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The Micro-Ribonucleic Acid (miRNA) miR-206 Targets the Human Estrogen Receptor-α (ERα) and Represses ERα Messenger RNA and Protein Expression in Breast Cancer Cell Lines

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Micro-RNAs are small noncoding RNAs, which diminish the stability and/or translation of mRNAs. This study examined whether miR-206, previously shown to be elevated in estrogen receptor (ER)α-negative breast cancer, regulates the expression of ERα. Two putative miR-206 sites, (hERα1 and hERα2), were found *in silico* within the 3′-untranslated region of human ERα mRNA. Transfection of MCF-7 cells with pre-miR-206 or 2′-*O*-methyl antagomiR-206 specifically decreased or increased, respectively, ERα mRNA levels. Overexpression of pre-miR-206 reduced ERα and β-actin protein levels, with no effect on ERβ, E-cadherin, or glyceraldehyde-3-phosphate dehydrogenase. Reporter constructs containing the hERα1 or hERα2 binding sites inserted into the 3′-untranslated region of the luciferase mRNA conferred a 1.6- and 2.2-fold repression of luciferase activity, respectively, in HeLa cells. Both miR-206 sites responded accordingly to exogenous hsa-pre-miR-206 and 2′-*O*-methyl antagomiR-206, and both sites were rendered inactive by mutations that disrupted hybridization to the 5′-seed of miR-206. A C→T single nucleotide polymorphism in the hERα1 site increased repression of luciferase activity to approximately 3.3-fold in HeLa cells. MiR-206 levels were higher in ERα-negative MB-MDA-231 cells than ERα-positive MCF-7 cells, but only the ERα1 site mediated significantly more repression in reporter constructs. MiR-206 expression was strongly inhibited by ERα agonists, but not by an ERβ agonist or progesterone, indicating a mutually inhibitory feedback loop. These findings provide the first evidence for the posttranscriptional regulation of ERα by a micro-RNA in the context of breast cancer. (*Molecular Endocrinology* 21: 1132–1147, 2007)

THE STEROID HORMONE, 17β-estradiol (E_2), regulates numerous developmental and physiological processes in both sexes (1). In female reproductive organs, which grow and regress in a cyclical manner, E_2 exerts a robust mitogenic action. E_2 promotes, directly and/or indirectly, the proliferation of pituitary lactotropes, breast ductal epithelium, uterine endometrial epithelia, and ovarian follicular cells. Moreover, this mitogenic action of E_2 underlies the ability of the hormone to promote the development and/or progression of cancers of the female reproductive tract, including uterine and breast cancer. This is emphasized by the finding that unopposed estrogen treatments in postmenopausal women severely increase the risk of endometrial hyperplasia and carcinoma (2, 3).

The results of animal studies, including mouse estrogen receptor (ER)α (αERKO) and ERβ (βERKO) knockouts, indicate that the proliferative effects of estrogen are mediated primarily by ERα (4, 5). For example, αERKO mice, but not βERKO mice, show extremely stunted mammary ductal growth. In this respect, ERα acts as a ligand-activated oncogene product in reproductive tissues and indeed the overexpression of ERα correlates with a higher risk for breast and endometrial cancers (6, 7). As such, it is not surprising that ERα expression and activity are tightly regulated by numerous mechanisms. Expression of the human ERα gene (ESR1) is controlled at the transcriptional level by seven different promoters used in a cell-specific manner (8). Furthermore, this complex transcriptional unit can undergo alternative splicing, which has been shown to generate shorter ERα isoforms (9). The ERα protein is also subjected to posttranslational regulation, including phosphorylation, acetylation, sumoylation, and polyubiquitination by HECT domain-containing E3 ligases (*e.g.* E6-AP, Rsp5/Rpf1) (10–15). Posttranslational regulation of ERα activity also involves ERα-protein interactions with calmodulin, cyclin

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Abbreviations: Ago2, Argonaute2; CMV, cytomegalovirus; DPN, diarylproprionitrile; E_2, 17β-estradiol; ER, estrogen receptor; FBS, fetal bovine serum; β-gal, β-galactosidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; miRNA, microRNA; nt, nucleotide; PPT, propyl pyrazole triol; qRT-PCR, quantitative RT-PCR; RT, reverse transcriptase; SNP, single nucleotide polymorphism; UTR, untranslated region.

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D1, BRCA1, metastasis-associated protein-2, transcriptional factors (*e.g*. c-*fos*/c-*jun* heterodimers), and coregulatory proteins (*e.g*. steroid receptor coregulator 1, cAMP response element-binding protein-binding protein/p300) (16–18).

A hallmark of most human breast cancers is the dysregulation of ERα expression. Approximately 70% of diagnosed primary breast cancers overexpress ERα, which can have profound implications for the treatment and prognosis of breast cancer. ERα-positive ($ER\alpha^+$) breast cancer cells are dependent on E_2 for their survival and progression (19). Consequently, $ER\alpha^+$ breast cancers respond favorably to adjuvant therapy with selective estrogen modulators (20), which act as ERα antagonists in breast tissue. Additionally, inhibitors of CYP19-aromatase are effective adjuvant drugs for the treatment of $ER\alpha^+$ breast cancers in postmenopausal women (21).

In contrast, about one-third of breast cancer patients are diagnosed with a form of cancer that does not express ERα. These ERα-negative ($ER\alpha^-$) breast cancer cells are typically more transformed and undergo proliferation independently of estrogenic stimulation (7, 19). Thus, the inability to use established endocrine/hormonal therapies mentioned above limits the treatment options for these patients. Multiple mechanisms involved in the silencing of ERα in these breast tumor types have been identified, including mutations within the open reading frame of the ESR1 gene (9), as well as the loss of ERα expression through transcriptional silencing by promoter-proximal DNA methylation at CpG islands in the ESR1 gene (7, 22–24). However, given the complex regulation of ERα in normal cells (see above), it is likely that other mechanisms lead to or reinforce the loss of ERα in some cancer cells. A better understanding of these mechanisms might uncover causal links between the loss of ERα and the gain of transforming signaling pathways, as well as provide insight into the physiological regulation of ERα density among tissues in the face of changing reproductive states.

One mechanism for the posttranscriptional silencing of gene expression is the targeting of mRNAs by one or more specific micro-RNAs (miRNAs). miRNAs are a novel class of regulatory molecules that have been shown to control gene expression at the posttranscriptional level (25). The genes for these small noncoding RNAs, positioned along intragenic and intergenic regions within the human genome, are transcribed by RNA polymerase II and in some cases RNA polymerase III (26–28). The primary miRNAs are then processed by a complex of both nuclear and cytosolic RNase III enzymes to form mature miRNAs, which are approximately 19–24 nucleotides in length (29–31). The RNaseIII-like nuclease Dicer, along with other RNA-binding/modifying proteins, engineers the dissociation of the two strands and the selection of the mature (guide) miRNA strand (32–34). The single-stranded miRNA becomes incorporated into a ribonucleoprotein complex, the core component of which is the Argonaute protein family (35–37), and forms imprecise hybrids with the 3′-untranslated region (3′-UTR) of a particular target mRNA. The transcript is then directed toward a process of mRNA cleavage or alternatively undergoes translational inhibition (38–41).

Dysregulation of miRNA expression might occur through either a genetic or epigenetic alteration of a miRNA gene, or by the presence of single-nucleotide polymorphisms (SNPs) that reside within the 3′-UTR of miRNA target mRNAs (42, 43). Dysregulation of miRNA expression is currently under intense investigation, which has revealed that miRNAs can act as oncogenes (so-called "oncomiRs") or as tumor-suppressors (25). For instance, *let-7* functions as a tumor suppressor in the lungs, because its down-regulation in lung carcinoma allows for increased expression of the Ras protein (44). In some cancers, tumor suppressor miRNAs are completely absent due to gene deletion. The miRNA cluster harboring miR-15a and miR-16–1 at chromosome 13q14 exists within a deleted region associated with chronic lymphocytic leukemia (45). These two miRNAs target and repress the antiapoptotic BCL-2 transcript, thereby allowing for unchecked survival/proliferation of lymphocytes. Conversely, some miRNAs are up-regulated in cancer and appear to act as oncogenes. The miRNAs, miR-221, miR-222, and miR-146, are up-regulated in papillary thyroid carcinomas (46). These miRNAs target c-*kit*, which may play a selective role in the pathogenesis of this type of cancer.

The evidence for miRNA regulation of ERα in human breast cancer is limited. A key study by Kenealy *et al.* (47) determined that segments of the hERα 3′-UTR lead to the destabilization of ERα, potentially through interactions with certain regulatory elements. These regions within the extremely long hERα 3′-UTR are likely to be miRNA binding sites, which would provide a novel mechanism for the posttranscriptional regulation of ERα. Microarray studies on miRNA levels in various human breast cancer tissues have revealed that some miRNAs are up-regulated in breast cancer *vs.* normal breast tissue, and a smaller cohort of miRNAs are up-regulated in $ER\alpha^-$ *vs.* $ER\alpha^+$ tumors (48). One miRNA observed to be up-regulated in $ER\alpha^-$ breast cancer was miR-206. In the current study, we demonstrate that miR-206 decreases endogenous ERα mRNA and protein levels in human MCF-7 breast cancer cells by acting through two specific miR-206 target sites within the 3′-UTR of the human ERα transcript. These findings provide the first evidence of a specific miRNA involved in the regulation of the human ERα in breast cancer cell lines.

