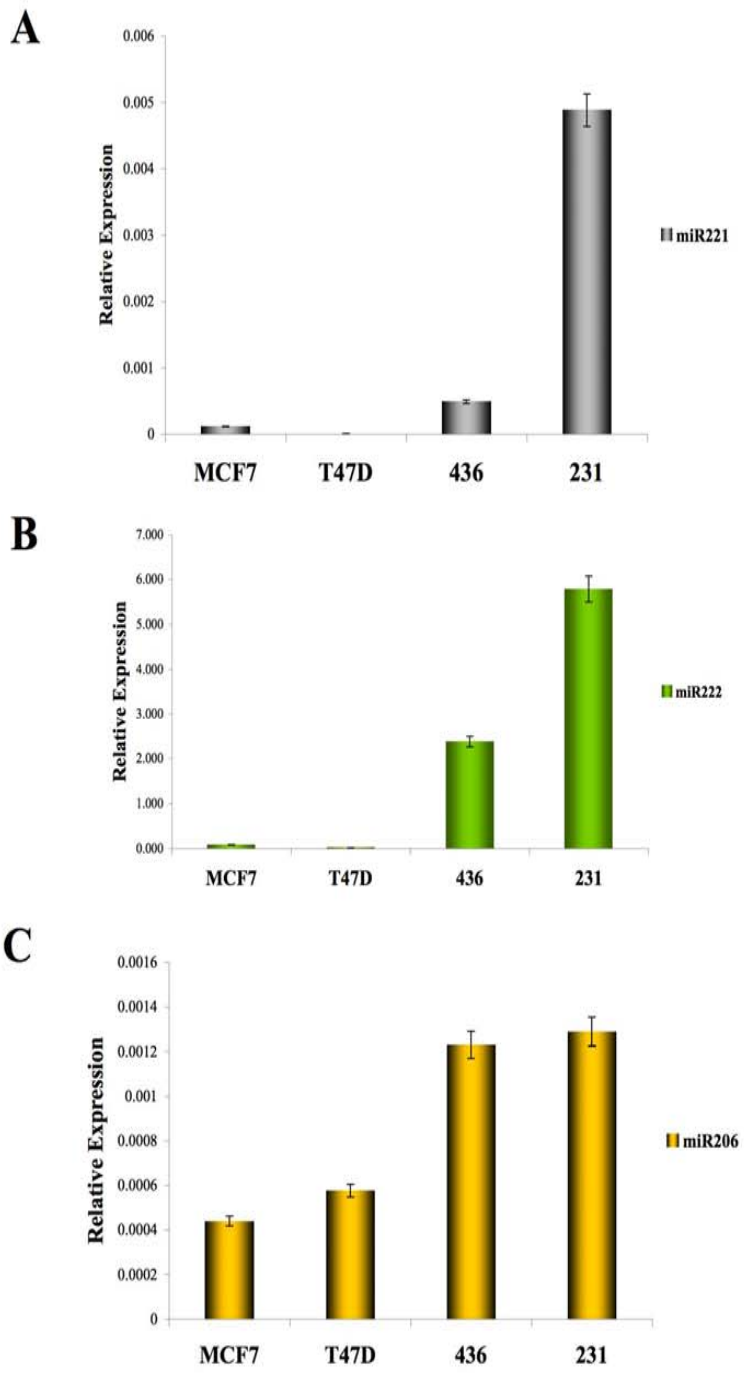
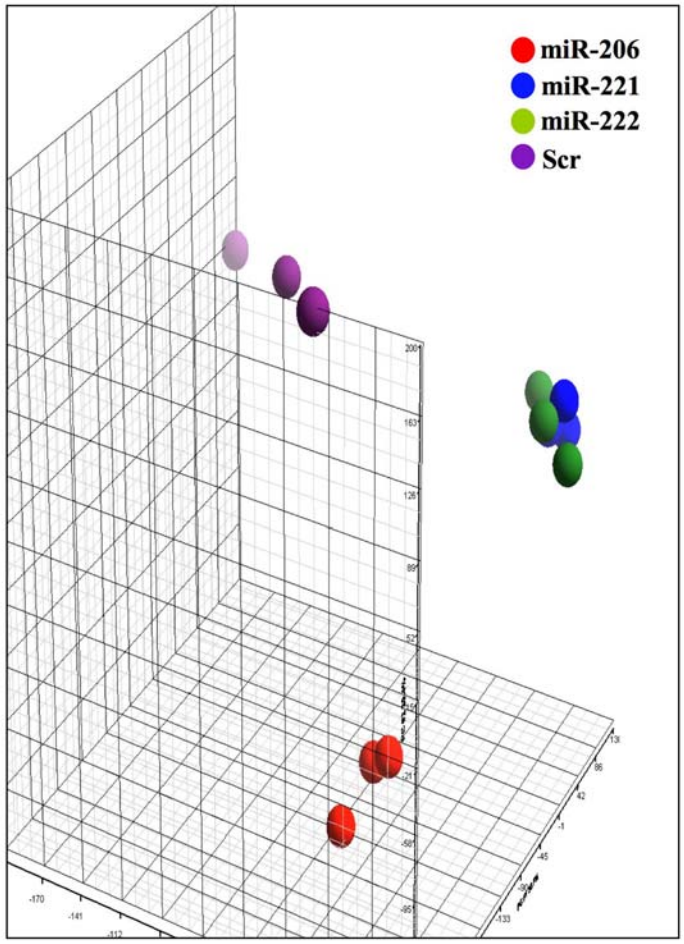
Supplementary Figures

