-TK-*Renilla* luciferase reporter (Promega) containing the 3′-UTR of PDCD4 gene (Wickramasinghe *et al*., 2009), was performed using FuGENE HD Transfection Reagent (Roche, Indianapolis, IN) with Opti-MEM I Reduced Serum Medium (Invitrogen). Twenty-four hours after transfection, triplicate wells were starved with phenol red–free IMEM supplemented with 5% DCC-FBS for 24 h, then treated with DMSO (vehicle control), 10nM E_2, 100nM fludioxonil, or 100nM fenhexamid. The cells were harvested 24 h posttreatment using Promega's Passive Lysis buffer. Luciferase and *Renilla* luciferase activities were determined using Promega's Dual Luciferase assay. *Renilla* luciferase was normalized by Firefly luciferase to correct for transfection efficiency, and values were normalized by the DMSO-antisense (AS)-control value within that experiment.

Wound-healing experiments. MCF-7 cells were plated in six-well plates in phenol red–free IMEM + 5% DCC-FBS for 48 h until ~80% confluent. Cells were wounded by scratching with a p200 pipette tip and then washed with medium to remove displaced cells. Cells were treated with IMEM + 5% DCC-FBS or with added vehicle control (DMSO, 0.1%), 10nM E_2, 100nM fludioxonil, or 100nM fenhexamid and cultured for 3 days. Images were captured at ×4 magnification using an EVOS microscope (AMG, Bothell, WA), and NIH Image J software was used to analyze the percent of wound area at each time point. Values were averaged from two separate readings at each time point.

Statistical analyses. Data were examined by Student's *t*-test or one way ANOVA followed by Kruskal-Wallis and/or Dunnett's Multiple Comparison Tests using GraphPad Prism software. $p < 0.05$ was considered statistically different.

RESULTS

Fludioxonil and Fenhexamid Increase miR-21 Expression in MCF-7 Cells

Because the role of EDC in regulating oncomiR miR-21 expression in breast cancer is largely undefined, we determined the effect of fludioxonil and fenhexamid on mature miR-21 expression in MCF-7 breast cancer cells. Both fludioxonil and fenhexamid increased miR-21 expression in MCF-7 cells (Fig. 1A). Fludioxonil showed a concentration-dependent increase with an apparent saturation of the response at 1–10μM and then miR-21 expression returned to basal at 50μM (Fig. 1A). The maximum activity detected was similar to the EC_{50} values of 3.7 and 9.0μM, respectively, for fludioxonil and fenhexamid for ERα in a yeast reporter assay (Jungbauer, unpublished data). Similar to other EDC (Vandenberg *et al*., 2012), fenhexamid did not show a linear or saturation response although, like fludioxonil, the peak induction of miR-21 was detected at 10μM and was reduced at 50μM (Fig. 1A). The fenhexamid response appears biphasic with an initial response peak at 10–100nM and a second peak at 10 μM. Importantly, neither fludioxonil nor fenhexamid altered the expression of RNU38 or RNU48 that was used as controls for qPCR for miRNA expression (Supplementary fig. 2 and data not shown).

As reported previously (Maillot *et al*., 2009; Tilghman *et al*., 2012; Wickramasinghe *et al*., 2009), E_2 inhibits miR-21 expression in MCF-7 cells. Cotreatment with E_2 and either fludioxonil or fenhexamid reduced the response seen with either fungicide alone, suggesting a competitive mechanism for interaction with ER. Neither fludioxonil nor fenhexamid affected ERα protein expression (Supplementary fig. 3). Likewise, pretreatment with pure ER antagonist/selective ER downregulator fulvestrant (ICI 182,780, ICI) attenuated the fludioxonil- or fenhexamid-induced increase in miR-21 expression, indicating a role for ER (Fig. 1B). ICI alone significantly increased miR-21 expression, perhaps by relieving repression mediated by unliganded ERα (Metivier *et al*., 2004).

Crosstalk between the AHR and ERα has been implicated in some EDC activities (Madak-Erdogan and Katzenellenbogen, 2012; Matthews and Gustafsson, 2006; Swedenborg *et al*., 2009). Pretreatment with the AHR antagonist α-napthoflavone inhibited fenhexamid, but not fludioxonil, and stimulated miR-21 expression (Fig. 1B). These results suggest a possible role for AHR in the activation of miR-21 by fenhexamid (Fig. 1B).

In addition to genomic activity, ER activates membrane-initiated phosphorylation cascades involving activation of mitogen-activated protein kinase (MAPK) and PI3K/AKT (Hammes and Levin, 2011; Levin, 2009; Watson and Lange, 2005). Pretreatment of MCF-7 cells with PD98059, a MEK1/2 inhibitor, or wortmannin, an inhibitor of PI3K, abrogated the fludioxonil- and fenhexamid-mediated increase in miR-21 expression (Fig. 1B). These results suggest the involvement of MAPK and PI3K pathways for miR-21 activation by fludioxonil and fenhexamid.

DHT Increased miR-21 Expression in MCF-7 Cells

DHT increased miR-21 in both LNCaP and VCaP prostate cancer cells (Waltering *et al*., 2011), but the effect of DHT on miR-21 expression in MCF-7 cells is unknown. MCF-7 cells express AR (Horwitz *et al*., 1975) although not all investigators find AR protein in MCF-7 cells (Robinson *et al*., 2011). Because fludioxonil and fenhexamid act as antiandrogens in a genetically engineered breast cancer cell line and inhibited MMTV luciferase reporter activity, we examined the effect of DHT alone or with fludioxonil or fenhexamid cotreatment. DHT increased miR-21 expression in MCF-7 cells and neither fludioxonil nor fenhexamid blocked this response (Fig. 1B). The antiandrogen bicalutamide inhibited the increase in miR-21 stimulated by DHT, fludioxonil, and fenhexamid, suggesting that AR is involved in induction of miR-21 expression (Fig. 1B). These results indicate that fludioxonil and fenhexamid are acting as AR agonists, not antagonists.