RESULTS

The ERα 3′-UTR Contains Two Potential miR-206 Target Sites via *in Silico* Analysis

To determine both putative miR-206 (Fig. 1A) and other miRNA target sites within the 3′-UTR of ERα, *in silico* analysis was performed using RNA hybrid (49)

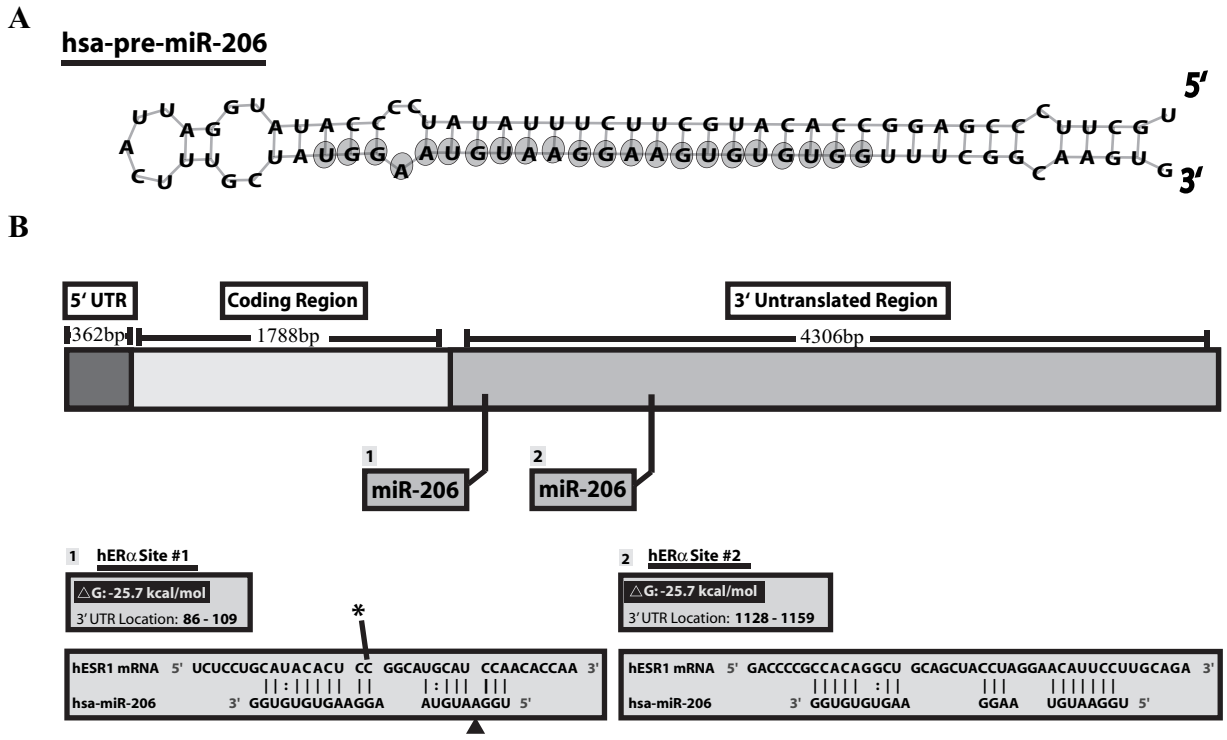

Fig. 1. Discovery of Two Potential miR-206 Target Sites within the 3′-UTR of ERα

A, Mature miR-206 is processed from pre-miR-206 through Dicer and targets the ERα mRNA. The pre-miR-206 sequence, determined by RNAFold (81), is shown here. B, Scaled diagram representing the two putative target sites for miR-206 in the 3′-UTR of the hERα transcript, including the RNA hybrid free energy calculations and the theoretical miRNA-mRNA duplex pairing. Specific locations of the binding sites are calculated from the beginning of the 3′-UTR. ▲, Position of T residue insertion in pIS-ERα1-Tins constructs; *, location of a C→U nucleotide switch in the pIS-ERα1-SNP construct; :, locations of G/U wobbles which destabilize miRNA-mRNA interactions.

and miRanda (50). Because ERα has an extremely long 3′-UTR of 4.3 kb, an attempt was made to verify and constrain our results for the most favorable miRNA binding locations. Discovery of miRNA target sites via miRanda are determined by the degree of complementarity of a sequence to the guide strand of a specific miRNA, and whether this sequence resides within a conserved region. The RNA hybrid program uses a free energy (ΔG) algorithm to calculate favorable binding interactions between a miRNA and potential sites within the mRNA of interest.

The analysis identified 65 potential miRNA target sites with a ΔG of not more than −25 kcal/mol, that tend to cluster in isolated regions of the 3′-UTR of ERα. These 65 sites were selected to contain fewer than three potential hairpin formations and have minimal G:U basepairing. Importantly, this *in silico* data revealed the existence of two putative miR-206 target sites within the 3′-UTR of ERα (Fig. 1B). Putative target sites for miR-185 and miR-212, two other miRNAs found to be up-regulated in ERα-negative breast cancer (48), were not identified in our analysis of the ERα 3′-UTR. Both miR-206 sites have a similar free energy of −25.7 kcal/mol. The first site (hERα1) is approximately 95% conserved from human to rat, whereas the second site (hERα2) was found only in humans and was determined through RNA hybrid.

MiR-206 Regulates Endogenous ERα mRNA and Protein Expression in Breast Cancer Cells

Regulation by miRNAs of protein translation can be due to translational repression and/or mRNA degradation. To assess whether miR-206 has a functional role in the down-regulation of endogenous ERα expression by either of these mechanisms, MCF-7 cells were transfected with synthetic miR-206-specific RNAs, and 24 h after transfection the cells were assayed for ERα mRNA expression (Fig. 2).

Transfection with various concentrations of pre-miR-206, a synthetic RNA duplex that mimics endogenous miR-206, induces a dose- and time-dependent repression of ERα mRNA levels (Fig. 2A; and Fig. S1 published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). ERα was repressed by approximately 2.5-fold with 100 nM of hsa-pre-miR-206 and was specific because no changes were noted in RPL-19 mRNA levels. Synthetic pre-miR-neg (a scrambled miRNA sequence) did not alter ERα, RPL-

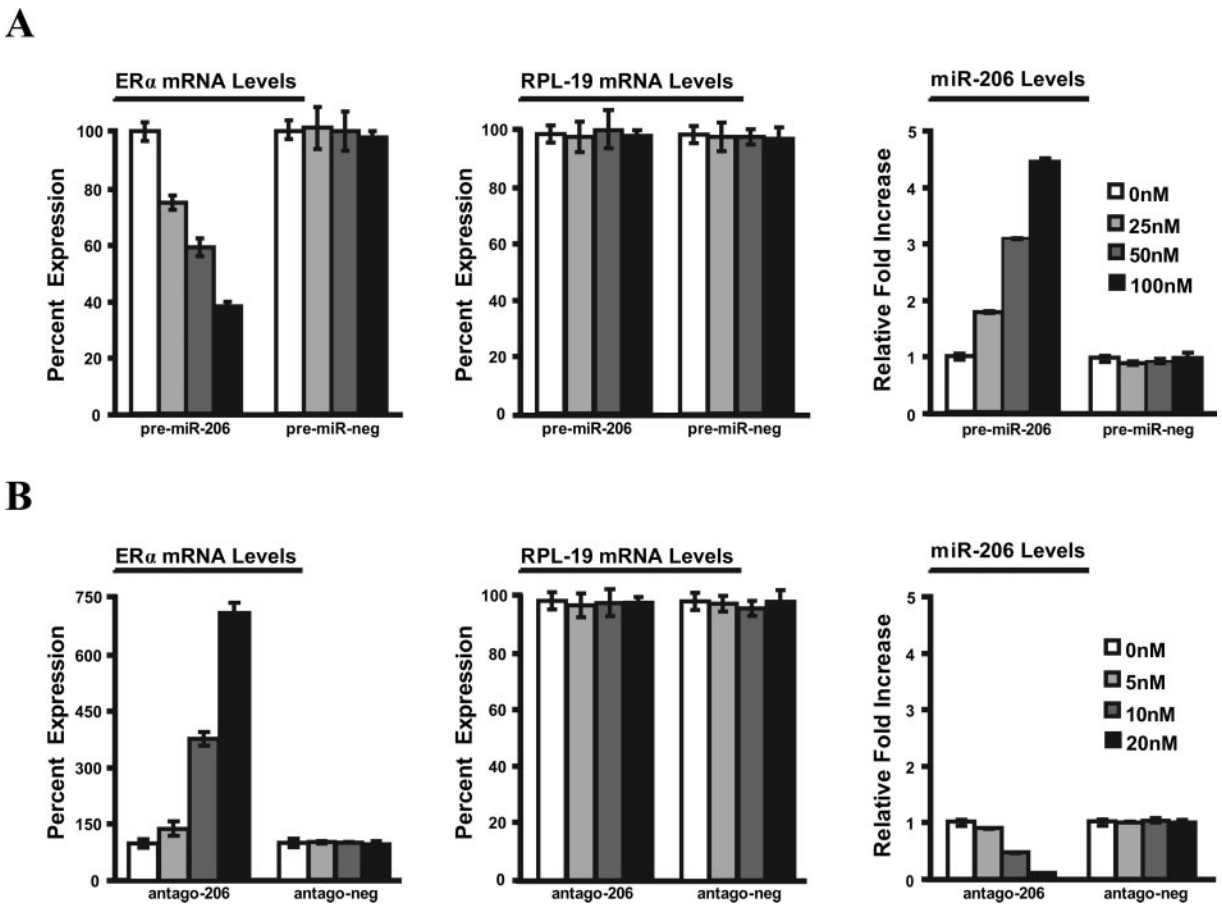

Fig. 2. miR-206-Specific RNAs Regulate Endogenous ERα Expression in MCF-7 Cells

A, MCF-7 cells were treated with 0–100 nM of either hsa-pre-miR-206 or hsa-pre-miR-neg (scrambled sequence) (panel A) or 0–20 nM of 2′-*O*-methyl antagomiR-206 (panel B) for 24 h, and total RNA was harvested for subsequent qRT-PCR analysis of ERα, RPL-19, or miR-206 levels. Values for miR-206 were normalized to 5s and reported relative to untreated samples, whereas ERα mRNAs are depicted as percent expression. Both experiments (A and B) are reported as the average of three independent experiments with five replicates per experiment ± SEM. Also, a scrambled RNA sequence (pre-miR-neg) had no significant effect on any RNAs assayed.

