Widespread Estrogen-Dependent Repression of microRNAs Involved in Breast Tumor Cell Growth

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Abstract
Altered expression of microRNAs (miRNA), an abundant class of small nonprotein-coding RNAs that mostly function as negative regulators of protein-coding gene expression, is common in cancer. Here, we analyze the regulation of miRNA expression in response to estrogen, a steroid hormone that is involved in the development and progression of breast carcinomas and that is acting via the estrogen receptors (ER) transcription factors. We set out to thoroughly describe miRNA expression, by using miRNA microarrays and real-time reverse transcription-PCR (RT-PCR) experiments, in various breast tumor cell lines in which estrogen signaling has been induced by 17β-estradiol (E2). We show that the expression of a broad set of miRNAs decreases following E2 treatment in an ER-dependent manner. We further show that enforced expression of several of the repressed miRNAs reduces E2-dependent cell growth, thus linking expression of specific miRNAs with estrogen-dependent cellular response. In addition, a transcriptome analysis revealed that the E2-repressed miR-26a and miR-181a regulate many genes associated with cell growth and proliferation, including the progesterone receptor gene, a key actor in estrogen signaling. Strikingly, miRNA expression is also regulated in breast cancers of women who had received antiestrogen neoadjuvant therapy. Overall, our data indicate that the extensive alterations in miRNA regulation upon estrogen signaling pathway play a key role in estrogen-dependent functions and highlight the utility of considering miRNA expression in the understanding of antiestrogen resistance of breast cancer. [Cancer Res 2009;69(21):8332–40]

Introduction
MicroRNAs (miRNA) are a class of small noncoding RNAs for which functions in regulating gene expression at the posttranscriptional level is now established in multicellular eukaryotes. These small (18–24 nucleotides in length) RNA molecules regulate numerous biological processes. The base-pairing interactions between miRNAs and their target mRNAs, often within the 3′ untranslated regions (UTR) of target genes, results in degradation and/or translation inhibition of the target mRNAs (1, 2). miRNA, generally transcribed by RNA polymerase II, are initially made as large RNA precursors, called pri-miRNAs (3). Transcription of miRNA-containing genes has been shown to be regulated in the same way as that of protein-coding genes through the modulation of the synthesis/activity of various transcription factors, e.g., c-Myc (4), cyclic AMP-response element binding protein (5), MyoD (6), or p53 (7–9).

In cancer, a large set of data shows that the expression of miRNAs is largely altered (10, 11). Specifically, several genome-wide miRNA expression studies have shown differential expression between normal tissue and breast tumors or between breast tumor subgroups (12–16). Of note, the differential expression of several miRNAs correlates with the status of estrogen receptors (ERs and ERβ; refs. 13, 15), which are members of the nuclear hormone receptor transcription factor family. This is particularly interesting because experimental, clinical, and epidemiologic data largely suggest that ERs, through their binding to the steroid hormone E2, contribute to the growth of many breast cancers. Furthermore, ERs is considered as one of the most valuable targets for endocrine-based therapies as it is expressed in 70% to 75% of early breast cancers (17–19). E2 binding to ERs leads to transcriptional regulation of genes involved in the control of cell growth. ERs exert many of their effect by interacting directly or through tethering to other transcription factors bound to DNA elements located in target gene promoters (20). However, the set of genes that directly mediate ERs effects on cell growth has not been fully defined.

Some recent reports have shown a link between E2 and/or ERs and miRNA expression. E2 treatment of human endometrial stromal cells, endometrial glandular epithelial cells, myometrium, or leiomyoma smooth muscle cells leads to altered expression of some miRNAs (21, 22). E2 also regulates miRNA expression in the adult zebrafish (23). Finally, two miRNAs (miR-206 and miR-21) have recently been identified as being downregulated by E2 in the MCF-7 breast tumor cell line (24, 25). However, a high throughput analysis of E2-regulated miRNAs in breast tumor cell lines is lacking and the role of miRNAs in estradiol-dependent functions is poorly documented.