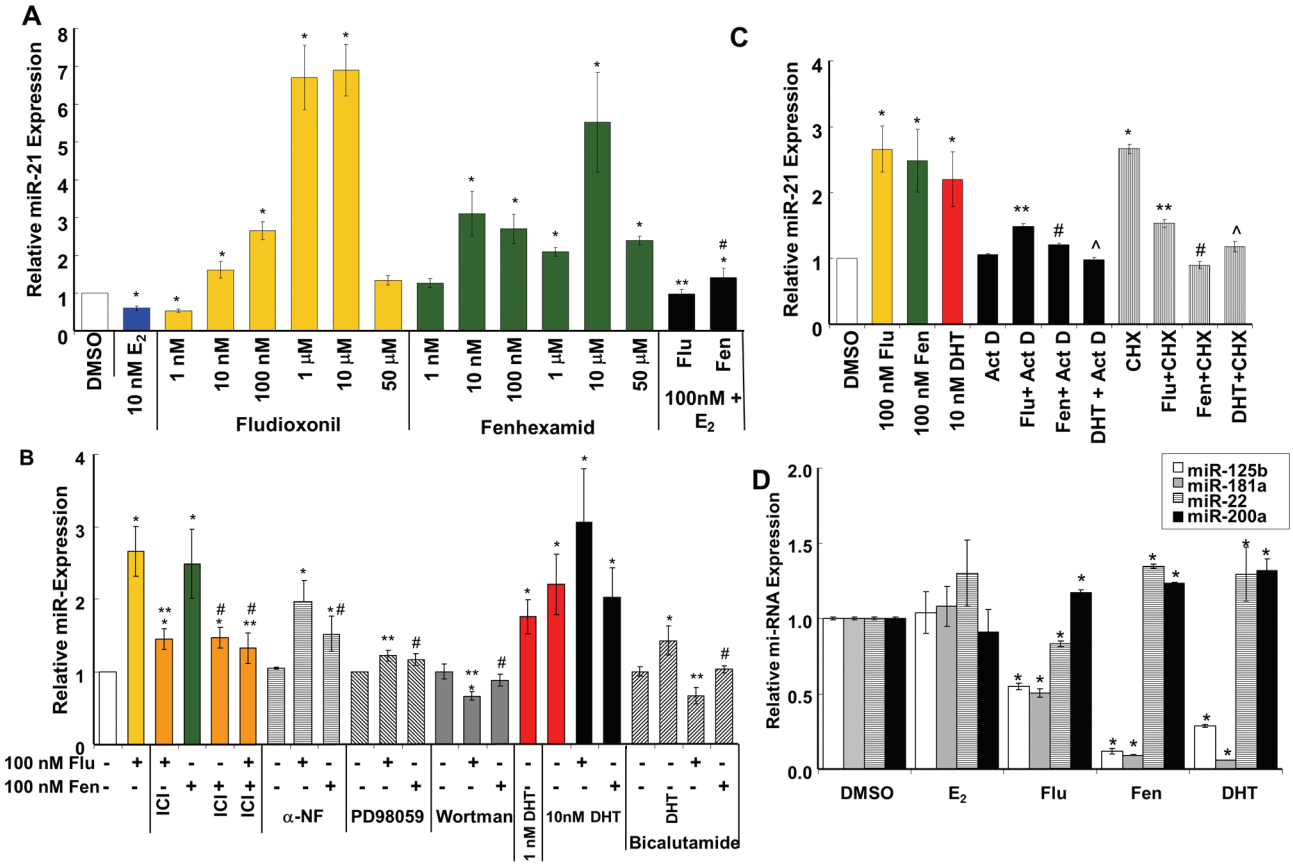

FIG. 1. Fludioxonil and fenhexamid increase miR-21 in MCF-7 cells. MCF-7 cells were "serum starved" for 72 h prior to treatment as indicated for 6 h. Quantitative real-time PCR was used to examine miR-21 expression. (A) Where indicated, the cells were cotreated with 10nM E_2 + 100nM of either fludioxonil (flu) or fenhexamid (fen). (B) Where indicated, the cells were preincubated with inhibitors as described in Materials and Methods section prior to the treatment indicated. (C) Where indicated, the cells were preincubated with ActD or CHX for 1 h followed by treatment as indicated for 6 h. (D) Cells were incubated with DMSO, 10nM E_2, 100nM Flu, 100nM Fen, or 10nM DHT for 6 h, and quantitative real-time PCR was used to examine miR-125b, miR-181a, miR-22, and miR-200a expression. For all panels, values are the average of 3–6 separate experiments ± SEM. Statistical analyses used one way ANOVA followed by Kruskal-Wallis and Dunnett's Multiple Comparison Tests. *$p < 0.05$ versus DMSO vehicle. **$p < 0.05$ versus 100nM fludioxonil; #$p < 0.05$ versus 100nM fenhexamid; ^$p < 0.05$ versus 10nM DHT.

Actinomycin D and Cycloheximide Block Fludioxonil, Fenhexamid, and DHT-Mediated miR-21 Expression

To determine whether the increase in miR-21 expression detected with fludioxonil, fenhexamid, and DHT is a direct effect at the genomic level or requires synthesis of a secondary protein, MCF-7 cells were pretreated with the transcriptional inhibitor actinomycin D (ActD) or the protein synthesis inhibitor cycloheximide (CHX) and then treated with fludioxonil, fenhexamid, and DHT for 6 h (Fig. 1C). CHX alone increased miR-21 expression to a level similar to that induced by fludioxonil, fenhexamid, and DHT (Fig. 1C). CHX superinduction of gene expression is a well-established phenomenon mediated by a reduction in turnover of mRNA, an increase in p38MAPK kinase, and NFκB activation although the mechanisms responsible for this cell-specific and gene-specific effect are not understood (Hershko *et al*., 2004). Pretreatment with ActD and CHX blocked fludioxonil-, fenhexamid-, and DHT-mediated miR-21 expression, suggesting that the mechanism by which they increase miR-21 involves both transcriptional (primary genomic) and secondary mechanisms.

Fludioxonil and Fenhexamid Affect the Expression of Other miRNAs

To determine whether the increase in miR-21 by fludioxonil and fenhexamid was attributable to a general increase in all miRNAs, we examined the effect of these fungicides on the expression of miR-125b, which has tumor suppressor (O'Day and Lal, 2010) and oncomiR (Rajabi *et al*., 2010; Wang *et al*., 2012) activities, and three tumor suppressor miRNAs: miR-181a, miR-22, and miR-200a. As reported (Manavalan *et al*., 2011), E_2 does not affect the expression of these miRNAs (Fig. 1D). Fludioxonil and fenhexamid inhibited miR-125b and miR-181 expression in MCF-7 cells (Fig. 1D). Fludioxonil reduced whereas fenhexamid increased miR-22. Both fludioxonil and fenhexamid increased miR-200a. These data indicate that these fungicides are not stimulating the expression of all

miRNAs and that they are likely to regulate miRNAs in a gene-specific manner. Because there are no published studies on DHT regulation of miRNAs in breast cancer cells and fludioxonil and fenhexamid were reported to have antiandrogenic activity (Orton *et al*., 2011), we also examined the effect of 10nM DHT on the expression of these four miRNAs. Interestingly, the effect of DHT was similar to that of fenhexamid: inhibition of miR-125b and miR-181a expression and stimulation of miR-22 and miR-200a expression (Fig. 1D). Because of its role as an oncomiR in breast cancer, we focused on miR-21 regulation by fludioxonil and fenhexamid in further studies.

Fludioxonil and Fenhexamid Increase miR-21 Expression in T47D but not in ERα-Negative MCF-10A and MDA-MB-231 Cells

To examine whether the induction of miR-21 expression with fludioxonil and fenhexamid is unique to MCF-7 or can be detected in another luminal breast cancer cell line, we examined miR-21 expression in T47D cells (Fig. 2A). Fludioxonil and fenhexamid increased miR-21 in T47D cells in a concentration-dependent manner. E_2 repressed miR-21 expression in T47D cells. In contrast, E_2, fludioxonil, and fenhexamid did not affect miR-21 expression in normal, ERα- MCF-10A breast epithelial cells or in basal-like, triple negative MDA-MB-231 breast cancer cells.

miR-21 is located in the 10th intron of the *TMEM49/VMP1* gene (Fujita *et al*., 2008). Previous results indicate that miR-21 and TMEM49 are independently regulated in HL-60 cells treated with phorbol 12-myristate 13-acetate (PMA) to activate AP-1 (Fujita *et al*., 2008), in XG-1 cells treated with IL-6 to activate Stat3 (Löffler *et al*., 2007), and in MCF-7 cells treated with E_2 (Wickramasinghe *et al*., 2009). Recently a read-thru transcript from VMP1 including miR-21 was identified as a source for miR-21 expression in MCF-7 breast, DU-145 prostate, and HL-60 promyelocytic leukemia cells (Ribas *et al*., 2012). Fludioxonil and fenhexamid increased pri-miR-21, but not *TMEM49* in T47D cells (Figs. 2B and 2C). Fludioxonil and fenhexamid increased *TMEM49* in MDA-MB-231 cells, and this increase was reflected in increased pri-miR-21 in fludioxonil-treated cells but not in fenhexamid-treated cells. These data indicate that the increase in pri-miR-21 may result from a read through of *TMEM49* transcription in fludioxonil-treated MDA-MB-231 cells. Overall, these data indicate that increase in miR-21 in response to fludioxonil and fenhexamid detected in MCF-7 cells (Fig. 1) is not cell-line specific but may be related to ERα expression.

Fludioxonil and Fenhexamid Increase pri-miR-21 Expression in MCF-7 Cells

Pri-miRNAs are the primary transcripts of miRNAs that are sequentially processed first to pre-miRNAs by Drosha in the nucleus and then by Dicer in the cytoplasm to miRNA (Gregory *et al*., 2004; Klinge, 2012). Fludioxonil and fenhexamid

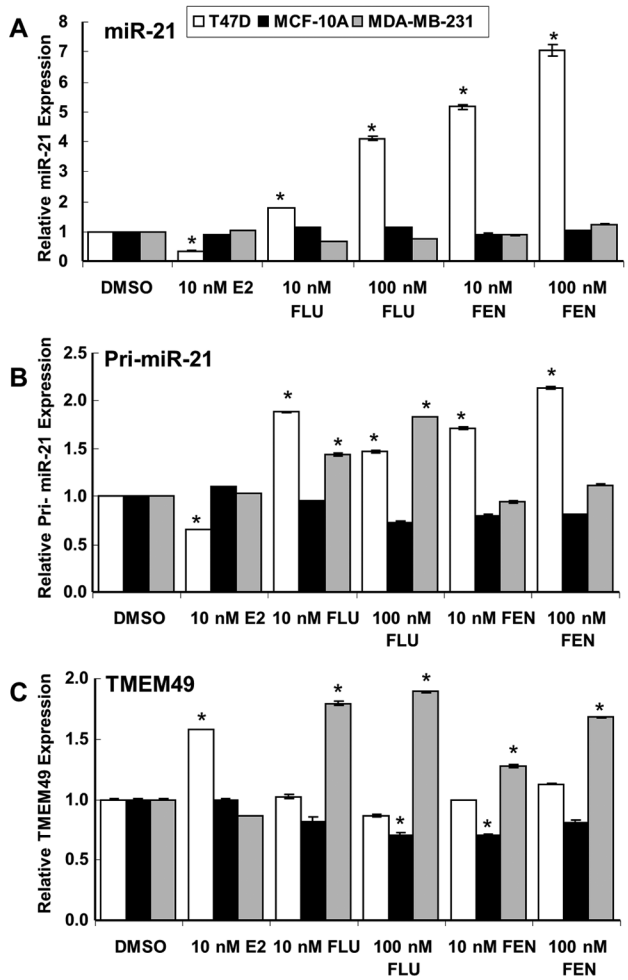

FIG. 2. Fludioxonil and fenhexamid increase miR-21 expression in T47D luminal A breast cancer cells, but not MDA-10A normal breast or MDA-MB-231 triple negative breast cancer cells. T47D, MCF-10A, and MDA-MB-231 cells were serum starved, as described in Figure 1, and then treated for 6 h with DMSO, 10nM E2, 10 or 100nM Flu, and 10 or 100nM Fen. qPCR for miR-21 (A) pri-miR-21 (B) and TMEM49 (C) expression. For all panels, values are the average of 3–6 separate experiments ± SEM. Statistical analysis used one way ANOVA followed by Dunnett's Multiple Comparison Test. *$p < 0.05$ versus DMSO vehicle.