19, or miR-206 RNA levels. To further validate these results, MCF-7 cells were treated with 2′-*O*-methyl antagomiRs, which are single-stranded modified RNAs that have a complimentary sequence to mature miRNAs and function by sequestering/degrading endogenous miRNAs (51, 52). When 20 nM antagomiR-206 was transfected into MCF-7 cells, ERα mRNA increased as much as 7.5-fold (Fig. 2B). These results indicate that miR-206 regulates endogenous ERα mRNA levels in MCF-7 cells, probably through a mechanism of mRNA degradation/cleavage.

The effects of miR-206 on ERα protein levels in MCF-7 cells were examined by constructing a miR-206 expression construct (PS-206). The pre-miR-206 along with 38 bp of endogenous flanking sequence was inserted downstream of the cytomegalovirus (CMV) promoter in the p*Silencer* 4.1-CMV neo vector (Fig. 3A). Transfection of 5 μg PS-206 into duplicate dishes of MCF-7 cells for 72 h increased miR-206 levels by approximately 50-fold, as assayed by real-time PCR and Northern blot analysis (Fig. 3B). Duplicate dishes of MCF-7 and T47D cells were then transfected with 5, 10, and 20 μg of PS-206 or PS-neg for 48 h (Fig. 3C). PS-206 induced a dose-dependent decrease in ERα, reducing it by approximately 70% at 20 μg of vector in both cell lines. This effect was highly specific, in that elevated miR-206 had no effect on ERβ, E-cadherin, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PS-neg construct, which expressed a small interfering RNA against GAPDH, effectively repressed GAPDH levels by about 50–75% after 48 h with the 20-μg treatment. These results demonstrate that miR-206 has the ability to reduce ERα expression in human breast cancer cells. PS-206 induced a dose-dependent decrease in non-muscle β-actin as well. Subsequent TargetScan analysis indicated that a potential miR-206 target site is located at position 223–255 in the 3′-UTR of β-actin. This finding is likely related to the role of miR-206 in striated muscle differentiation from myoblasts (see *Discussion*).

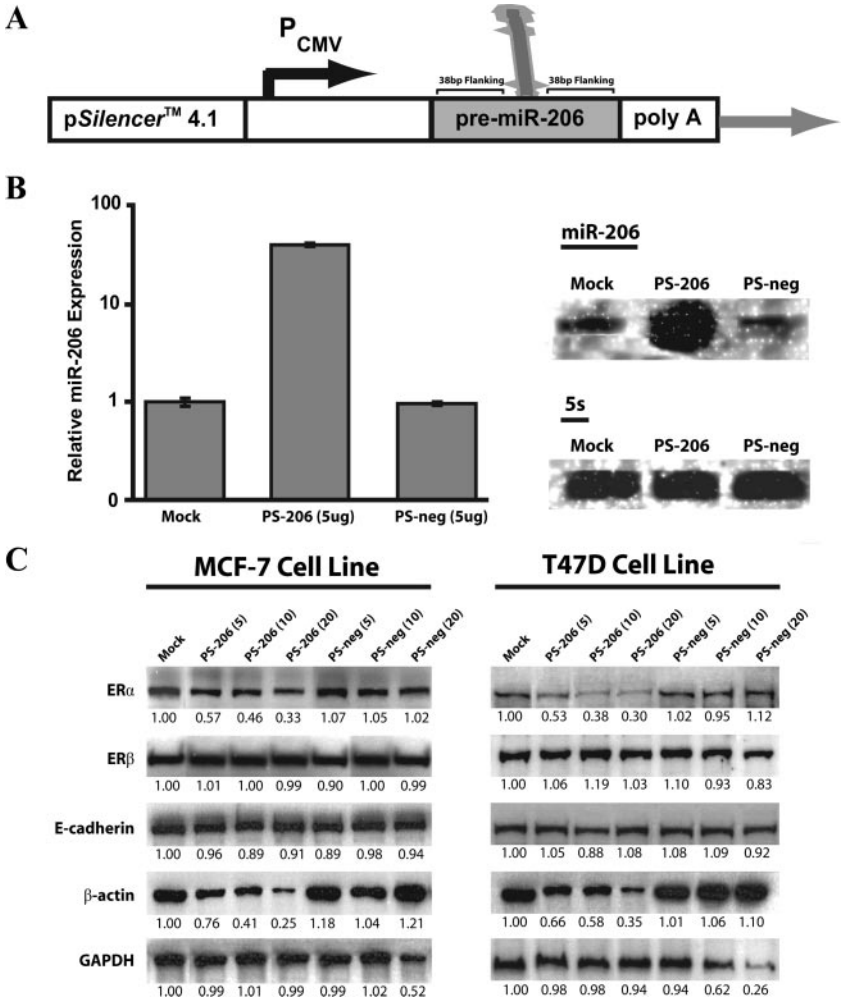

Fig. 3. Overexpression of miR-206 (PS-206) Knocked Down ERα Protein Expression

A, Diagram of the PS-206 overexpression construct made (see *Materials and Methods*). The endogenous miR-206 stem loop and flanking sequence was inserted into a vector that contains a CMV promoter. B, The PS-206 and PS-neg constructs, which produce a siRNA against GAPDH, were transfected in the MCF-7 cell line at 5 μg for 72 h. MiR-206 levels were assayed from triplicate samples from duplicate cultures by real-time PCR and Northern hybridization analysis. C, Effect of miR-206 overexpression on ERα protein levels. MCF-7 and T47D cell lines were transfected with a titration of 5–20 μg of PS-206 or PS-neg at 48 h. The *number below the band* represents the mean value from densitometry reading, relative to the mock transfection control, which was set at 1.00. Densitometry readings are reported as averages from triplicate samples of two independent experiments.

Validation of the Putative miR-206 Target Sites in the 3′-UTR of ERα mRNA in HeLa Cells

We next tested whether the regulation of ERα by miR-206 occurred through direct targeting of the ERα 3′-UTR. A luciferase reporter assay (53) was used to determine whether miR-206 could bind to the two putative miR-206 target sites (Fig. 1B) within the ERα 3′-UTR. In this assay, levels of luciferase activity correlated to translational repression and/or destabilization of the luciferase mRNA. Luciferase reporter constructs were made to contain either the miR-206 target site-1 (pIS-ERα1) or miR-206 target site-2 (pIS-ERα2) in the 3′-UTR of luciferase (Fig. 4A).

Transfection of either the pIS-ERα1 or pIS-ERα2 constructs in HeLa cells for 24 h conferred repression of luciferase activity as compared with the control (pIS-0) construct (Fig. 4B). The pIS-ERα1 and pIS-ERα2 constructs yielded a 1.6- and 2.2-fold repression in luciferase activity, respectively, as compared with pIS-0. In the miR-206 site-1, insertion of a T residue allowed for complementation with the fourth nucleotide of the miRNA (see *arrowhead* in Fig. 1B, conserved site). This modification is predicted to increase the stability of miRNA:mRNA hybrids within the critical "seed sequence." The T insertion raised the efficacy of this site to further repress luciferase activity by approximately 1.3-fold. Mutation of the 3′-ends of both miRNA target sites, (named "5′mut," because it disrupts hybridization to the 5′-seed sequence of miR-206), rendered the sites inactive as shown by the rescue of luciferase activity (Fig. 4C). In contrast, a

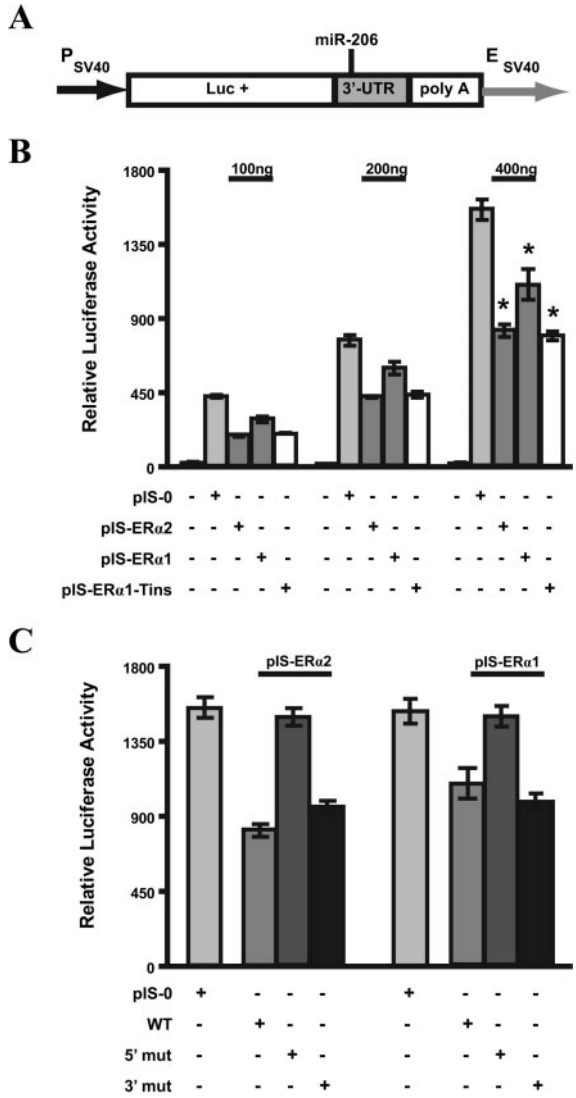

Fig. 4. Validation of miR-206 Target Sites via Luciferase Reporter System

A, Diagram depicting the pIS-0 luciferase reporter constructs, containing an SV140 promoter and enhancer element, which was used to verify the putative miR-206 binding sites (see *Materials and Methods*). B, HeLa cells were cotransfected with 100, 200, and 400 ng of pIS-ERα1 or pIS-ERα2 DNA, along with a β-gal reporter plasmid. Cells were also transfected with an ERα luciferase reporter containing a T insertion (see Fig. 1B), which would enhance the hybridization of the miR-206 seed sequence at the ERα1 site. After 24 h the luciferase activity was measured and normalized to β-gal. Results are reported as relative luciferase activity and as mean activity ± SEM from three independent transfection experiments with treatments performed in duplicate (*, $P < 0.05$ *vs.* mock pIS-0). C, Effects of 4-nt mutations in the pIS-ERα constructs (see Table 1) to abrogate binding of the 5′- and 3′-ends of miR-206 to the ERα 3′-UTR (noted as *5′mut* and *3′mut*, respectively). pIS-ERα wild-type and mutant constructs (400 ng) were transfected into HeLa cells for 24 h. Results and statistics are shown as above, and *P* values were determined from Student's *t* test. SV140, Simian virus; WT, wild type.