Supplementary Figure 1. miR-221, miR-222, and miR-206 Expression in a Small Set of Breast Cancer Cells. Expression levels of miR-221, 222 and 206 were determined by qRT-PCR in a small set of breast cancer cells with different ERα status (ER+: MCF7, T47D; ER-: MDA-MB-436, MDA-MB-231). Experiments were performed in triplicate and the results are expressed as mean expression levels, relative units (**bars**) with 95% confidence intervals (CIs) (**error bars**). All *P* values were determined using the two-sided Student's *t* test. **A) miR-221**: MCF7: 0.0001, 95% CI = 0.00009 to 0.00012; T47D: 5.36418E-06, 95% CI = 5.01E-06 to 5.5E-6; MDA-MB-436: 0.00048, 95% CI= 0.00045 to 0.00052; MDA-MB-231: 0.00488, 95% CI = 0.0043 to 0.0054; $P < .05$**, B) miR-222**: MCF7: 0.0788, 95% CI= 0.0075 to 0.0086; T47D: 0.012, 95% CI = 0.09 to 0.015; MDA-MB-436: 2.38, 95% CI= 2.2 to 2.6; MDA-MB-231: 5.8, 95% CI = 5.67 to 5.89; $P < .05$. **C) miR-206**: MCF7: 0.00044, 95% CI = 0.00038 to 0.00048; T47D: 0.00058, 95% CI = 0.00049 to 0.00063; MDA-MB-436: 0.00123, 95% CI = 0.0010 to 0.00154; MDA-MB-231: 0.00129, 95% CI = 0.0010 to 0.0015; $P < .05$.).