increased pri-miR-21 transcript levels in MCF-7 cells (Fig. 3A). No significant change was seen with E_2, but DHT increased pri-miR-21 (Fig. 3A). The increase in pri-miR-21 in cells treated with fludioxonil and fenhexamid was blocked by ActD, suggesting a direct transcriptional response. ActD only partially inhibited DHT-induced pri-miR-21 expression. These data indicate that the increase in mature miR-21 in response to fludioxonil and fenhexamid, but not DHT, appears to be a primary transcriptional event.

Fenhexamid Increases TMEM49 Transcription

In MCF-7 cells, Fenhexamid and DHT increased *TMEM49* but fludioxonil and E_2 did not (Fig. 3B). ActD reduced basal

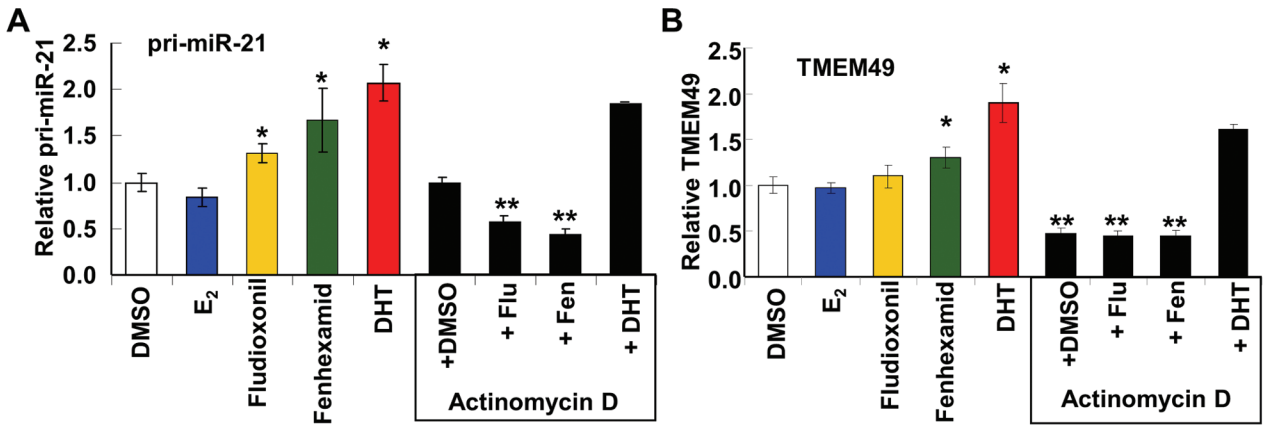

FIG. 3. Fludioxonil and fenhexamid increase primary miR-21 and fenhexamid, and DHT increase TMEM49 expression in MCF-7 cells. MCF-7 cells were serum starved, as described in Figure 1, and then treated for 6 h with DMSO, 10nM E2, 100nM Flu, 100nM Fen, or 10nM DHT. Where indicated, the cells were preincubated with ActD. qPCR: pri-miR-21 (A) and TMEM49 (B). Values are the average of 3–4 separate experiments ± SEM. Statistical analysis used one way ANOVA followed by Kruskal-Wallis test. *$p < 0.05$ versus DMSO vehicle; **$p < 0.05$ versus the same treatment without ActD.

TMEM49 expression and ablated the induction by fludioxonil and fenhexamid but did not affect DHT-induced *TMEM49* expression. These data indicate that the increase in *TMEM49* expression by fenhexamid results from increased transcription, whereas the mechanism by which DHT increases *TMEM49* expression is independent of new transcription. Testosterone increased the expression of *Tmem49* in the meibomian glands of orchidectomized BALB/c mice treated for 14 days with testosterone, but the mechanism was not examined (Sullivan *et al*., 2009). Because the focus of this study was the effects of fludioxonil and fenhexamid on miR-21 expression, the DHT observations were not further dissected.

Effects of Fludioxonil and Fenhexamid on mRNA and Protein Targets of miR-21

miR-21 regulates the translation of PDCD4, PTEN, and BCL2 (Wickramasinghe *et al*., 2009). To determine whether the increase in miR-21 in response to fludioxonil and fenhexamid correspondingly reduced the expression of these miR-21 target mRNAs, qPCR was performed for *PDCD4, PTEN,* and *BCL2*. Cells were treated with E_2 in parallel as a positive control (Wickramasinghe *et al*., 2009). Fludioxonil and fenhexamid decreased the mRNA levels of *PDCD4* and *PTEN*, in agreement with the increase in miR-21 expression (Fig. 4A). However, like E_2, fludioxonil and fenhexamid increased antiapoptotic *BCL2* expression. This effect agrees with the decrease in miR-21 by E_2 but is the opposite of the increase in miR-21 by fludioxonil and fenhexamid. The E_2-mediated increase in mRNA levels *PDCD4* and *BCL2*, but not *PTEN*, agrees with our previous report (Wickramasinghe *et al*., 2009). Fludioxonil and fenhexamid reduced and E_2 increased the levels of *PDCD4* mRNA in T47D cells (Fig. 4B) in inverse correlation with miR-21 (Fig. 2A). E_2 had no effect on *PDCD4* expression in MCF-10A cells (Fig. 4B), reflecting the lack of impact of E_2 on miR-21 (Fig. 2A). Fludioxonil and fenhexamid, 10nM, but not 100nM, reduced *PDCD4* mRNA expression ~15% in MCF-10A, an effect that appears to be independent of miR-21 regulation because neither of these fungicides affected miR-21 expression (Fig. 2A). E_2 increases *BCL2* transcription in MCF-7 cells via ERα-Sp1 and AP1 interactions (Dong *et al*., 1999); thus, the increase in *BCL2* mRNA with E_2 is not solely mediated by a decreased miR-21 (Wickramasinghe *et al*., 2009). The increase in *BCL2* mRNA with fludioxonil and fenhexamid appears to be independent of their stimulation of miR-21 expression.

In agreement with their upregulation of miR-21, both fludioxonil and fenhexamid reduced Pdcd4 (Fig. 4B) and Bcl-2 (Fig. 5B) protein expression. These data are consistent with the inhibition of *PDCD4* mRNA; however, fludioxonil and fenhexamid increased *BCL2* mRNA. Thus, the reduced Bcl-2 protein is consistent with increased miR-21 and inhibition at the translational level. Because miRNAs can reduce translation while not modifying mRNA levels (Iorio and Croce, 2012), this finding is consistent with an increase in miR-21 reducing Bcl-2 at the protein and not mRNA level. Pdcd4 and Bcl-2 protein expression was increased in MCF-7 cells treated with E_2, in agreement with previous findings (Wickramasinghe *et al*., 2009) (Figs. 4B and 5B).