5′-mutation in the target sites (noted as "3′mut") does not significantly affect luciferase activity when compared with pIS-ERα1 and pIS-ERα2, which is expected. Collectively, these data indicate the presence of two specific miR-206 binding sites within the 3′-UTR of the ERα transcript.

To confirm that miR-206 is the specific miRNA repressing the activity of our constructs, we performed experiments in which the endogenous pool of miR-206 was altered in the HeLa cells. Transfection of pre-miR-206 further repressed luciferase activity in a dose-dependent manner in both ERα constructs (Fig. 5A). In contrast, pre-miR-206 had no effect on the pIS-0 and pIS-33 constructs [which does not contain a miRNA binding site or includes a nonspecific sequence containing a miR-19a binding site in phosphatase and tensin analog mRNA (53), respectively]. Conversely, 2′-*O*-methyl antagomiRs restored luciferase activity in both the pIS-ERα1 and pIS-ERα2 constructs by approximately 1.5-fold at the highest dose of 40 nM (Fig. 5B). Neither pre-miR-206 nor antagomiR-206 had an effect on the 5′-mutant constructs (pIS-ERα1–5′ and pIS-ERα2–5′) (Fig. 5C). A *let-7*-specific modified RNA was then used to determine whether miR-206 specifically targeted the predicted miR-206 sites within ERα. Whereas the *let-7* specific RNAs were confirmed as biologically active (data not shown), no effect was seen on either pIS-ERα1 or pIS-ERα2 (Fig. 5D). These results indicate that both miR-206 target sites identified in the human ERα 3′-UTR specifically interact with miR-206, which in turn represses the corresponding ERα mRNA and protein expression.

An Identified SNP in miR-206 Binding Site-1 Enhances miR-206 Repression in HeLa Cells

Further *in silico* analysis using the University of California Santa Cruz (UCSC) Genome Browser (54) identified a human C→T SNP within the miR-206 target site-1 of the ERα 3′-UTR (Fig. 6A and see *asterisk* in Fig. 1B). This C→T functional variant alters the positions of the compliment base pairing between miR-206 and the ERα 3′-UTR, such that nucleotides 10 and 11 of miR-206 would bind to the 3′-UTR as opposed to nucleotides 11 and 12. According to Elbashir *et al.* (55), one would predict that this shift would allow for enhanced Argonaute2 (Ago2)-mediated mRNA degradation through the intrinsic slicer actions of the Ago2 protein. To determine whether the functional variant affected the binding capability and the function of miR-206, luciferase constructs with the variant (pIS-ERα1-SNP) were transfected into HeLa cells as mentioned above. When compared with the pIS-0 and pIS-ERα1 constructs, reporter activity in the p-206-SNP constructs was significantly lowered by approximately 3.6- and 2.2-fold, respectively (Fig. 6B, *left panel*). The observed repression was further enhanced when pIS-ERα1-SNP-transfected cells were treated with 200 nM pre-miR-206 (186.42 ± 6.04 light units/μl; $P < 0.005$) as compared with the untreated

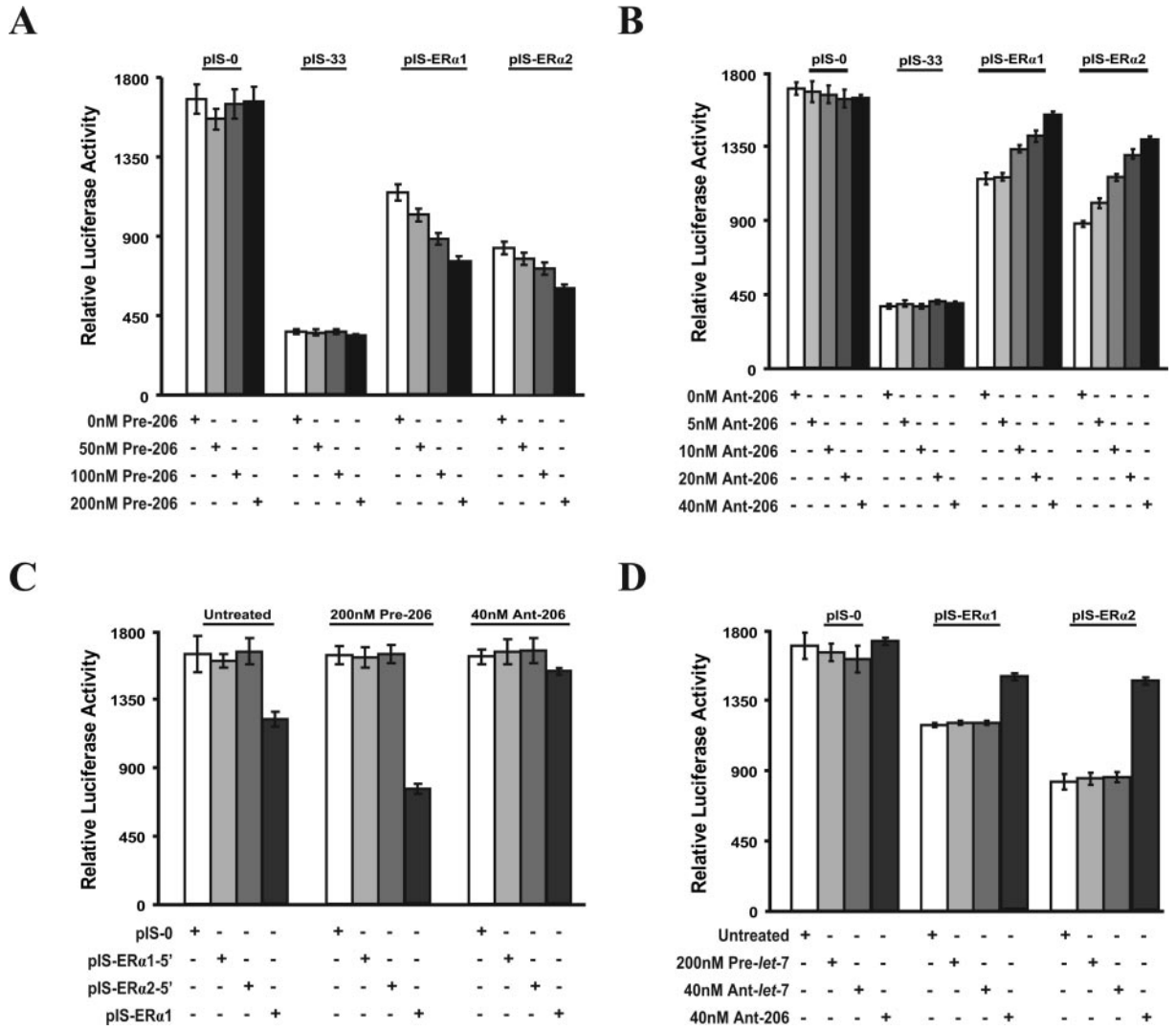

Fig. 5. Putative miR-206 Binding Sites Respond to Pre-miR-206 and AntagomiR-206

The ERα constructs (400 ng) were transfected into HeLa cells, treated with the indicated doses of pre-miR-206 (A), or antagomiR-206 (B) and assayed for luciferase activity 24 h after transfection. pIS-0 (empty vector) and pIS-33 [nonspecific sequence containing a miR-19a binding site in phosphatase and tensin analog (PTEN) mRNA (53)] constructs were used as controls. C, Effects of pre-miR-206 and antagomiR-206 on luciferase reporter constructs with pIS-ERα1 and pIS-ERα2 sites containing mutations to abrogate hybridization to the 5′-seed sequence of miR-206 and denoted here as pIS-ERα1–5′ and pIS-ERα2–5′, respectively. pIS-ERα1 is shown here as a positive control. D, Luciferase activity in the pIS-ERα constructs was not influenced when treated with pre-miR-*let*-7d and antagomiR-let7a-1.

pIS-ERα-SNP levels (440 ± 9.01 light units/μl) (Fig. 6B, *right panel*). As expected, partial recovery of luciferase reporter activity occurred after treatment with 40 nM miR-206-specific antagomiR (833.50 ± 20.57 light units/μl; $P < 0.005$). The above results indicate that the functional variant is able to increase miRNA repressive actions, probably through enhanced binding interaction between miR-206 and the ERα 3′-UTR.