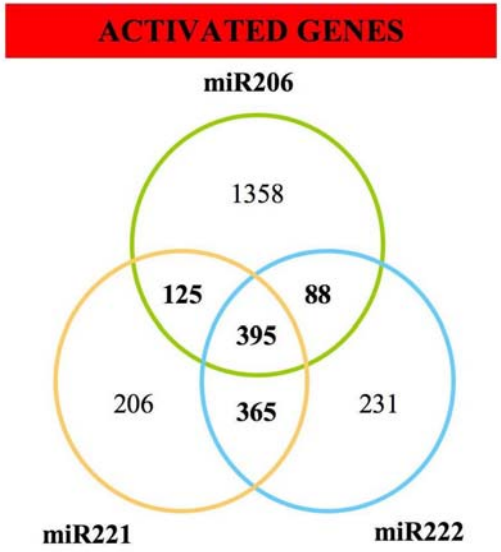
Supplementary Figure 1

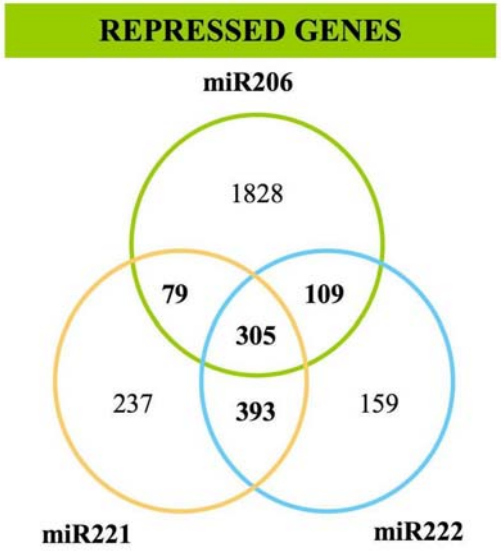
Supplementary Figure 2. Singular Value Decomposition (SVD) Analysis of miR-221, miR-222, and miR-206-overexpressing MCF7 cells. MCF7 cells were transfected in triplicate with miR-221, miR-222, miR-206, and scrambled control (100nM). 72 h after transfection, total RNAs were collected and used for microarray analyses. SVD analysis was performed as reported in Methods.



Supplementary Figure 2

Supplementary Figure 3. Intersection of genes regulated by different miRNAs. The associations between genes modulated by two microRNAs were examined using a two-sided Fisher's exact test, which assumes a hypergeometric distribution under the null hypothesis. Overall, the associations between the modulation by any two microRNAs were statistically significant ($P < .001$).

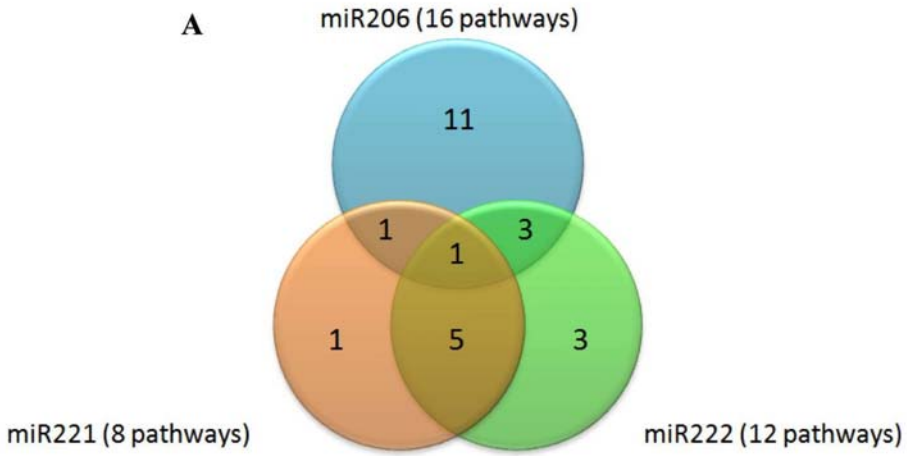




Supplementary Figure 3

Supplementary Figure 4. Modulation of Biological Pathways by **miR-221-222 and miR-206**. **A)** Venn diagram of biological processes in MCF7 cells modulated by overexpression of miR-206, miR-221, and miR-222 vs scrambled control. Two-sided Fisher's exact tests were performed to test the statistical significance of overlaps between significant pathways (**206 vs 221**: P = .628; **206 vs 222**: P = .215; **221 vs 222**: P < .001). **B)** List of biological processes modulated by the overexpression of miR-206, miR-221, and miR-222 (*P* < .001). Pathways in **red** are shared in common by all three miRNAs; in **green**, by miR-206 and miR-222; in **blue**, by miR-206 and miR-221; in **orange** by miR-221 and miR-222.