AS-miR-21 Inhibits Fludioxonil and Fenhexamid-Mediated Inhibition of Pdcd4 Protein Expression

To determine whether the inhibition of Pdcd4 and Bcl-2 protein expression in MCF-7 cells by fludioxonil and fenhexamid was mediated directly by miR-21, cells were transfected with antisense-miR-21 oligonucleotide (AS-miR-21) or an AS control for 48 h and then treated with E_2, fludioxonil, or fenhexamid for 6 or 24 h for RNA and protein studies, respectively (Fig. 5). AS-miR-21 completely ablated miR-21 expression

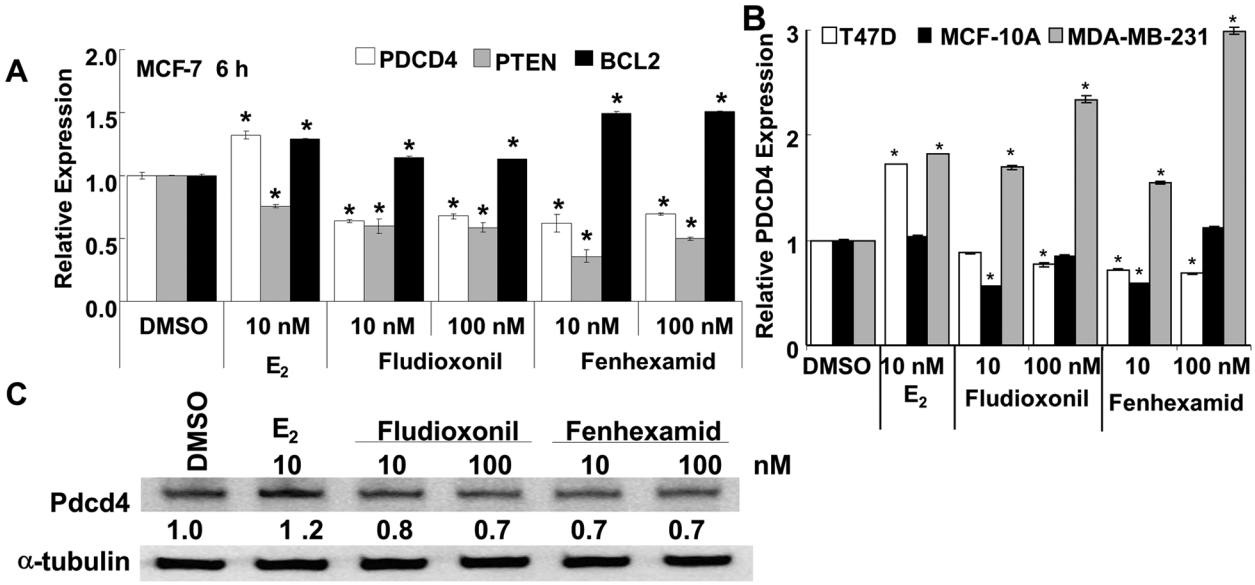

FIG. 4. Effects of fludioxonil and fenhexamid on miR-21 targets mRNA expression in MCF-7 cells. MCF-7 cells were serum starved, as described in Figure 1, and then treated with the indicated concentrations of E_2, fludioxonil, and fenhexamid for 6 h (A) or 24 h (B). (A) The mRNA expression of *PDCD4*, *PTEN*, and *BCL2* was determined by qPCR. Values are the average of 3–4 separate experiments ± SEM. *$p < 0.05$ versus DMSO (control). (B) T47D and MCF-10A cells were serum starved and then treated for 6 h with DMSO, 10nM E_2, 10 or 100nM Flu, and 10 or 100nM Fen. Values are the average triplicates ± SEM. Statistical analysis used one way ANOVA followed by Kruskal-Wallis test. *$p < 0.05$ versus DMSO vehicle. (C) Whole cell lysates were prepared from MCF-7 cells treated for 24 h, and Pdcd4 and Bcl-2 were analyzed by Western blot. The same blot was used for all Western blots. Band intensities were analyzed and expressed relative to α-tubulin, and values are expressed relative to the DMSO value that was set to 1.

(Fig. 5A). As expected, E_2 increased Pdcd4 and Bcl-2 protein and AS-miR-21 further increased Pdcd4 and Bcl-2 protein (Fig. 5B). AS-miR-21 increased basal Pdcd4 protein expression in MCF-7 cells but had no effect on basal Bcl-2 protein (Fig. 5B). AS-miR-21 blocked the repression of Pdcd4 and Bcl-2 protein by fludioxonil and fenhexamid (Fig. 5B). These data are commensurate with a direct stimulation of miR-21 by fludioxonil and fenhexamid that in turn reduces Pdcd4 and Bcl-2 protein levels.

Fludioxonil and Fenhexamid Directly Regulate miR-21 Repression of Pdcd4 Through the 3′UTR

To determine whether fludioxonil and fenhexamid directly regulate miR-21 interaction with the 3′UTR of Pdcd4, MCF-7 cells were transiently transfected with a PDCD4-3′UTR construct cloned downstream of the *Renilla* luciferase reporter. Cells were also transfected with AS-miR-21 or an AS control and then treated with E_2, fludioxonil, or fenhexamid for 24 h (Fig. 5C). In agreement with E_2 suppression of miR-21, E_2 increased *Renilla* luciferase activity. In agreement with the increase in miR-21 by fludioxonil and fenhexamid, PDCD4-3′UTR *Renilla* luciferase activity was inhibited. Transfection with AS-miR-21 increased basal *Renilla* luciferase activity and blocked regulation by E_2, fludioxonil, and fenhexamid. These data conclusively demonstrate that the increase in miR-21 by fludioxonil and fenhexamid targets PDCD4 through the 3′UTR.

Fludioxonil and Fenhexamid Inhibit MCF-7 Cell Viability

To determine the functional consequence of fludioxonil and fenhexamid's induction of miR-21 in MCF-7 cells, MCF-7 cells were treated with a range of concentrations of fludioxonil and fenhexamid, and cell viability was measured by an MTT assay after 48-h treatment (Fig. 6A). E_2 was included as a positive control and increased MCF-7 viability. Fludioxonil had a biphasic effect, first increasing proliferation at 10nM and then inhibiting cell viability at higher concentrations. Fenhexamid had no effect on cell viability at concentrations from 1nM to 10μM. Fludioxonil and fenhexamid inhibited E_2-induced cell viability, a finding in agreement with their ability to block E_2 inhibition of miR-21 expression (Fig. 1A). There was no evidence that fludioxonil and fenhexamid induced apoptosis in MCF-7 cells (Fig. 6B), even after 96-h treatment (Supplementary fig. 4). With 48-h treatment, fludioxonil increased intact poly(ADP-ribose) polymerase (PARP), a finding that reflects the lack of effect of fludioxonil on cell viability.

Fludioxonil and Fenhexamid Inhibit E_2-Regulated Gene Transcription in MCF-7 Cells

To determine whether fludioxonil and fenhexamid exhibit antiestrogenic activity on ERα-regulated gene targets, the expression of E_2-regulated genes cyclin D1 (*CCND1*) and progesterone receptor (PGR) was measured by quantitative real-time PCR along with the expression of ERα (*ESR1*) and ERα (*ESR2*). As a positive control, E_2 increased the expression of

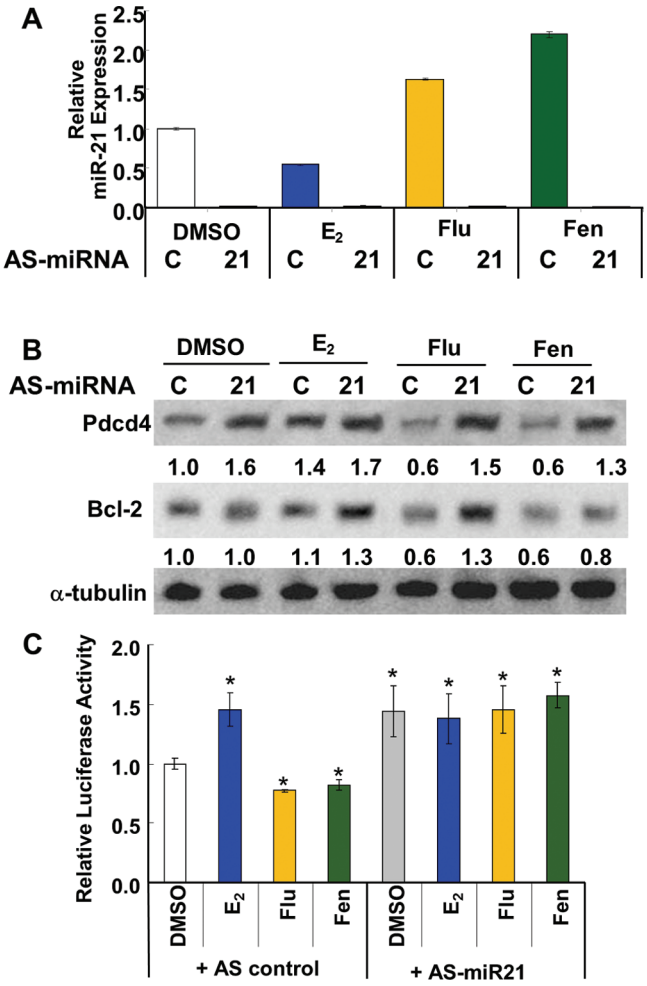

FIG. 5. AS-miR-21 inhibits fludioxonil- and fenhexamid-mediated inhibition of Pdcd4 and Bcl-2 protein expression and inhibition of PDCD4-3′UTR luciferase reporter activity. MCF-7 cells were transfected with control nonspecific antisense (AS) RNA (C) or AS-miR-21 (21) duplexes. Cells were transfected and treated for 6 h as described in Materials and Methods section with DMSO, 10nM E_2, or 100nM fludioxonil or fenhexamid for RNA (A) and 24 h for protein (B). (A) qPCR for miR-21. Values are the average of triplicate determinations ± SEM. (B) Whole cell lysates were prepared from MCF-7 cells transfected with control or AS-miR-21 for 48 h and then treated for 24 h as indicated. The same blot was used for all Western blots shown (Pdcd4, Bcl-2, and α-tubulin). The values are Pdcd4/α-tubulin or Bcl-2/α-tubulin ratio with the AS-control-DMSO value set to 1 for comparison. (C) MCF-7 cells were transiently transfected with *Renilla* luciferase reporter containing the 3′-UTR of *PDCD4* cloned 3′ to *Renilla*, pGL3-pro-luciferase (firefly) as a transfection control, and either AS control or AS-miR-21. Cells were treated with DMSO, 10nM E_2, 100nM fludioxonil, or 100nM fenhexamid for 24 h. Relative luciferase activity determined relative to the DMSO AS-control transfected cells. Values are the average of triplicate determinations ± SEM. Statistical analysis used one way ANOVA followed by Kruskal-Wallis test. $*p < 0.05$ versus DMSO-AS-control.