The ERα miR-206 Binding Sites Confer Luciferase Repression in Breast Cancer Cells

The levels of miR-206 expression and activity were assayed in ERα$^+$ MCF-7 cells and ERα$^-$ MDA-MB-231 cells. Quantitative real-time and end-point PCR analysis revealed that miR-206 levels were significantly higher (1.8-fold ± 0.41; $P < 0.05$) in MDA-MB-231 cells than in MCF-7 cells (Fig. 7A). Both cell lines had high miR-206 activity, as determined by repression of luciferase activity, when transfected with either the pIS-ERα1, pIS-ERα2, or pIS-ERα1-SNP construct (Fig. 7B). However, only the pIS-ERα2 site displayed significantly greater repression in the MDA-231 cells *vs.* the MCF-7 cells (1.6-fold ± 0.34; $P < 0.05$). No change in activity was observed in the mutant construct (pIS-ERα1–5′) when compared with pIS-0. To show that the decrease in reporter activity was due to

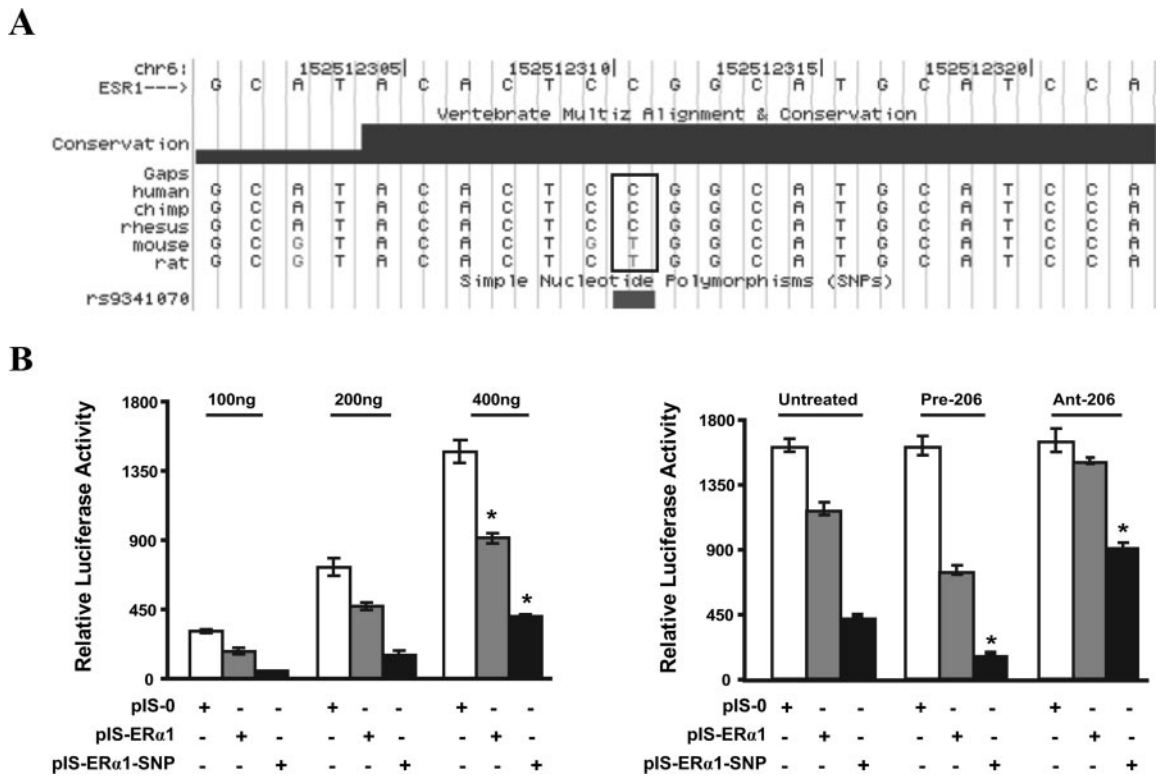

Fig. 6. An Identified C→T Functional Variant Enhances Repressive Qualities of miR-206

A, Using the UCSC Genome Browser, we discovered a SNP (rs9341070) within the ERα gene that correlates to the first miR-206 binding site in the ESR1 3′-UTR (see Fig. 1B). This C→T switch seems only to occur in nonprimate species, specifically in mouse and rat. B, To assess the function of the identified C→T SNP as a functional variant, a luciferase reporter construct denoted as pIS-ERα1-SNP was made, which harbored a T residue as opposed to the wild-type C residue. The indicated amounts of pIS-ERα1-SNP and pIS-ERα1 were transfected into HeLa cells and assayed after 24 h (*left panel*). Relative responsiveness of pIS-ERα1 and pIS-ERα1-SNP luciferase reporter constructs to 200 nM pre-miR-206 or 40 nM antagomiR-206 (*right panel*).

a loss of luciferase mRNA, MCF-7 cells were transfected with β-gal and various pIS-ERα1 constructs. RT-PCR was then employed with primers directed toward β-gal and luciferase mRNA (Fig. 7C). Luciferase mRNA levels were repressed in cells containing either the pIS-ERα1 or the pIS-ERα1-SNP constructs, which was subsequently rescued in cells treated with 40 nM antagomiR-206. It was observed that cells transfected with pIS-ERα1-SNP had the lowest levels of luciferase mRNA. The higher levels of miR-206 and its activity in the $ER\alpha^-$ MDA-231 cells, as compared with the $ER\alpha^+$ MCF-7 cells, raises the possibility that miR-206 could contribute to the loss of ERα expression in these cells.

E_2 Can Down-Regulate Endogenous miR-206 Levels in MCF-7 Cells

A separate, although related, question was whether or not miR-206 could be regulated by estrogen as part of a regulatory feedback mechanism. To test this hypothesis, MCF-7 cells were cultured in estrogen-depleted media for 48 h before treatment with various hormones (Fig. 8A). When the cells were treated with either 1 nM E_2 or 10 nM propyl pyrazole triol (PPT) (an ERα-selective agonist), miR-206 levels dropped 4- to 5-fold within a 24-h period. Interestingly, the 10 nM diarylproprionitrile (DPN) (an ERβ-selective agonist) treatment slightly enhanced miR-206 expression, whereas 1 nM progesterone had no effect. The luciferase assay was then used as a functional assay to confirm these results (Fig. 8B). MCF-7 cells were cultured in estrogen-depleted media for 48 h and transfected with pIS-0, pIS-ERα1, pIS-ERα1–5′, or pIS-ERα1-SNP luciferase constructs for 24 h with media containing 0, 0.1, or 1.0 nM E_2. When the luciferase expression was normalized to β-gal, all of the expression constructs had an enhanced luciferase activity with the increased concentrations of estrogen. However, when each construct was then compared with the relative pIS-0 values, both pIS-ERα1 and pIS-ERα1-SNP constructs had a higher expression of luciferase with increased doses of estrogen. The pIS-ERα1–5′-construct served as a negative control and was unresponsive to the estrogen treatments. These findings indicate that ERα-selective agonists decrease miR-206 levels and activity in MCF-7 cells.

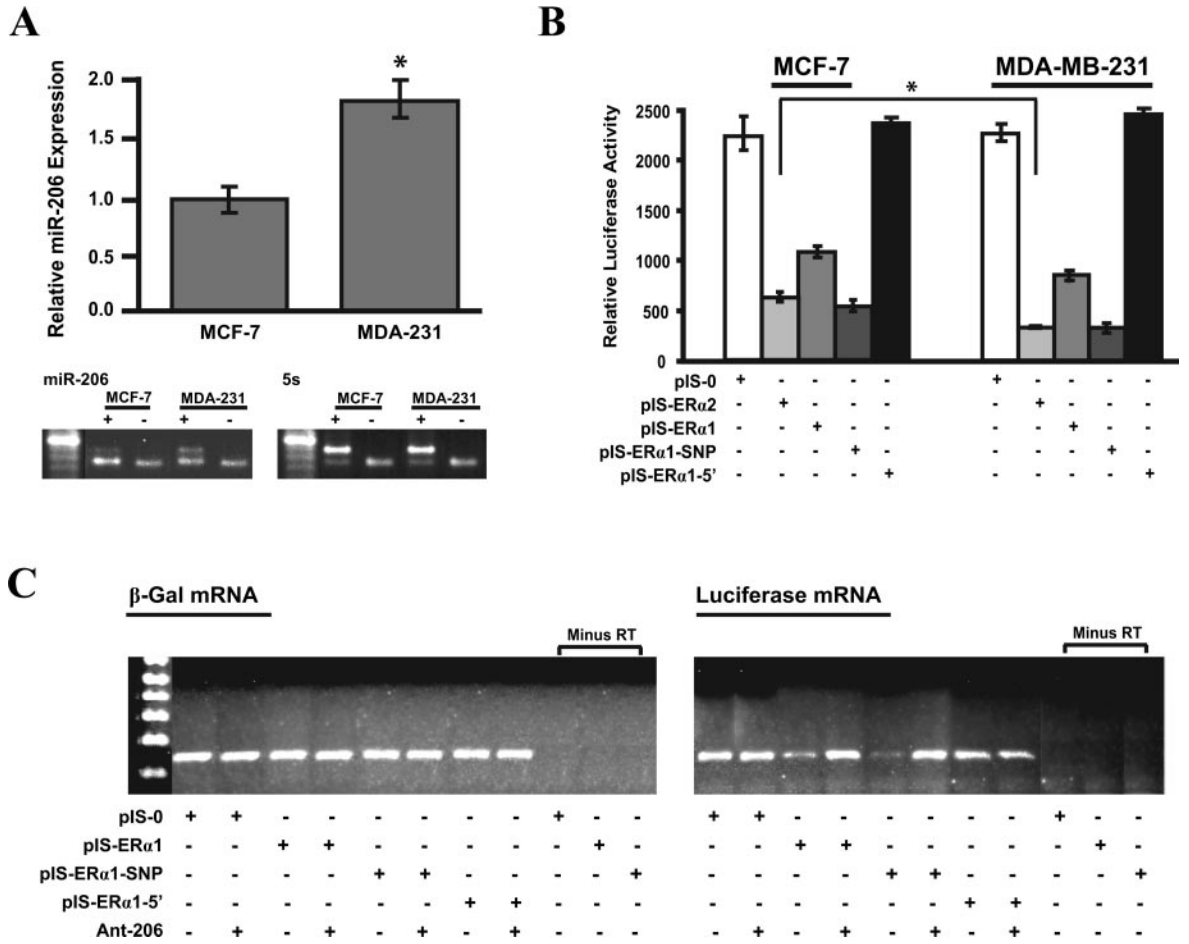

Fig. 7. Effects on Luciferase Reporter Activity and miR-206 Levels in Breast Cancer Cells

A, Relative levels of endogenous miR-206 in MCF-7, and MDA-MB-231 cells as assayed by real-time PCR in triplicate samples (*upper panel*). Levels were normalized to 5s RNA, and values presented as the mean ± SEM. Compared with the MCF-7 cell line, MDA-MB-231 cells contained significantly higher miR-206 expression (*, $P < 0.05$). End-point PCR for miR-206 and 5s was also performed to assay the endogenous miR-206 levels in the real-time assay as shown above (*lower panel*). The (+/−) symbols indicates positive or minus RT reactions and the lower bands common to both + and −RT lanes is indicative of primer-dimer. B, Effects of endogenous miR-206 levels in breast cancer cell lines on luciferase reporter activity (400 ng of pIS-ERα luciferase reporter construct was used in the transfections). MiR-206 activity was significantly higher in the MDA-MB-231 cells transfected with the pIS-ERα2 construct compared with the MCF-7 cells (*, $P < 0.05$). C, Effects of endogenous miR-206 on luciferase mRNA levels, as assayed by end-point RT-PCR. MCF-7 cells were transfected with 200 ng β-gal, 400 ng of the indicated luciferase construct, and 40 nM of antagomiR-206 for 24 h. The luciferase RNA was also assayed in a repeat experiment by qRT-PCR (data not shown), which confirmed the end-point data shown here.