A



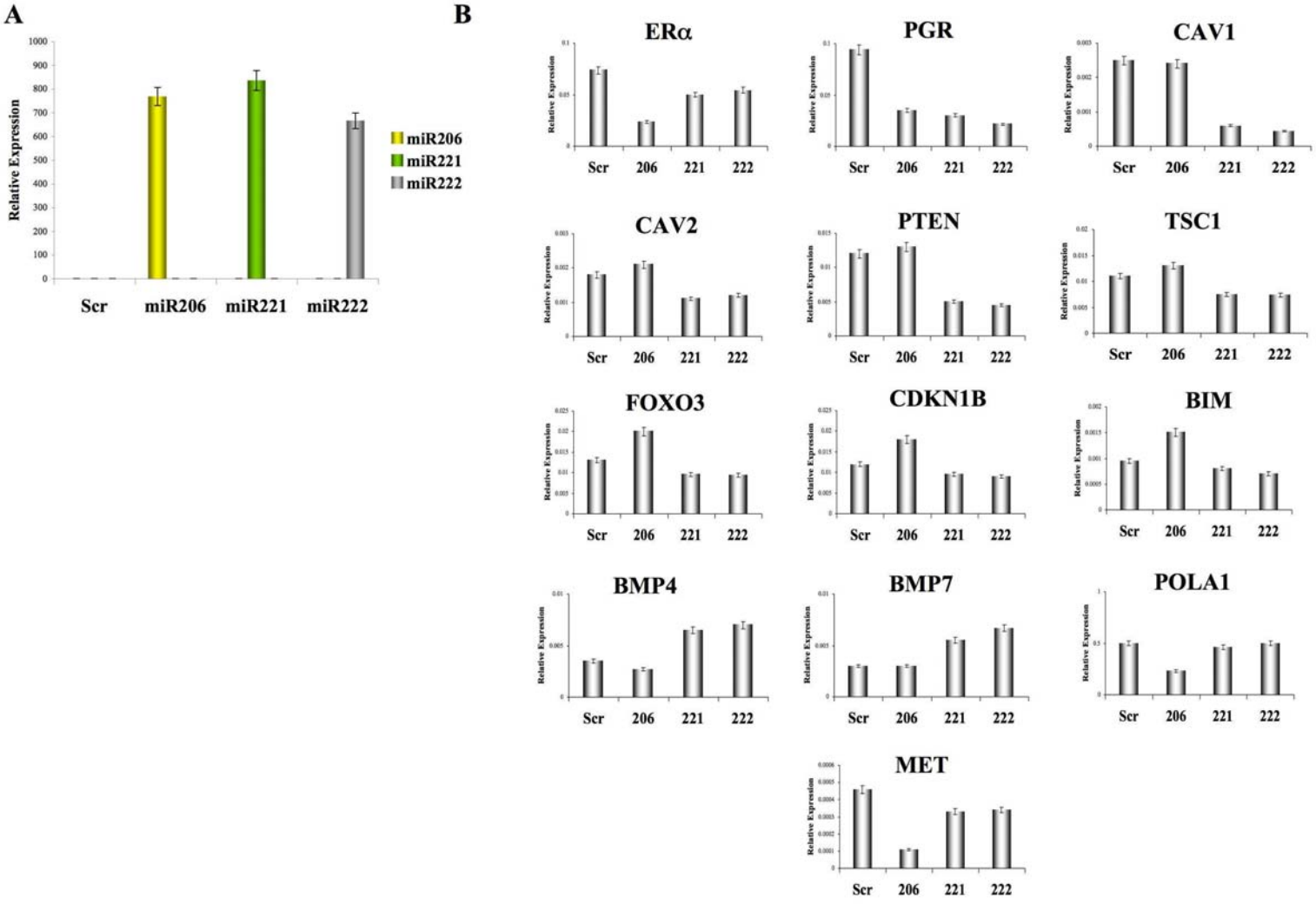
B

miR206	miR221	miR222
Cell adhesion molecules (CAMs)	TGF-beta signaling pathway	Adherens junction
DNA replication	MAPK signaling pathway	Colorectal cancer
Cell cycle	Adherens junction	TGF-beta signaling pathway
Homologous recombination	Apoptosis	Apoptosis
Adherens junction	Colorectal cancer	Cell cycle
p53 signaling pathway	Natural killer cell mediated cytotoxicity	Pancreatic cancer
Base excision repair	Antigen processing and presentation	MAPK signaling pathway
Mismatch repair	Focal adhesion	p53 signaling pathway
Nucleotide excision repair		Axon guidance
Circadian rhythm		Focal adhesion
Ubiquitin mediated proteolysis		ErbB signaling pathway
Pancreatic cancer		Chronic myeloid leukemia
Antigen processing and presentation		
Non-small cell lung cancer		
Glioma		
Prostate cancer		