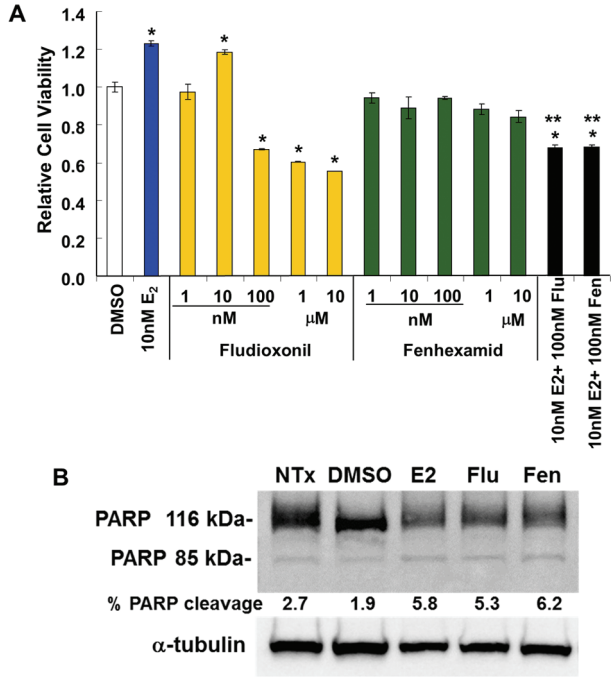

FIG. 6. Fludioxonil and fenhexamid differentially affect MCF-7 cell viability but do not stimulate apoptosis. For both (A) and (B), MCF-7 cells were serum starved for 72 h, as in Figure 1, and then treated with (A) DMSO (vehicle control), 10nM E_2 or 1nM–10μM fludioxonil or fenhexamid as indicated for 48 h. MTT assays were performed and results are the average ± SEM of quadruplicate determinations. $*p < 0.05$ versus DMSO (control). $**p < 0.05$ versus E_2. (B) MCF-7 cells were treated with DMSO, 10nM E_2, 100nM fludioxonil (Flu), 100nM fenhexamid (Fen), or 100nM 4-OHT for 48 h. Thirty micrograms of protein from whole cell lysates were separated by 10% SDS-PAGE and immunoblotted for PARP. The membrane was stripped and reprobed for α-tubulin as a loading control. Immunoreactive bands were quantified, and the ratio of intact PARP/α-tubulin normalized to the DMSO control is indicated.

CCND1, PGR, and *ESR2* (Supplementary fig. 5). Fludioxonil and fenhexamid inhibited expression of *CCND1, PGR*, and *ESR1*. Fludioxonil inhibited *ESR2* and fenhexamid had no effect on *ESR2*. 18S rRNA control gene expression was not altered by any treatment (Supplementary fig. 6).

Fludioxonil and Fenhexamid Regulate BCL2 Gene Expression in ERα-Negative MDA-MB-231 Cells

In addition, we tested the effect of fludioxonil and fenhexamid on three well-established E_2-ERα-induced genes: *PGR, CCND1,* and *BCL2* in ERα-negative, ERβ-positive (Dotzlaw *et al.*, 1999), MDA-MB-231 cells. *PGR* was not expressed in MDA-MB-231 cells (CT > 38, data not shown). Neither fludioxonil nor fenhexamid increased the expression of cyclin D1 (*CCND1*) (Supplementary fig. 7). Fludioxonil inhibited the expression of *BCL2*, whereas fenhexamid increased *BCL2* (Supplementary fig. 7). Fludioxonil did not inhibit *BCL2* expression in MCF-7 cells, whereas fenhexamid increased *BCL2* in MCF-7 cells (Fig. 4), indicating cell-specific regulation of *BCL2* expression by these fungicides.

Fludioxonil and Fenhexamid Inhibit MCF-7 Cell Mobility in a Wound-Healing Assay

To address the dichotomy of the increase in miR-21 expression by fludioxonil and fenhexamid with the inhibition of MCF-7 cell viability by fludioxonil, we examined the effect

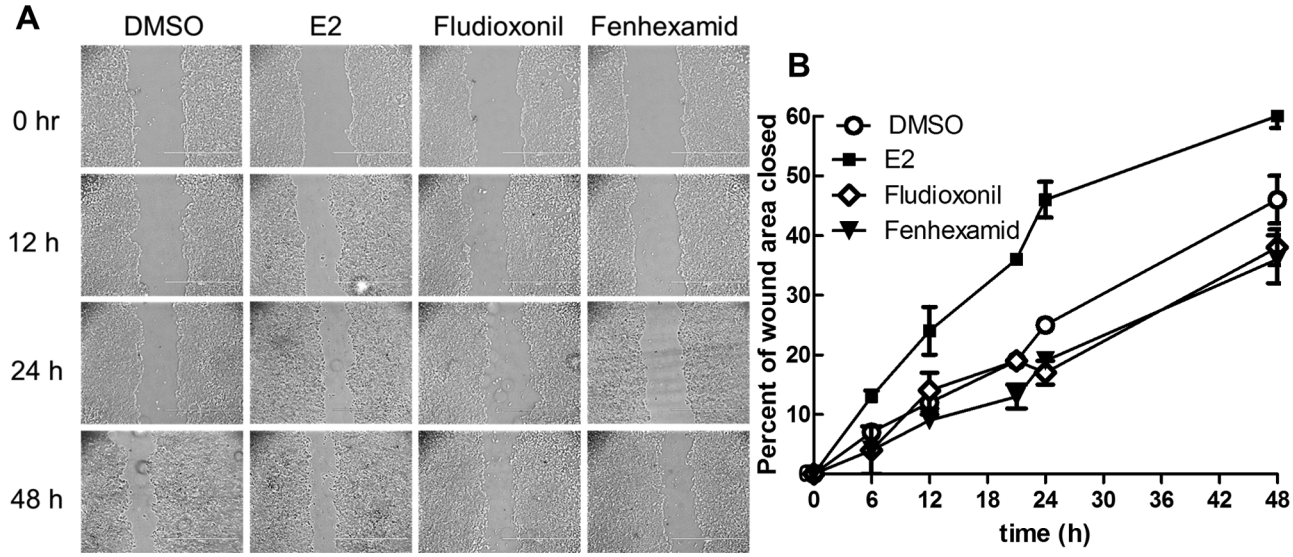

FIG. 7. Fludioxonil and fenhexamid inhibit MCF-7 cell migration in a wound-healing assay. MCF-7 cells were seeded in six-well plates and incubated overnight in phenol red–free IMEM + 5% DCC-FBS before wounding with a plastic pipette tip. The cells were treated with DMSO (vehicle control), 10nM E_2, 100nM fludioxonil, or 100nM fenhexamid, as indicated, for 48 h total. (A) Cells were photographed at the indicated times, the bar is 1000 µm. (B) The wound closure was analyzed at the indicated times using NIH Image J software. Values are the mean ± SD of duplicate measurements.

of these fungicides on MCF-7 cell motility in a wound-healing assay (Figs. 7A and B). E_2 increased MCF-7 cell motility. Fludioxonil and fenhexamid slowed cell migration compared with DMSO at the 24- and 48-h time points.

DISCUSSION

A role for EDCs in breast cancer is suspected but unproven in humans (Weyandt *et al*., 2008). The mechanisms by which EDC might stimulate breast tumorigenesis and disease progression involve potential effects on a wide range of processes involved in hormone action. Here we report for the first time that the commonly used fungicides fludioxonil and fenhexamid increase the expression of miR-21 in MCF-7 breast cancer cells at the gene transcription level. Similar results were observed in another luminal A breast cancer cell line, T7D, but not in two ERα-negative cell lines: MCF-10A and MDA-MB-231 cells.