DISCUSSION

The existence of miRNAs was discovered through genetic and molecular biological approaches in *Caenorhabditis elegans* (56, 57). Since those seminal studies, more than 1000 miRNAs have been identified in numerous species of plant and animals, 474 of which are currently within the human genome (Sanger Registry: http://microrna.sanger.ac.uk/sequences). MiRNAs have a direct role in regulating the processes of development and differentiation in many organisms, and miRNA dysregulation has been correlated with the progression and aggressiveness of several forms of cancer (44, 58). However, only a small percentage of the identified miRNAs have a specific physiological function. In this study, we focused on the possibility that miR-206 regulates ERα because it was one of three miRNAs with enhanced expression in ERα$^-$ *vs.* ERα$^+$ breast cancer, and for which *in silico* analysis revealed two sites within the 3′-UTR of the ERα transcript (48). We demonstrate herein that miR-206 represses endogenous ERα expression in both human MCF-7 and T47D breast cancer cells. This regulation is specific to ERα, because overexpression of miR-206 has no effect on the levels of ERβ, E-cadherin, or GAPDH. We also show that miR-206 regulates ERα mRNA through two specific miR-206 target sites harbored within the first 1200 bp of the 3′-UTR of the ERα transcript. These findings provide the first evidence of a specific miRNA that may contribute to the silencing of ERα in human breast cancer cells.

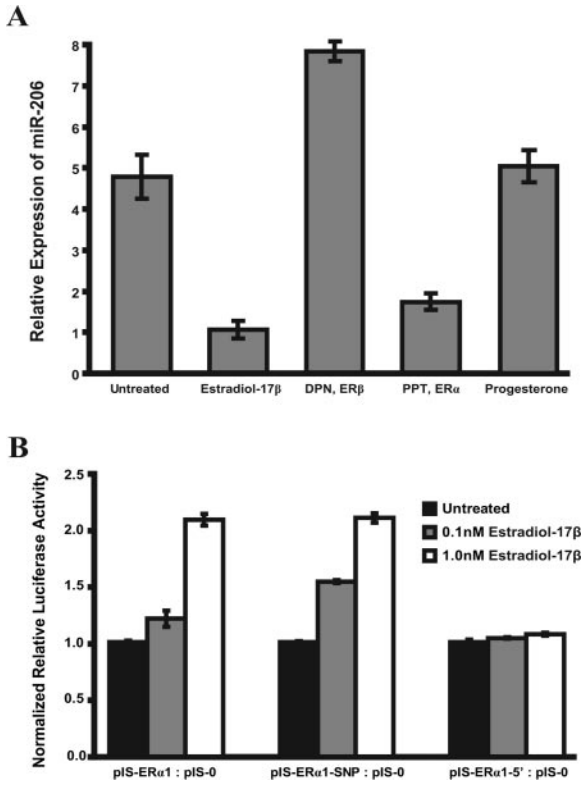

Fig. 8. Estrogen and Other ERα Agonists Repress miR-206 Expression in MCF-7 Cells

A, MCF-7 cells were cultured under normal conditions until 80% confluent and switched to estrogen-free conditions for 48 h. Cells were then treated with 1 nM E_2, 10 nM DPN (ERβ-selective agonist), 10 nM PPT (ERα-selective agonist), or 1 nM progesterone for 24 h. Cells were harvested for total RNA, and real-time PCR was performed to monitor miR-206 levels. All experiments were performed in replicates of five, with the mean ± SEM of three independent experiments shown. B, Luciferase assays were used as a bioassay to confirm that E_2 altered endogenous miR-206 activity. MCF-7 cells were cultured as noted above and transfected with 400 ng of the pIS-ERα1 constructs. After 4–5 h, media containing 0, 0.1, or 1 nM E_2 were added to the appropriate cells. After 24 h, cells were lysed and monitored for luciferase activity. Data were normalized to both β-gal expression and to pIS-0 values. This assay was performed three times, each with duplicate treatments and reported as the average ± SEM.

Recently, other receptors have been found to be regulated by miRNAs. These include the mammalian angiotensin II type I receptor, the *Drosophila melanogaster* DAF-16 insulin/IGF-1 receptor, and the two nuclear hormones receptors, *nhr-23* and *25*, in *C. elegans* (59–61). In addition to the regulation of receptors, miRNAs have been shown to regulate downstream effector proteins, including Sma- and Mad-related protein 3, Ras, and the coactivator protein, amplified in breast cancer 1 (AIB-1; also called steroid receptor coregulator 3) that interacts with ERα in the promotion of proliferation in breast cancer (44, 62, 63).

Previous functional studies on miR-206 have focused on its role in muscle differentiation, because tissue arrays have shown that miR-206 is highly abundant in skeletal and cardiac muscle across several species (64, 65). Induction of the C_2C_{12} mesenchymal stem cell line to form myotubes involves the increase in myogenic transcription factors, MyoD and myogenin, which in turn, bind to the putative promoter region of the miR-206 gene and stimulate miR-206 expression (66). MiR-206 directly decreases the expression of several mRNAs, including the p180 subunit of DNA polymerase α (Pola1), while indirectly decreasing antimyogenic factors such as Id1–3 and MyoR (64). Our findings also raise the possibility that miR-206 could be involved in the clearing of nonmuscle β/γ-actin during muscle development because only α-actin is present within mature muscle. This hypothesis is supported by our observation that overexpression of miR-206 led to a dramatic decrease in β-actin protein levels in MCF-7 levels, and that the TargetScan database found miR-206 sites in both β- and γ-actin.

When myogenesis was induced in the C2C12 cell line, other myogenic miRNAs, including miR-1 and miR-133, were also up-regulated (66, 67). It is worth noting that miR-1 has a sequence similar to miR-206 and is predicted to regulate some of the same target mRNAs. Although we did not examine miR-1 here because it was apparently not detected previously in breast tissue (48), miR-1 could potentially regulate ERα at the same sites as miR-206. It should be emphasized that we detected at least 65 putative miRNA target sites in the 3′-UTR of the ERα transcript. Thus, it is likely that multiple miRNAs regulate ERα expression in a combinatorial manner in different cell types. In support of this, our preliminary studies utilizing luciferase assays and antagomiRs, as previously described, indicate that the miRNA, *let-7d*, also directly regulates ERα mRNA (data not shown).

In seminal studies, the miRNAs, *let-7* and *lin-4*, were shown to function as negative regulators of gene expression by mediating translation repression of the target mRNAs, lin-14 and lin-41, respectively (38, 40, 68, 69). Recently it has been shown that miRNA/mRNA hybridization can also mediate mRNA cleavage through the slicer activity of Ago2, a protein within the RNA-induced silencing complex (36, 70, 71). In our experiments miR-206 reduced the presence of both the ERα mRNA and protein, indicating that Ago2 could induce the cleavage of miR-206-ERα mRNA hybrids. An interesting observation was that the C/T SNP could potentially modulate miR-206 binding to the conserved target site within the ERα 3′-UTR by shifting nucleotides 11/12 to 10/11 of the miRNA, selectively enhancing Ago2-induced slicing of the ERα message.

The abundance of miR-206 in skeletal muscle is well established, but the expression of miR-206 in other tissues is less studied. Human tissue screens failed to detect miR-206 in any female reproductive organs (64). In contrast, Iorio *et al.* (48) found in a panel of 29

differentially expressed miRNAs (normal *vs.* cancer) that miR-206 was the seventh most abundant in normal tissue and was further increased in breast cancer. These conflicting findings might be explained by the fact that miR-206 expression is under hormonal regulation, and thus miR-206 levels may vary according to a woman's endocrine status. We observed that the ERα-agonists, E_2 and PPT, strongly repressed miR-206 expression in MCF-7 cells, whereas the ERβ-specific agonist, DPN, and progesterone had no effect on miR-206 expression. Thus, miR-206 expression in the normal breast tissue may vary depending upon the time at which the sample was obtained during the menstrual cycle, and whether the donor was taking oral contraceptives, pregnant, or was pre- or postmenopausal.

Given the role of miR-206 in myogenic differentiation (64), and its ability to target a nuclear hormone receptor, ERα, which is vital for proliferative signaling in breast tissue, one would place miR-206 into the category of a tumor suppressor. In fact, we observed a decrease in cell proliferation as well as an increase in cell death when cells were treated with the PS-206 construct compared with those transfected with the PS-negative construct (data not shown). Given the apparent antiproliferative action of miR-206, it is somewhat counterintuitive that miR-206 expression is elevated in more transformed breast cancers (48). However, the role of miR-206 during the progression of breast cancer may shift from one as a classic tumor suppressor to one that contributes to the tumorigenic process known as "oncogenic addiction" (72). By this mechanism, cells progress to a cancerous state by altering gene expression through selective mutations allowing for increased growth and metastasis. It is likely that breast cells acquire genetic and epigenetic alterations that lead to diminished ERα expression. Presumably, those cells that have also acquired oncogenic mutations will be able to continue growth in the presence of attenuated ERα signaling. These cells would also be expected to have increased levels of miR-206, which would further inhibit expression of any residual ERα mRNA. In the face of other up-regulated oncogenic signaling pathways, this would allow the cell to undergo a proliferative switch from a stage tightly controlled by estrogen-dependent growth to a more transformed estrogen-independent status.