Therefore, we sought to identify E2-regulated miRNAs. We showed that E2 stimulation of various breast/ovarian tumor cell lines resulted in a widespread repression of miRNA expression that was needed for E2-dependent in vitro cell growth. We also showed that the most potent miRNAs in repressing cell growth, miR-26a and miR-181a, regulated the gene encoding the progesterone receptor and many other genes associated with cell proliferation. Lastly, we found that the expression of several miRNAs was
regulated by antiestrogen treatment in breast tumors, highlighting the potential use of miRNAs as surrogate markers of response to antiestrogens.

Materials and Methods

Cell culture. Description of cell lines is provided in the Supplementary Materials and Methods. For E2 induction experiments, cells, plated at 20% to 30% confluency, were grown for 3 d in phenol red–free DMEM containing dextran-coated charcoal–treated serum. E2 (Sigma) treatments were at 10 nmol/L (E2 diluted in ethanol) for the period of time indicated in the text. IC182,780 (Sigma; 200 nmol/L) or tamoxifen (Sigma; 5 nmol/L) were added directly to the E2-containing medium. Control cells received equal amounts of vehicle ethanol. When indicated, cycloheximide or actinomycin D (both at 10 μg/mL) were added 1 h before E2 treatment.

miRNAs microarrays. miRNAs microarray analysis was performed as described by Castoldi and colleagues (26). More information can be found in the Supplementary Materials and Methods section.

Affymetrix Exon Array was performed as described in the Supplementary Materials and Methods.

Real-time reverse transcription-PCR quantification, transfection of miRNAs, cell proliferation, and [3H]-thymidine incorporation. See Supplementary Materials and Methods.

Patient selection, design of the clinical study, tissue collection, and immunohistochemistry. See Supplementary Materials and Methods.

Results

Identification of estrogen-regulated miRNAs. To identify estrogen-regulated miRNAs, we initially focused on the ERα-positive MCF-7 breast cancer cell line as it has been extensively used as a model of hormone-dependent breast cancer (e.g., refs. 27, 28). We used a sensitive array for miRNA expression profiling based on locked nucleic acids–modified capture probes (26). This allowed us to apply a straightforward RNA isolation procedure devoid of RNA size selection and/or amplification steps. Strikingly, the predominant consequence of E2 treatment was a widespread repression of miRNA expression. Indeed, we observed that 23 miRNAs (of 125 miRNAs whose expression is detectable on the array according to our criteria for inclusion; see Materials and Methods) were significantly (P ≤ 0.05) downregulated following E2 treatment (Table 1; Fig. 1A). No upregulated miRNA candidates were obtained following the same criteria. To confirm the expression changes detected by microarray analysis, real-time reverse transcription-PCR analysis was performed on eight candidate miRNAs (miR-181a, miR-21, miR-181b, miR-26a, miR-26b, miR-200c, miR-27b, miR-23b). The expression of these miRNAs decreased upon E2 treatment (Fig. 1B). To exclude the possibility that E2 treatment led to an artifactual change in miRNA recovery, we also analyzed the expression of two miRNA (miR-18a and miR-92) that were not regulated by E2 according to our microarray data. As expected, miR-18a and miR-92 did not change their expression upon E2 treatment (Fig. 1B).

As E2 treatment leads to an increase in cell proliferation, and because miRNA expression is regulated by cell density (29), we asked whether the observed effects might be linked to differential cell confluence in the E2-treated cells compared with the vehicle-treated cells. We observed no change in miR-26a expression at different confluence, demonstrating that the difference in the expression of miR-26a following E2 treatment is not linked to the state of confluence (Supplementary Fig. S1).

We next analyzed by real-time RT-PCR the expression of the above-mentioned eight E2-regulated miRNAs in several human ER-positive breast (T-47D, ZR-75-1, BT-474) or ovarian (BG1) cell lines. We found that, in all these cell lines, the expression of the analyzed miRNAs was downregulated by E2 treatment (Fig. 1C). Therefore, E2-dependent regulation is not restricted to the MCF-7 cell line. Interestingly, E2-dependent repression of the expression of tested miRNAs was absent in the ER-negative SK-BR-3 breast tumor cell line (Fig. 1C), suggesting that ER is involved in E2-dependent miRNA regulation.