The rationale for examining miR-21 is that it is the most overexpressed miRNA in breast tumors and correlates with poor prognosis (Yan *et al*., 2008). The increase in oncomiR miR-21 expression induced by fludioxonil and fenhexamid was blocked by fulvestrant and bicalutamide, suggesting that these fungicides act through ERα and AR to increase miR-21. Overexpression of miR-21 in breast tumors has been associated with reduced survival time, and knockdown of miR-21 in breast cancer cell lines has been associated with increased responses to taxol *in vitro* (Corcoran *et al*., 2011). Hence, it is possible that women exposed, perhaps repeatedly through ingestion of fruits with residual fludioxonil and fenhexamid pesticides, might have an increase in miR-21 in breast tumor cells, which would be expected to increase cell proliferation and reduce responses to anticancer agents.

In contrast to results from a reporter assay in stably transformed MDA-MB-231 cells (Orton *et al*., 2011), fludioxonil and fenhexamid did not act as antiandrogens in MCF-7 cells, suggesting cell-specific responses to these fungicides. We note that fludioxonil and fenhexamid reduced the expression of tumor suppressors miR-125b and miR-181a but stimulated the expression of tumor suppressor miR-200a. Increased miR-200a would be predicted to inhibit cell motility, commensurate with our observations in the wound-healing assay, but further detailed studies are necessary to elucidate the mechanisms involved. miR-22 is a tumor suppressor and downregulates *ESR1* (ERalpha) (Klinge, 2012). Interestingly, the small increase and decrease in ERα protein in MCF-7 cells treated with fludioxonil and fenhexamid, respectively (Supplementary fig. 3), reflect the decrease and increase, respectively, in miR-22 seen with these fungicides. Overall, the fungicides fludioxonil and fenhexamid regulate miRNA transcription in a gene-specific manner.

Our study also shows for the first time that 1 and 10nM DHT increase miR-21, miR-22, and miR-200a and reduce miR-125b and miR-181a in MCF-7 cells. Although full dissection of the mechanism and meaning of these results will require further investigation, it appears that DHT, like fludioxonil and fenhexamid, regulates miRNA expression in a gene-specific manner. The increase in tumor suppressor miR-22 and miR-200a is in agreement with the ability of DHT to inhibit MCF-7 cell proliferation (Macedo *et al*., 2006). There is currently much interest in the role of androgens in breast cancer because epidemiological studies suggest an association between higher circulating

levels of androgens, as well as estrogens, in postmenopausal breast cancer (Kotsopoulos and Narod, 2012).

OncomiR miR-21 downregulates PDCD4, BCL2, and PTEN as well as other targets in breast cancer cells (O'Day and Lal, 2010). As expected, commensurate with their ability to increase miR-21, fludioxonil and fenhexamid reduced PTEN and BCL2 protein levels. Fludioxonil and fenhexamid also reduced *PDCD4* and *PTEN* mRNA but not *BCL2* mRNA in MCF-7 cells. We also detected reduced *PDCD4* in T47D cells but not in ERα-negative MCF-10A cells. These results indicate, as modeled in Fig. 8, that fludioxonil and fenhexamid activate ERα and AR to increase miR-21 expression, which, in turn, directly downregulated PDCD4, PTEN, and BCL2. In agreement with this model, we demonstrated that AS-miR-21 blocked fludioxonil and fenhexamid upregulation of miR-21 and thus derepressed PDCD4 and BCL2 protein expression.

Although previous studies reported that miR-21 inhibits apoptosis by reducing Bcl-2 in a breast cancer mouse model (Si *et al*., 2007), here fenhexamid inhibited MCF-7 cell proliferation despite increasing miR-21 expression. This observation is similar to other paradoxical data showing that all-*trans*-retinoic acid (ATRA) increased miR-21 in MCF-7 and other ERα-expressing cells and inhibited cell proliferation and decreased cell motility (Terao *et al*., 2011). In contrast, fludioxonil had no effect on cell viability and increased the amount of intact PARP seen at 48 h, an effect that was ablated at 96 h, although no increase in cleaved PARP was detected. These data indicate different mechanisms for fludioxonil and fenhexamid in MCF-7 cells and suggest a potentially beneficial role for the observed increase in miR-21 with fludioxonil and fenhexamid, i.e., reducing cell motility, an event adversely affecting breast cancer prognosis.

Fenhexamid and DHT, but not fludioxonil or E_2, increased expression of the *TMEM49* in which miR-21 is encoded in MCF-7 cells, and fludioxonil and fenhexamid increased *TMEM49* in MDA-MB-231 cells. Although exploring the significance of increased *TMEM49* expression is beyond the scope of this study, *TMEM49/VMP1* has been suggested to be a 7-transmembrane protein (Sauermann *et al*., 2007). When overexpressed in HEK-293 cells, VMP1 is located in the endoplasmic reticulum and vacuoles and plays a role in the plasma membrane by initiating maturation of tight junctions and adherens junctions (Sauermann *et al*., 2007). Knockdown of VMP1 in HEK-293 cells revealed that VMP1 plays a role in cell-cell adhesion and that cells with low VMP1 showed increased invasiveness. VMP1 expression was reported to be reduced with increasing tumor grade in invasive ductal breast carcinomas. The authors concluded that VMP1 plays a role in maintaining cell-cell interaction similar to that of E-cadherin, and when VMP1 is reduced, cells become more migratory and metastatic (Sauermann *et al*., 2007). Because fenhexamid increased *TMEM49/VMP1* and reduced cell migration in MCF-7 cells, these results are consistent with the role of overexpressed VMP1 in HEK-293 cells (Sauermann *et al*., 2007).

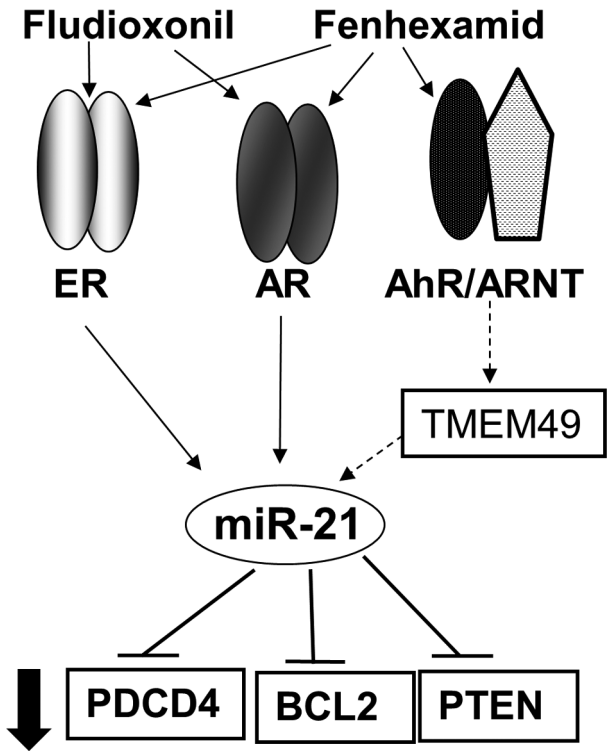

FIG. 8. Model of fludioxonil and fenhexamid regulation of miR-21 expression and downstream effects in MCF-7 cells. Fludioxonil and fenhexamid increase pri-miR-21 and miR-21 expression in and ER- and AR-mediated manner. Fenhexamid also increases miR-21 via AhR activation. In turn, the increase in miR-21 reduces *PDCD4* and *PTEN* mRNA and Pdcd4 protein. Fenhexamid, not fludioxonil, reduces Bcl-2 protein. AS-miR-21 blocks the fludioxonil- and fenhexamid-mediated reduction in Pdcd4 and Bcl-2 proteins.

The AHR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) reduced *Tmem49* expression in fetal mouse hearts in a dose-dependent manner (Thackaberry *et al*., 2005). We observed that treating MCF-7 cells with AHR antagonist α-NF increased *TMEM49* expression ~sevenfold (data not shown). The finding that α-NF had a small, but statistically significant, effect inhibiting fenhexamid-stimulated miR-21 expression is consistent with some activity of fludioxonil via AHR but is inconsistent with the reported repressive effect of AHR on E_2-ERα regulated genes (Madak-Erdogan and Katzenellenbogen, 2012). Knockdown of AHR increases the expression of some ERα-mediated genes in response to E_2 treatment by dismissing the corepressor RIP140 from the gene promoters (Madak-Erdogan and Katzenellenbogen, 2012). Given the Geoprofiles data GDS2744 and GDS2745 (GSE7765) showing that a 16-h treatment of MCF-7 cells with 100nM TCDD increased miR-21 and *TMEM49* expression, it is possible that the increase in miR-21 is a consequence of a read through of AHR regulation of *TMEM49* transcription. This hypothesis is indicated as a possibility in Figure 8 and requires further exploration. Overall, we

conclude that like other EDCs (Casals-Casas and Desvergne, 2011), it is likely that fludioxonil and fenhexamid target multiple receptors and pathways, albeit with relatively lower affinity than the natural ligands for these pathways.