The C/T SNP (rs9341070) identified within the conserved hERα1 site1 appears to be an evolutionary remnant, in that the T residue is found in rodents, but has changed to a C in primates. Our data indicate that this SNP is a functional variant, in that the T residue at this position allows miR-206 to target and down-regulate the hERα1 site more effectively. One might predict that this polymorphism would result in lower levels of ERα expression, and thus, inhibit the viability and progression of estrogen-dependent cancer cells. Clearly, many factors contribute to different rates of a disease among different ethnic groups. However, it is noteworthy to mention that Hispanics and Utah residents with ancestry from northern and western Europe have a higher incidence of the C/T SNP, and lower rates of breast cancer compared with the general population, as determined from the HapMap, SNP500Cancer, and SEER databases (73–75).

The hormonal regulation of miRNAs has been barely studied but clearly has major implications for normal development and other physiological processes. The insect hormone, ecdysone, increases miR-100, miR-125, and *let-7*, and decreases miR-34, during metamorphosis in *Drosophila*, and juvenile hormone opposed the effects of ecdysone in insect cells *in vitro* (76). Our findings revealed the existence of an apparent feedback loop between E_2 and miR-206, with mutual repression between the two factors, which may contribute to the ability of estrogen-responsive tissues to alternate between mitotically quiescent and proliferative states. This loop is reminiscent of the mutually repressive interaction between the transcription factor, Yan, and miR-7 in *Drosophila* photoreceptors (77). In the absence of epidermal growth factor receptor signaling, Yan is expressed and represses miR-7 gene transcription in progenitor cells. Epidermal growth factor receptor signaling induces the rapid degradation of Yan, allowing another transcription factor, Pointed-P1, to compete for the miR-7 promoter and increase miR-7 expression. MiR-7 then binds to the 3′-UTR of Yan, to ensure stable down-regulation of Yan, which allows for photoreceptor differentiation. Another example of the mutual repression between a miRNA and a transcription factor involves the role of miR-223 in granulopoiesis (78).

The specific function of miR-206 in the normal breast tissue, if any, will require further study. MiR-206 could function in a mutually negative feedback loop to temporally regulate ERα expression and ductal/lobuloalveolar proliferation. Placental estrogen production is high during pregnancy and induces ductal growth and ductal branching within the breast (79). Estrogen-induced repression of miR-206 may facilitate ductal growth. In contrast, the abrupt loss of placental estrogens at parturition may allow miR-206 levels to rise sharply, thereby terminating growth and promoting a differentiated function (as it does in muscle). In the continued absence of estrogen during lactation, miR-206 may be one of several factors that inhibit ERα gene expression at the posttranscriptional level, leading to loss of both ERα mRNA and protein (79). Elevated miR-206 levels would also inhibit proliferation directly (*e.g*. Pola1 expression) and indirectly (*e.g*. ERα expression). Further characterization of the mechanism by which E_2 regulates miR-206 expression, as well as in what endocrine states and in what tissues E_2 regulates miR-206 *in vivo*, will undoubtedly allow for a more complete understanding of the role of miR-206 in mammary function and breast cancer.

Table 1. Sequences of Inserts Used in miR-206 Luciferase Reporter Constructs

Construct	Symbol	Primer Sets Used to Create Reporter Constructs	Length
miR-206 (Site #1)	pIS-ERα1	F: 5′ -- 3′ ***C***AGCCAAATTCTGTCTCCTGCATACACTCCGGCATGCATCCAACACCAATGGCTTTCTAGA***G*** R: 3′ -- 5′ ***TCGAG***TCGGTTTAAGACAGAGGACGTATGTGAGGCCGTACGTAGGTTGTGGTTACCGAAAGATCT***CGATC***	62mer 70mer
miR-206 (Site #1) SNP C/T	pIS-ERα1-SNP	F: 5′ -- 3′ ***C***AGCCAAATTCTGTCTCCTGCATACACTC**T**GGCATGCATCCAACACCAATGGCTTTCTAGA***G*** R: 3′ -- 5′ ***TCGAG***TCGGTTTAAGACAGAGGACGTATGTGAG**A**CCGTACGTAGGTTGTGGTTACCGAAAGATCT***CGATC***	62mer 70mer
miR-206 (Site #1) 5′ Mutant	pIS-ERα1-5′	F: 5′ -- 3′ ***C***AGCCAAATTCTGTCTCCTGCATACACTCCGGCATGC**CGAT**AACACCAATGGCTTTCTAGA***G*** R: 3′ -- 5′ ***TCGAG***TCGGTTTAAGACAGAGGACGTATGTGAGGCCGTACG**GCTA**TTGTGGTTACCGAAAGATCT***CGATC***	62mer 70mer
miR-206 (Site #1) 3′ Mutant	pIS-ERα1-3′	F: 5′ -- 3′ ***C***AGCCAAATTCTGTCTCCTG**GTCG**CACTCCGGCATGCATCCAACACCAATGGCTTTCTAGA***G*** R: 3′ -- 5′ ***TCGAG***TCGGTTTAAGACAGAGGAC**CAGC**GTGAGGCCGTACGTAGGTTGTGGTTACCGAAAGATCT***CGATC***	62mer 70mer
miR-206 (Site #1) T-insert	pIS-ERα1-Tins	F: 5′ -- 3′ ***C***AGCCAAATTCTGTCTCCTGCATACACTCCGGCATGCAT**T**CCAACACCAATGGCTTTCTAGA***G*** R: 3′ -- 5′ ***TCGAG***TCGGTTTAAGACAGAGGACGTATGTGAGGCCGTACGTA**A**GGTTGTGGTTACCGAAAGATCT***CGATC***	63mer 71mer
miR-206 (Site #2)	pIS-ERα2	F: 5′ -- 3′ ***C***GCTCAGGGTGGCCCTGCCACAGGCTGCAGCTACCTAGGAACATTCCTTGCAGACCCCGCA***G*** R: 3′ -- 5′ ***TCGAG***CGAGTCCCACCGGGACGGTGTCCGACGTCGATGGATCCTTGTAAGGAACGTCTGGGGCGT***CGATC***	62mer 70mer
miR-206 (Site #2) 5′ Mutant	pIS-ERα2-5′	F: 5′ -- 3′ ***C***GCTCAGGGTGGCCCTGCCACAGGCTGCAGCTACCTAGGAAC**TAAG**CTTGCAGACCCCGCA***G*** R: 3′ -- 5′ ***TCGAG***CGAGTCCCACCGGGACGGTGTCCGACGTCGATGGATCCTTG**ATTC**GAACGTCTGGGGCGT***CGATC***	62mer 70mer
miR-206 (Site #2) 3′ Mutant	pIS-ERα2-3′	F: 5′ -- 3′ ***C***GCTCAGGGTGGCCCTGC**GTGT**GGCTGCAGCTACCTAGGAACATTCCTTGCAGACCCCGCA***G*** R: 3′ -- 5′ ***TCGAG***CGAGTCCCACCGGGACG**CACA**CCGACGTCGATGGATCCTTGTAAGGAACGTCTGGGGCGT***CGATC***	62mer 70mer

The table lists the oligonucleotides used to create the miR-206 luciferase reporter constructs. These sequences (shown as paired duplexes) are the endogenous ESR1 regions that encompass the two miR-206 putative target sites mentioned earlier. Flanked *Sac*I/*Nhe*I restriction sites are depicted in *bold, underlined text*. The *light gray region* highlights the miR-206 binding sites, whereas mutations are illustrated in *large, bold text*. F, Forward; R, reverse.

MATERIALS AND METHODS

Cell Lines and Culture

All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA) and Sigma (St. Louis, MO). MDA-231 and MCF-7 cell lines were obtained through American Type Culture Collection (Manassas, VA) whereas the HeLa cells were a gift from Dr. C. Heinen (University of Connecticut Health Center, Farmington, CT). HeLa cells used for the luciferase reporter assays were cultured in MEM supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, and 1% Pen/Strep. Human breast cancer cell lines (MDA-231 and MCF-7) were maintained in DMEM/F12 supplemented with 10% FBS, and 1% Pen/Strep.

Luciferase Constructs

Basic molecular biology-based reagents were obtained from Invitrogen and New England Biolabs (Ipswich, MA). Deoxyoligonucleotides synthesized by Integrated DNA Technology (Coralville, IA) spanned 60 nucleotide (nt) regions of the endogenous ERα 3′-UTR sequence containing the putative miR-206 target sites. Each of the forward and reverse deoxyoligonucleotide paired inserts (see Table 1) were annealed and treated with DNA T4 kinase. The pIS-0 luciferase reporter vector (53), a kind gift from Dr. D. Bartel (Massachusetts Institute of Technology, Cambridge, MA), was cut with *Sac*I/*Nhe*I restriction endonucleases, treated with calf intestinal alkaline phosphatase and purified using the QIAquick PCR Purification Kit (QIAGEN,Valencia, CA). The deoxyoligonucleotide inserts, which contained partial *Sac*I and *Nhe*I flanking regions (see Table 1), were ligated into the pIS-0 vector and transformed into DH5α competent cells. Colonies were screened for presence of the insert via colony PCR and standard sequencing. DNA from positive colonies was purified using the QIAGEN Plasmid Midi Kit and titrated onto a 1% agarose gel to determine accurate concentrations of the constructs to be used in the luciferase assays.