Several pri-miRNAs are primary transcriptionally repressed targets of ER. We noticed that, in most cases, clustered miRNAs decreased their expression similarly (cluster miR-181a∼181b; miR-23b∼27b−24; miR-23a∼27a−24; miR-98∼let-7f-2; Table 1), suggesting a regulation at the transcriptional level. To determine whether estrogen-dependent transcriptional regulation of miRNA expression is mediated through ER, the ability of several ER antagonists to block the observed E2 repression was tested. We found that preincubation of MCF-7 cells with ICI 182,780 (ICI), a pure antagonist of ER genomic action, or with 4-hydroxytamoxifen blocked the E2-dependent decrease in the expression of the tested miRNAs (Supplementary Fig. S2; Fig. 2A).

The results presented up to now show that a 48-hour-long E2 treatment downregulates the expression of several miRNA-containing genes. We next asked whether a modulation in the abundance of miRNAs could also occur at earlier time points. Although no difference in the amount of the tested miRNAs was observed after

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NOTE: P value ≤ 0.05 for each miRNA listed. Clustered miRNAs are in silico information released from miRBase (miRNAs located at <10 kb of the E2-regulated miRNA are indicated); (chr) indicates the chromosome number.
6 hours of E2 treatment, a weak but reproducible effect was observed after 18 hours of treatment (Supplementary Fig. S3). This shows that the E2-dependent effects on the abundance of miRNAs is relatively late, starting after 18 hours of treatment, compared with the known E2-dependent immediate transcription targets that can be modulated after 3 hours of treatment.

Of note, the miRNA abundance is the result of (a) the transcription of the miRNA-containing gene as a pri-miRNA, (b) the processing of the transcribed pri-miRNA into the mature miRNA, and (c) the stability of the miRNA. It is therefore possible that the regulation of the transcriptional activity of miRNA-containing genes occurs at early time points but that the effect on miRNA abundance occurs at later time points due to inefficient processing of the pri-miRNA or an important stability of the mature miRNA. We therefore decided to evaluate E2-dependent regulation of pri-miRNA expression.

We focused on the two pri-miRNAs containing the most repressed miRNAs (miR-21 and miR-181a∼miR-181b). We found a 3- to 4-fold reduction in the expression of these pri-miRNAs upon a 3-hour-long E2 treatment (Fig. 2B). This early E2-dependent transcriptional repression was partially lost in the presence of ICI (Fig. 2B), showing the participation of ER in this effect. This repression was also lost upon treatment with the transcriptional inhibitor actinomycin D (Fig. 2C), showing that the effect is linked to transcriptional regulation. To next determine whether these pri-miRNAs were primary targets of ER signaling (i.e., genes regulated in the absence of de novo protein synthesis), the effect of the protein synthesis inhibitor cycloheximide on E2 repression was examined. Notably, pretreatment of MCF-7 cells with cycloheximide did not change the early E2-mediated transcriptional repression of pri-miR-21 and pri-miR-181a∼b-1 synthesis (Fig. 2C). However, cycloheximide impaired the E2-dependent regulation of pri-miR 17∼92, a known target of the E2-regulated c-Myc transcription factor (Fig. 2C; ref. 4). These results indicate that pri-miRNA-21 and pri-miRNA-181a∼b-1 are primary targets of ER transcriptional action, this notion being also supported by the presence of ERα binding.
sites located at <50 kb around the miR-21 (25) or miR-181a–b-1 sequences [as reported by Chip-chromatin immunoprecipitation experiments (30); Supplementary Fig. S4].