We also report that inhibitors of MAPK and PI3K pathways inhibit fludioxonil- and fenhexamid-induced miR-21 expression. However, because TRBP is regulated by MAPK phosphorylation which increases Dicer activity, the ablation of miR-21 induction by the MEK1 inhibitor PD98059 may reflect inhibition of Dicer activity and thus not be informative of the mechanism by which these fungicides increase miR-21 (Klinge, 2012). Likewise, AKT was identified as a positive upstream regulator of miR-21 in rat cardiomyocytes because wortmannin upregulated FasL and downregulated miR-21 (Sayed *et al*., 2010). However, that study used 5μM wortmannin for 24 h compared with 100nM wortmannin for 7 h total in our experiments. Nonetheless, we cannot conclude a role for the PI3K/AKT pathway in mediating the increase in miR-21 by fludioxonil and fenhexamid in MCF-7 cells.

In summary, the fungicides fludioxonil and fenhexamid increase the expression of miR-21 in an ER- and AR-dependent manner in MCF-7 breast cancer cells. The requirement for ERα was reflected in similar findings in T47D luminal A breast cancer cells, but not in ERα-negative MCF-10A normal breast epithelial cells or MDA-MB-231 triple negative breast cancer cells. Fenhexamid also appears to increase miR-21 expression through AHR activation. The fludioxonil- and fenhexamid-induced decrease in Pdcd4 and Bcl-2 proteins was mediated by an increase in miR-21. Fludioxonil inhibited MCF-7 cell viability, whereas fenhexamid had no impact on cell viability at concentrations from 1nM to 10μM. This is in agreement with results of Jungbauer (unpublished data) although in that report 100μM fenhexamid inhibited MCF-7 cell viability. Both fungicides reduced cell motility. Although the relationship of the concentrations of fludioxonil and fenhexamid as residuals in fruits and vegetables and the 100nM concentration used in most of these experiments is unknown, we suggest that depending on the time of harvest after spraying and subsequent washing procedures, a 100nM concentration might be found. Rats gavaged with fludioxonil at 5 mg/kg bw had serum levels of 0.065 and 0.027 mg/l for males and females, respectively, 15 min after treatment (World Health Organization, 2006). These serum levels are ~250–100nM, similar to the concentrations used in this study. Likewise, rats gavaged with fenhexamid at 1 mg/kg bw showed serum levels of 0.065 and 0.027 μg/ml for males and females, respectively (UN FAO, 2005), which is ~200-100nM, again similar to concentrations used here. Although studies of fludioxonil sprayed on tomatoes showed no fludioxonil 24 days after field spraying (Cabizza *et al*., 2012), further testing of human exposure levels may be warranted. The ability of fenhexamid and fludioxonil to act as endocrine disruptors by activating endogenous ERα and AR and increasing expression of the oncomiR miR-21 in human breast cancer cells requires further study for its significance for fetal development and human health.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

National Institutes of Health (CA138410 to C.M.K.).

ACKNOWLEDGMENT

We thank Brandie J. Radde for performing some Western blots.

REFERENCES

Angioni, A., Porcu, L., and Dedola, F. (2012). Determination of famoxadone, fenamidone, fenhexamid and iprodione residues in greenhouse tomatoes. *Pest Manag. Sci.* **68,** 543–547.

Biswas, R., and Vonderhaar, B. K. (1987). Role of serum in the prolactin responsiveness of MCF-7 human breast cancer cells in long-term tissue culture. *Cancer Res.* **47,** 3509–3514.

Cabizza, M., Dedola, F., and Satta, M. (2012). Residues behavior of some fungicides applied on two greenhouse tomato varieties different in shape and weight. *J. Environ. Sci. Health B* **47,** 379–384.

Casals-Casas, C., and Desvergne, B. (2011). Endocrine disruptors: From endocrine to metabolic disruption. *Annu. Rev. Physiol.* **73,** 135–162.

Corcoran, C., Friel, A. M., Duffy, M. J., Crown, J., and O'Driscoll, L. (2011). Intracellular and extracellular microRNAs in breast cancer. *Clin. Chem.* **57,** 18–32.

Diamanti-Kandarakis, E., Bourguignon, J. P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., Zoeller, R. T., and Gore, A. C. (2009). Endocrine-disrupting chemicals: An Endocrine Society scientific statement. *Endocr. Rev.* **30,** 293–342.

Dong, L., Wang, W., Wang, F., Stoner, M., Reed, J. C., Harigai, M., Samudio, I., Kladde, M. P., Vyhlidal, C., and Safe, S. (1999). Mechanisms of transcriptional activation of bcl-2 gene expression by 17beta-estradiol in breast cancer cells. *J. Biol. Chem.* **274,** 32099–32107.

Dotzlaw, H., Leygue, E., Watson, P. H., and Murphy, L. C. (1999). Estrogen receptor-beta messenger RNA expression in human breast tumor biopsies: Relationship to steroid receptor status and regulation by progestins. *Cancer Res.* **59,** 529–532.

Fillinger, S., Leroux, P., Auclair, C., Barreau, C., Al Hajj, C., and Debieu, D. (2008). Genetic analysis of fenhexamid-resistant field isolates of the phytopathogenic fungus Botrytis cinerea. *Antimicrob. Agents Chemother.* **52,** 3933–3940.

Fu, X., Han, Y., Wu, Y., Zhu, X., Lu, X., Mao, F., Wang, X., He, X., Zhao, Y., and Zhao, Y. (2011). Prognostic role of microRNA-21 in various carcinomas: A systematic review and meta-analysis. *Eur. J. Clin. Invest*. **41,** 1245–53.

Fujita, S., Ito, T., Mizutani, T., Minoguchi, S., Yamamichi, N., Sakurai, K., and Iba, H. (2008). miR-21 gene expression triggered by AP-1 is sustained through a double-negative feedback mechanism. *J. Mol. Biol*. **378,** 492–504.

Gill, P. G., Tilley, W. D., De Young, N. J., Lensink, I. L., Dixon, P. D., and Horsfall, D. J. (1991). Inhibition of T47D human breast cancer cell growth by the synthetic progestin R5020: Effects of serum, estradiol, insulin, and EGF. *Breast Cancer Res. Treat.* **20,** 53–62.

Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., and Shiekhattar, R. (2004). The Microprocessor complex mediates the genesis of microRNAs. *Nature* **432,** 235–240.

Hammes, S. R., and Levin, E. R. (2011). Minireview: Recent advances in extranuclear steroid receptor actions. *Endocrinology* **152,** 4489–4495.

Hatley, M. E., Patrick, D. M., Garcia, M. R., Richardson, J. A., Bassel-Duby, R., van Rooij, E., and Olson, E. N. (2010). Modulation of K-Ras-dependent lung tumorigenesis by microRNA-21. *Cancer Cell* **18,** 282–293.

Hershko, D. D., Robb, B. W., Wray, C. J., Luo, G. J., and Hasselgren, P. O. (2004). Superinduction of IL-6 by cycloheximide is associated with mRNA stabilization and sustained activation of p38 map kinase and NF-kappaB in cultured Caco-2 cells. *J. Cell. Biochem.* **91,** 951–961.

Horwitz, K. B., Costlow, M. E., and McGuire, W. L. (1975). MCF-7; a human breast cancer cell line with estrogen, androgen, progesterone, and glucocorticoid receptors. *Steroids* **26,** 785–795.

Iorio, M. V., and Croce, C. M. (2012). MicroRNA dysregulation in cancer: Diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Mol. Med.* **4,** 143–159.

JMPR. (2005). Pesticide residues in food-2005. The Joint FAO/WHO Meeting on Pesticide Residues (JMPR), Rome.

Klinge, C. M., Riggs, K. A., Wickramasinghe, N. S., Emberts, C. G., McConda, D. B., Barry, P. N., and Magnusen, J. E. (2010). Estrogen receptor alpha 46 is reduced in tamoxifen resistant breast cancer cells and re-expression inhibits cell proliferation and estrogen receptor alpha 66-regulated target gene transcription. *Mol. Cell. Endocrinol.* **323,** 268–276.

Klinge, C. M. (2012). miRNAs and estrogen action. *Trends Endocrinol. Metab.* **23,** 223–233.

Kotsopoulos, J., and Narod, S. A. (2012). Androgens and breast cancer. *Steroids* **77,** 1–9.

Koturbash, I., Beland, F. A., and Pogribny, I. P. (2012). Role of microRNAs in the regulation of drug metabolizing and transporting genes and the response to environmental toxicants. *Expert Opin. Drug Metab. Toxicol.* **8,** 597–606.

Levin, E. R. (2009). Plasma membrane estrogen receptors. *Trends Endocrinol. Metab.* **20,** 477–482.

Li, T., Li, D., Sha, J., Sun, P., and Huang, Y. (2009). MicroRNA-21 directly targets MARCKS and promotes apoptosis resistance and invasion in prostate cancer cells. *Biochem. Biophys. Res. Commun.* **383,** 280–285.