Luciferase Reporter Assays

Reagents for the transfection were purchased from Invitrogen, and the luciferase and β-galactosidase (β-gal) assays were purchased from Promega Corp. (Madison, WI). The appropriate 2′-*O*-methyl antagomiRs were provided by Dharmacon (Lafayette, CO), and the hsa-pre-miRs were obtained through Ambion (Austin, TX). HeLa, MCF-7, and MDA-231 cell lines were seeded at 1.5×10^5 cells per well in 12-well tissue culture plates and incubated overnight in appropriate growth media. Cells were washed with PBS and switched to antibiotic-free growth media for 24–48 h before transfection. Using Lipofectamine 2000, cell lines were cotransfected with various amounts of the pIS-0 and pIS-ERα constructs for 24 h. β-Gal construct (200 ng), purchased from Promega, was used for normalization. The small synthetic RNAs were transfected using the methods mentioned above. Cells were then treated with trypsin, lysed in 100 μl of Reagent Lysis Buffer per well, and centrifuged at 14,000 rpm for 15 min. The supernatant was assayed for luciferase and β-gal activity following Promega's protocol. Values were reported as relative light units normalized to β-gal and standard errors are reported as ± SEM.

Quantitative RT-PCR (qRT-PCR) and Northern Blot Detection of miRNA

The *mir*Vana RNA Isolation Kit, qRT-PCR Detection Kit, and hsa-miR-206/hsa-miR-5s primer sets were purchased though Ambion. This assay was used to monitor levels of

mature miR-206 in various cell lines after various treatments mentioned above. Total RNA was isolated, diluted to a 100 ng/μl working dilution, and used in the reverse transcriptase (RT) reaction, using the protocol given by Ambion. In the real-time PCR assay, 0.5 μl of 50× Rox Reference Dye (Invitrogen) was added to the PCR mix as an internal reference, and all samples were run with a minus RT and normalized to 5s ribosomal control. The real-time PCR was performed in triplicates in three independent experiments with SEMs of the mean reported. Some of the samples were assayed by end-point PCR, using the same procedure, but run at 30 cycles and resolved on a 3% low-melt agarose gel.

Northern blots for miRNAs were performed as previously described (80); modifications are mentioned below. Total RNA (20 μg), isolated from MCF-7 cells via Trizol (Invitrogen), was denatured in an equal volume of deionized formamide at 65 C for 10 min and separated on a 15% denaturing urea-PAGE gel for 1–2 h. The gel was transferred to a nylon membrane (GE Healthcare Life Sciences, Piscataway, NJ) in 0.5× Tris-borate EDTA at 80 V for 1 h, and was UV cross-linked at 120 mJ. DNA for the miR-206 and 5s probes was obtained by isolating the 85-nucleotide (nt) band from an end-point PCR using the qRT-PCR Detection Kit mentioned above. The probe was labeled by a Random Primer DNA Labeling System from Invitrogen and incorporation of α-^{32}P dCTP (3000 Ci/mmol) from PerkinElmer (Boston, MA). Fresh probe was purified over a G-25 microspin column (GE Healthcare Life Sciences) and incubated with the nylon membrane in hybridization buffer (50% formamide, 5× Denhardt's solution, 5× sodium chloride sodium phosphate and EDTA (SSPE), 0.1% sodium dodecyl sulfate, and 0.01% ss Salmon Sperm DNA) at 35 C overnight. Consecutive washes were performed with 2× and 0.2× standard sodium citrate buffer, and blot was detected after 4–6 h exposures on x-ray film from Eastman Kodak Co. (Rochester, NY).

qRT-PCR Detection of mRNA

A standard SYBR Green real-time assay was performed to detect ERα and RPL-19 transcript levels after pre-miR-206 or antagomiR-206 treatments in the MCF-7 and GH3 cell lines. RNA was isolated as mentioned above and diluted to 100 ng/μl stock, and reverse transcribed using the Superscript III enzyme (Invitrogen). Real-time PCR was then performed on cDNA in an iQ SYBR Green Supermix by Bio-Rad Laboratories, Inc. (Hercules, CA) and with primers to either rat ERα (forward, 5′-CATCGATAAGAACCGGAGGA-3′; and reverse, 5′-AAGGTTGGCAG CTCTCATGT-3′)]; rat RPL-19 (forward, 5′-CATGGAGCACATCCACAAAC-3′; and reverse, 5′-CCATAGCCTGG CCACTATGT-3′); human ERα (forward, 5′-GCAGGGAGAGGAGTTTGTGT-3′; and reverse, 5′-ATGTGGGAG AGGATGAGGAG-3′); or human RPL-19 (forward, 5′-TCGATGCCGGAAAAACAC-3′; and reverse, 5′-TGAA CACATTCCCCTTCACC-3′). Analysis of data was done using GraphPad Prism 4.0 software (from GraphPad Software, Inc., San Diego, CA) and Microsoft Excel. Experiments were performed in triplicate in three independent experiments. Errors are reported as ± SEM, whereas values are reported as relative fold change as compared with untreated sample.

miR-206 Overexpression Constructs

All the basic molecular biology reagents used to make the miR-206 overexpression construct were obtained from New England Biolabs and Invitrogen. The 86-bp pre-miR-206 sequence along with 38 bp of flanking sequence were found using the UCSC genome browser. Deoxyoligonucleotides were synthesized by Integrated DNA Technology to contain 91 nt from the 5′-end of both forward and reverse templates, which allowed for a 20-bp overlap in sequence at the 3′-end of each template. The sequences of the templates are as follow: forward template (5′-GCTAGCCTCCGGGTGGTACCCTGGGG CCAGCGAGGAGGCACTTGCCGAAACCACACACTTCCTTACATTCCATAGCAAAGTAATCCATATG-3′) and the reverse template (5′-TATCGTTTCATTAGGTATACCCCTATATTTCTTCGTACACCGGAGCCCTTCGTCACAGTAGGTCTTCGTCGGGCGTAGAGTCTTCCTCGAG-3′). The deoxyoligonucleotides were mixed at a 1:1 ratio and heat annealed for 30 min. Addition of DNA polymerase I large (Klenow) fragment and dNTPs were used to fill in the 71-bp 5′-overhangs. The 162-bp product was gel purified and treated with *Taq* polymerase to facilitate cloning into the PCR 2.1 TOPO vector. Positive constructs were then digested with *Nhe*I/*Sac*I restriction endonucleases and gel purified to obtain the pre-miR-206 insert. This insert was ligated into a modified p*Si-lencer* 4.1-CMV neo vector from Ambion, Inc., and positive clones were identified by PCR screens and sequencing. The miR-206 CMV-driven expression construct (PS-206) was used to determine ERα protein knockdown as described below.

miR-206 Expression Systems in MCF-7 Cells

The miR-206 CMV construct (PS-206) construct along with the PS-neg construct (purchased from Ambion, Inc.) were transfected into MCF-7 cells to knock down ERα expression. Cells were seeded into 100-mm dishes, grown to 90% confluency, transfected with 5, 10, or 20 μg of the constructs for 48–72 h using Lipofectamine 2000, and monitored carefully for cell death. Cells were then lysed and analyzed for protein expression (see below). 2′-*O*-methyl antagomiRs 206, *let-7a*-1, and 16a, were provided by Dharmacon, and 50 nM amounts were transfected into MCF-7 cells as per normal protocol. Cells were lysed 48 h after transfection to determine ERα protein expression.

Western Blot Analysis

All reagents for Western blot analysis were supplied by Bio-Rad Laboratories, Invitrogen, and GE Healthcare Life Sciences. Protein lysates from MCF-7 and T47D cells treated with the miR-206 overexpression construct were prepared. Cells were washed with cold PBS, lysed in 500 μl RIPA buffer, sonicated, and centrifuged at 20,000 × *g* for 5 min at 4 C. A standard BCA assay was performed on the resultant supernatant, diluted to a working concentration of 1 μg/μl, and stored at −80 C. Protein (10 μg) was diluted 1:1 with Laemmli sample buffer and resolved on a 10% SDS-PAGE gel. The protein was transferred onto a nitrocellulose membrane, and Ponseau S stain was used to determine transfer efficiency. Blots were blocked in 5% milk in 0.1% Tris-buffered saline-Tween 20 for 0.5 h at room temperature. Probes to ERα (1:500) from Cell Signaling Technology (Danvers, MA), ERβ (1:000) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), GAPDH (1:1000) from Abcam Inc. (Cambridge, MA), E-cadherin (1:500) from Transduction Laboratories, Inc. (Lexington, KY), and β-actin (1:500) from Abcam Inc. were incubated with the blot overnight at 4 C. Membranes were washed with 0.1% Tris-buffered saline-Tween 20 and incubated with either antimouse or antirat IgG horseradish peroxidase-conjugated secondary antibody, both obtained from Santa Cruz Biotechnology. Protein expression was assessed by enhanced chemiluminescence and exposure to chemiluminescent film from Eastman Kodak Co. Image J software from National Institutes of Health was used to quantify band intensities.

Estrogen Regulation of miR-206 Experiments

MCF-7 cells were cultured in phenol red-free DMEM/F12 media containing dextran-treated charcoal-stripped FBS from Hyclone Laboratories, Inc. (Logan, UT), for 48 h before any experiment. After 48 h, 1 nM E_2, 10 nM DPN (ERβ-agonist), 10 nM PPT (ERα-agonist), and 1 nM progesterone

was added back to media for 24 h. All hormones were purchased from Tocris Bioscience (Ellisville, MO). Cells were lysed for total RNA by the Trizol protocol from Invitrogen, and qRT-PCR was performed, as described above, to measure miR-206 levels after the hormone treatments. Luciferase assays were performed as a functional assay for assessing miR-206 repression. MCF-7 cells were cultured in estrogen-free conditions 48 h before transfection to keep miR-206 levels high. Four hours after transfection of the luciferase constructs pIS-ERα1, pIS-ERα1–5′, pIS-ERα1-SNP, and pIS-0 with Lipofectamine 2000, cells were cultured in media containing 0, 0.1, or 1 nM E_2 for 24 h. Cells were then monitored for luciferase activity as mentioned earlier. All experiments were performed three times with SEM reported.

Statistical Analysis

Values reported in all analysis were expressed as the mean ± SEM. However, Western blot quantifications were noted only as averages. Differences between treatments and/or groups were analyzed using an unpaired Student's *t* test. Statistical significance was accepted at $P < 0.05$.

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