Specific E2-repressed miRNAs suppress estrogen-dependent cell proliferation. Because estrogen functions as a mitogen in ER-positive tumors, the observed downregulation of miRNA expression suggested a possible role for these miRNAs in cancer cell proliferation. We therefore investigated whether some E2-downregulated miRNAs were able to interfere with E2-dependent cell growth by analyzing the proliferation activity of MCF-7 cells after reintroduction of each of the candidate miRNA. E2-treated MCF-7 cells were transfected with either a control miRNA (miR-ctrl) or each of the eight candidate miRNAs. miR-181a and miR-26a strongly inhibited E2-dependent cell growth in a time-dependent manner, whereas MCF-7 cells transfected with miR-ctrl continued to grow during the period in response to E2 (Fig. 3A). miR-181b, miR-26b, miR-200c, miR-21, or miR-23b could also inhibit E2-dependent cell growth but to a lesser extent than miR-26a and miR-181a (Fig. 3A). Interestingly, miR-26a and miR-26b, but also miR-181a and miR-181b, differ from only three nucleotides in their sequence, highlighting the specificity of the effect. miR-27b had no effect on cell growth (Fig. 3A).

To further analyze these effects on MCF-7 cell growth, the level of DNA synthesis was measured after [3H]-thymidine incorporation. Introduction of miR-181a, miR-26a, or miR-200c, but not miR-ctrl or miR-27b, led to a strong decrease in DNA synthesis of E2-treated cells (Fig. 3B). Introduction of miR-181b, miR-26b, miR-21, or miR-23b led to a milder effect in reducing DNA synthesis of E2-treated MCF-7 cells (Fig. 3B). All together, these results show that among the eight tested E2-regulated miRNAs, seven of them are functionally involved, although to various extents, in E2-dependent cell growth.

We next extended our analysis to define the global changes in transcriptome induced by miR-26a, one of the most potent miRNA to inhibit proliferation in our study. We therefore decided not to look only for individual direct predicted mRNA targets of miR-26a but to analyze global changes in the transcriptome of the various treated cells. Thus, we transcriptionally profiled MCF-7 cells grown in the presence of E2 and transfected or not with miR-26a. We arrived at a set of 503 genes for which expression...
is regulated (>1.2-fold; \( P < 0.05 \)) by miR-26a (Supplementary Table S2; Fig. 4A). Interestingly, among the miR-26a–regulated genes, 178 (35%) genes corresponded to genes related to "cellular growth and proliferation" as determined using Ingenuity Pathways Analysis software (Supplementary Table S3).

We also described 343 genes for which expression was regulated in E2-treated compared with vehicle-treated MCF-7 cells (Supplementary Table S4; Fig. 4A). The overlap between this list and the list of miR-26a–regulated genes yielded a set of 78 genes (Supplementary Table S5; Fig. 4A). We found only 5 genes (among the set of 78) that contain \textit{in silico} predicted miR-26a binding sites (Supplementary Fig. S5).

We focused on the \textit{PGR} (progesterone receptor) gene for several reasons. First, PGR isoforms (-A and -B) are, as such as ERs, members of the steroid hormone receptor family of transcription factors and are key mediators of estrogen signaling (31). Second, we noticed that the extremely long (>9 kpb) PGR 3'UTR had very few conserved predicted miRNA binding sites (miRNA response elements; Fig. 4B). Moreover, six miRNA (miR-26a, miR-26b, miR-181a, miR-181b, miR-23a, miR-23b), of the seven miRNAs predicted to bind to
the PGR 3′UTR, were actually found to be repressed by E2 in our study (Table 1) including two miRNAs (miR-26a and miR-181a) that strongly inhibited cell growth (Fig. 3).

We therefore analyzed the expression of PGR in MCF-7 cells grown in the presence of E2 and transfected with miR-26a or miR-181a. We found that both miRNAs inhibited the expression of PGR at the RNA (Fig. 4C) and protein level (Fig. 4D). Conversely, anti-miRs against miR-26a and miR-181a derepressed PGR expression (Supplementary Fig. S6). Furthermore, the predicted miRNA response elements are critical for the direct and specific binding of miR-26a or miR-181a to the PGR mRNA, as judged by luciferase reporter assays (Supplementary Fig. S7). Overall, this set of results argues for the involvement of specific miRNAs in suppressing E2-dependent cell proliferation through their ability to regulate genes involved in the control of cell growth, such as the one encoding the progesterone receptor isoforms.