Löffler, D., Brocke-Heidrich, K., Pfeifer, G., Stocsits, C., Hackermüller, J., Kretzschmar, A. K., Burger, R., Gramatzki, M., Blumert, C., Bauer, K., *et al.* (2007). Interleukin-6–dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. *Blood* **110,** 1330–1333.

Lundin, K. B., Henningson, M., Hietala, M., Ingvar, C., Rose, C., and Jernström, H. (2011). Androgen receptor genotypes predict response to endocrine treatment in breast cancer patients. *Br. J. Cancer* **105,** 1676–1683.

Macedo, L. F., Guo, Z., Tilghman, S. L., Sabnis, G. J., Qiu, Y., and Brodie, A. (2006). Role of androgens on MCF-7 breast cancer cell growth and on the inhibitory effect of letrozole. *Cancer Res.* **66,** 7775–7782.

Madak-Erdogan, Z., and Katzenellenbogen, B. S. (2012). Aryl hydrocarbon receptor modulation of estrogen receptor α-mediated gene regulation by a multimeric chromatin complex involving the two receptors and the coregulator RIP140. *Toxicol. Sci.* **125,** 401–411.

Maillot, G., Lacroix-Triki, M., Pierredon, S., Gratadou, L., Schmidt, S., Benes, V., Roche, H., Dalenc, F., Auboeuf, D., Millevoi, S., *et al.* (2009). Widespread estrogen-dependent repression of microRNAs involved in breast tumor cell growth. *Cancer Res.* **69,** 8332–8340.

Manavalan, T. T., Teng, Y., Appana, S. N., Datta, S., Kalbfleisch, T. S., Li, Y., and Klinge, C. M. (2011). Differential expression of microRNA expression in tamoxifen-sensitive MCF-7 versus tamoxifen-resistant LY2 human breast cancer cells. *Cancer Lett.* **313,** 26–43.

Matthews, J., and Gustafsson, J. A. (2006). Estrogen receptor and aryl hydrocarbon receptor signaling pathways. *Nucl. Recept. Signal.* **4,** e016.

Metivier, R., Penot, G., Carmouche, R. P., Hubner, M. R., Reid, G., Denger, S., Manu, D., Brand, H., Kos, M., Benes, V., *et al.* (2004). Transcriptional complexes engaged by apo-estrogen receptor-alpha isoforms have divergent outcomes. *EMBO J.* **23,** 3653–3666.

Ni, M., Chen, Y., Lim, E., Wimberly, H., Bailey, S. T., Imai, Y., Rimm, D. L., Liu, X. S., and Brown, M. (2011). Targeting androgen receptor in estrogen receptor-negative breast cancer. *Cancer Cell* **20,** 119–131.

O'Day, E., and Lal, A. (2010). MicroRNAs and their target gene networks in breast cancer. *Breast Cancer Res.* **12,** 201.

Orton, F., Rosivatz, E., Scholze, M., and Kortenkamp, A. (2011). Widely used pesticides with previously unknown endocrine activity revealed as in vitro antiandrogens. *Environ. Health Perspect.* **119,** 794–800.

Rajabi, H., Jin, C., Ahmad, R., McClary, C., Joshi, M. D., and Kufe, D. (2010). Mucin 1 oncoprotein expression is suppressed by the miR-125b oncomiR. *Genes Cancer* **1,** 62–68.

Ribas, J., Ni, X., Castanares, M., Liu, M. M., Esopi, D., Yegnasubramanian, S., Rodriguez, R., Mendell, J. T., and Lupold, S. E. (2012). A novel source for miR-21 expression through the alternative polyadenylation of VMP1 gene transcripts. *Nucleic Acids Res.* **40,** 6821–6833.

Robinson, J. L., Macarthur, S., Ross-Innes, C. S., Tilley, W. D., Neal, D. E., Mills, I. G., and Carroll, J. S. (2011). Androgen receptor driven transcription in molecular apocrine breast cancer is mediated by FoxA1. *EMBO J.* **30,** 3019–3027.

Ross, J. A., and Leavitt, S. A. (2010). Analysis of the mutations induced by conazole fungicides in vivo. *Mutagenesis* **25,** 231–234.

Sauermann, M., Sahin, O., Sultmann, H., Hahne, F., Blaszkiewicz, S., Majety, M., Zatloukal, K., Fuzesi, L., Poustka, A., Wiemann, S., *et al.* (2007). Reduced expression of vacuole membrane protein 1 affects the invasion capacity of tumor cells. *Oncogene* **27,** 1320–1326.

Sayed, D., He, M., Hong, C., Gao, S., Rane, S., Yang, Z., and Abdellatif, M. (2010). MicroRNA-21 is a downstream effector of AKT that mediates its antiapoptotic effects via suppression of Fas ligand. *J. Biol. Chem.* **285,** 20281–20290.

Si, M. L., Zhu, S., Wu, H., Lu, Z., Wu, F., and Mo, Y. Y. (2007). miR-21-mediated tumor growth. *Oncogene* **26,** 2799–2803.

Sullivan, D. A., Jensen, R. V., Suzuki, T., and Richards, S. M. (2009). Do sex steroids exert sex-specific and/or opposite effects on gene expression in lacrimal and meibomian glands? *Mol. Vis.* **15,** 1553–1572.

Swedenborg, E., Rüegg, J., Mäkelä, S., and Pongratz, I. (2009). Endocrine disruptive chemicals: Mechanisms of action and involvement in metabolic disorders. *J. Mol. Endocrinol.* **43,** 1–10.

Terao, M., Fratelli, M., Kurosaki, M., Zanetti, A., Guarnaccia, V., Paroni, G., Tsykin, A., Lupi, M., Gianni, M., Goodall, G. J., *et al.* (2011). Induction of miR-21 by retinoic acid in estrogen receptor-positive breast carcinoma cells. *J. Biol. Chem.* **286,** 4027–4042.

Thackaberry, E. A., Jiang, Z., Johnson, C. D., Ramos, K. S., and Walker, M. K. (2005). Toxicogenomic profile of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the murine fetal heart: Modulation of cell cycle and extracellular matrix genes. *Toxicol. Sci.* **88,** 231–241.

Tilghman, S. L., Bratton, M. R., Segar, H. C., Martin, E. C., Rhodes, L. V., Li, M., McLachlan, J. A., Wiese, T. E., Nephew, K. P., and Burow, M. E. (2012). Endocrine disruptor regulation of microRNA expression in breast carcinoma cells. *PLoS ONE* **7,** e32754.

UN FAO. (2005). Pesticides Residue in Food: Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues Geneva, Switzerland, 20–29 September 2005, pp. 125. World Health Organization, Rome.

Vandenberg, L. N., Colborn, T., Hayes, T. B., Heindel, J. J., Jacobs, D. R., Lee, D.-H., Shioda, T., Soto, A. M., vom Saal, F. S., Welshons, W. V., *et al.* (2012). Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. *Endocr. Rev.* **33,** 378–455.

Waltering, K. K., Porkka, K. P., Jalava, S. E., Urbanucci, A., Kohonen, P. J., Latonen, L. M., Kallioniemi, O. P., Jenster, G., and Visakorpi, T. (2011). Androgen regulation of micro-RNAs in prostate cancer. *Prostate* **71,** 604–614.

Wang, H., Tan, G., Dong, L., Cheng, L., Li, K., Wang, Z., and Luo, H. (2012). Circulating MiR-125b as a marker predicting chemoresistance in breast cancer. *PLoS ONE* **7,** e34210.

Watson, C. S., and Lange, C. A. (2005). Steadying the boat: Integrating mechanisms of membrane and nuclear-steroid-receptor signalling. *EMBO Rep.* **6,** 116–119.

Weyandt, J., Ellsworth, R. E., Hooke, J. A., Shriver, C. D., and Ellsworth, D. L. (2008). Environmental chemicals and breast cancer risk–a structural chemistry perspective. *Curr. Med. Chem.* **15,** 2680–2701.

Wickramasinghe, N. S., Manavalan, T. T., Dougherty, S. M., Riggs, K. A., Li, Y., and Klinge, C. M. (2009). Estradiol downregulates miR-21 expression and increases miR-21 target gene expression in MCF-7 breast cancer cells. *Nucleic Acids Res.* **37,** 2584–2595.

Wiseman, R. A. (2004). Breast cancer: Critical data analysis concludes that estrogens are not the cause, however lifestyle changes can alter risk rapidly. *J. Clin. Epidemiol.* **57,** 766–772.

World Health Organization. (2006). Pesticide Residues in Food 2004: Evaluations 2004: Part II - Toxicological, pp. 461. WHO, Rome, Italy.

Yan, L. X., Huang, X. F., Shao, Q., Huang, M. Y., Deng, L., Wu, Q. L., Zeng, Y. X., and Shao, J. Y. (2008). MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. *RNA* **14,** 2348–2360.