**Regulation of miRNA expression by antiestrogen in breast cancer.** To investigate the regulation of miRNAs expression by antiestrogen in human breast tumor specimens, we used tumors from patients participating to a phase II pilot study. Fifteen postmenopausal women with ER-positive breast tumors (Supplementary Table S6) received, as a neoadjuvant therapy, a combination of exemestane (an inhibitor of CYP19 aromatases, enzymes catalyzing the last steps of estrogen biosynthesis) and tamoxifen during 4 months, daily before surgery. As expected, a decrease in the expression of biological markers involved in the estrogenic transduction pathway (ER, PGR), and in proliferation (Ki-67 and cyclin D1) was generally observed after treatment (Supplementary Table S7; Fig. 5, top). An example of the histopathologic response as well as cyclin D1 expression for one among the responsive tumors (patient 7) is shown in Fig. 5.

We isolated RNAs from the initial breast tumor biopsy and from the surgery specimen for 10 patients and analyzed the expression of several miRNAs by real-time RT-PCR. The expression of miR-92, which is not regulated by E2 (Fig. 1A), was unchanged upon treatment (Fig. 5, bottom). The expression of most of the candidate miRNAs (miR-21, miR-181b, miR-26a, miR-26b, miR-27b, miR-23b), except for miR-181a and miR-200c, was higher after treatment (Fig. 5, bottom), showing regulation of miRNA expression by tamoxifen combined with exemestane in breast cancer.

**Discussion**

Multiple lines of evidence provided here argue for the involvement of specific miRNAs in E2-dependent cellular response. A set of 23 miRNAs (including miR-181a, miR-21, miR-181b, miR-26a, miR-200c, miR-26b, miR-27b, miR-23b for which a more detailed study was carried out) are downregulated by E2 in a number of ER-containing human cell lines. The RNA precursors of miRNAs (i.e., pri-miR-21 and pri-miR-181a–b-1) are primary immediate transcriptionally repressed targets of ER. The expression of most of these miRNAs is upregulated by antiestrogen treatment in breast tumors. Several miRNAs, the most potent being miR-26a and miR-181a, counteract the E2-dependent increase in cell proliferation, through a global deregulation of genes (e.g., progesterone receptor) involved in the control of cell growth.

**Figure 4.** Regulation of gene expression by E2-repressed miRNAs. A, the Venn diagram shows the number of genes differentially expressed in E2-treated MCF-7 transfected with miR-26a compared with E2-treated MCF-7 transfected with miR-ctrl (miR-26a-regulated), in E2-treated MCF-7 compared with vehicle-treated MCF-7 (E2-regulated) and in both. B, schematic representation of the PGR 3′ UTR and the conserved miRNA target sites. Base-pairing between miR-26a and miR-181a and the PGR mRNA is also indicated. C, real-time RT-PCR analysis of PGR mRNA expression in E2-treated MCF-7 cells transfected with the indicated miRNA molecules. The results are normalized to the β-actin mRNA expression. D, Western blotting analysis of the expression of both PGR isoforms (PGR-A and PGR-B) in E2-treated MCF-7 cells transfected with the indicated miRNA molecules.
miRNAs are thus uncovered as another class of molecules that modulate E2-dependent functions.

Recently, two miRNAs (miR-206 and miR-21) were identified as being downregulated by E2 in MCF-7 (24, 25). In our miRNA microarray analysis, we indeed found an E2-dependent repression of miR-21 expression (Supplementary Table S1; also see Table 1 and Fig. 1). However, we could not find any evidence of E2-dependent miR-206 regulation of expression. This may be consistent with the apparent lack of miR-206 expression in MCF-7 cells (32) as also discussed by Pandey and Picard (33). For miR-21, our results differ from that of Wickramasinghe and colleagues (25) concerning the time at which the repression was observed. The reason for that is unknown but maybe due to slightly different cell conditions (e.g., serum starvation times, cell confluences). It was also recently shown that E2 induced the expression of several miRNAs, including miR-21, in MCF-7p cells, a derivative of MCF-7 cells that contain a bicistronic vector (34).

The simplest way to explain this discrepancy is to consider the difference in the cell line used, because we and others (25) have used the parental MCF-7 cell line.

In any cases, our work shows that a full set of miRNAs are actually downregulated by E2 in MCF-7 cells. It also provides evidence for regulation of miR-21 and several other miRNAs by an antiestrogen treatment (tamoxifen combined with exemestane) in breast cancer.

For two miRNAs (miR-21 and miR-181a), we have shown an early transcriptional inhibition of the expression of the corresponding pri-miRNAs. Interestingly, according to Carroll and colleagues (30), several ER binding sites can be found at 50 kb around the miR-21 sequence (Supplementary Fig. S4; ref. 25). Furthermore, chromatin immunoprecipitation assays have confirmed the direct binding of ERα to the miR-21 promoter sequence (34). The position of these sites may account for ER action as correlation of ER binding sites with estrogen-regulated genes showed a bias of binding sites...
within 50 kb of the transcription start sites of these genes (30). Strikingly, the E2 effects on all the tested miRNAs were ER dependent and 80% of the E2-repressed miRNA contained one or more ER binding sites in a region of 50 kb surrounding the miRNA sequence (Supplementary Fig. S4). Therefore, the regulation of these miRNAs may follow the same kind of mechanisms. 

Noteworthy, global downregulation of miRNA expression is common in cancer (12) and contributes to tumorigenesis (35). This can be achieved through widespread transcriptional repression (36) or impaired miRNA processing (35). Avoidance of gene regulation by miRNAs can also occur through regulation of the presence of miRNA binding sites in miRNAs. In this context, it has been recently shown that globally shifting gene expression to shorter 3′UTR isoforms, devoid of target sequences for specific miRNAs, is a mechanism to control proliferation during lymphocyte activation (37). Together with our data, these findings suggest that gene expression regulation by miRNAs generally tends to suppress cell proliferation/transformation. 

It will now be important to more deeply describe how estrogen genes modulate gene expression through downregulation of miRNA expression. The E2-regulated miR-206 was shown to directly downregulate the expression of ERF (24, 38), although this conclusion was contradicted by Pandey and Picard (33). We have found that the E2-dependent downregulation of miR-26a and miR-181a leads to upregulation of PGR, possibly through their direct binding to the PGR 3′ UTR. This leads to the interesting notion that, to be efficiently expressed, a gene that is directly upregulated at the transcriptional level by E2 (such as PGR) also has to avoid miRNA-dependent posttranscriptional repression. This can be achieved by E2-dependent downregulation of miRNA expression. Therefore PGR, and possibly several other genes, may be regulated at two levels by E2: a direct transcriptional effect and an indirect posttranscriptional effect through E2-dependent repression of miRNA expression. Nevertheless, it is unlikely that PGR alone can explain the effects of miR-26a and miR-181a on cell growth. It will therefore be essential to define novel targets for these miRNA.

Our data on regulation of miRNA expression in breast tumors from postmenopausal patients with ER-positive breast cancer that were administered an antiestrogen neoadjuvant treatment for several months before surgery leads to several interesting perspectives. One of the main aims of neoadjuvant therapy is the validation of predictive factors of response, which could allow optimizing the adjuvant therapy and then hopefully increasing the disease-free and overall survival. In this study, we assessed the use of miRNAs as potential predictive biomarkers. We observed a marked increase in the expression of several miRNAs (miR-21, miR-181b, miR-26a, miR-26b, miR-27b, miR-23b) upon antiestrogen treatment. Interestingly, several of these miRNAs (miR-21, miR-23b, miR-181b) were shown as being differentially expressed in tamoxifen-resistant MCF-7 (39). Therefore, the analysis of the expression of these candidate miRNAs in larger cohorts may certainly warrant further investigation to help in the evaluation of the response to hormonal therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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