Supplementary Figure 4

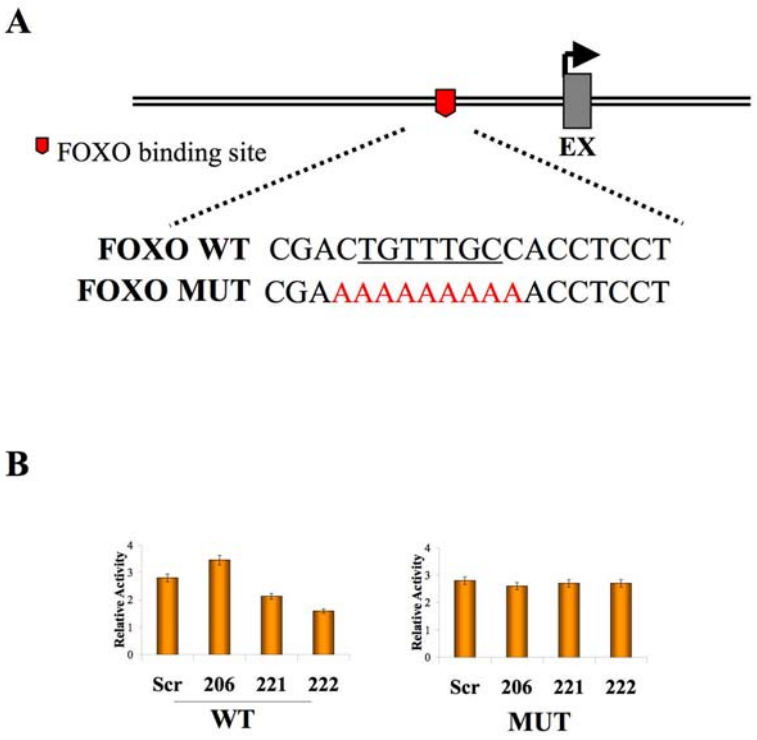
Supplementary Figure 5. Validation of Microarray Data in miR Over-expressing T47D cells. T47D cells were transfected with miR-221, miR-222, miR-206, and scrambled control (100 nM). **A)** 72 h after transfection, total RNAs were collected and used for qRT-PCR to test the overexpression of mature miR-206, miR-221 and miR-222. **B)** qRT-PCR was used to evaluate the expression levels of some of the miR-modulated genes. Experiments were performed twice in triplicate and data are expressed as the means of the two experiments with 95% confidence intervals (CIs). (Mean expression levels, relative units. **ERα**: miR-206: 0.0237, 95% CI = 0.02 to 0.0260; miR-221: 0.05, 95% CI = 0.046 to 0.05; miR-222: 0.0543, 95% CI = 0.047 to 0.060. **PGR**: miR-206: 0.035, 95% CI = 0.03 to 0.04; miR-221: 0.0302, 95% CI = 0.025 to 0.036; miR-222: 0.0214, 95% CI = 0.018 to 0.025. **CAV1**: miR-221: 0.0006, 95% CI = 0.0004 to 0.00067; miR-222: 0.00044, 95% CI = 0.00037 to 0.0006 **CAV2**: miR-221: 0.0012, 95% CI = 0.00095 to 0.0013; miR-222: 0.0011, 95% CI = 0.00089 to 0.00125. **PTEN**: miR-221: 0.005, 95% CI = 0.0035 to 0.006; miR-222: 0.0045, 95% CI = 0.0038 to 0.005. **TSC1**: miR-221: 0.0075, 95% CI = 0.0065 to 0.008; miR-222: 0.0073, 95% CI = 0.0068 to 0.0075. **FOXO3**: miR-206: 0.02, 95% CI = 0.018 to 0.0260; miR-221: 0.0095, 95% CI = 0.0086 to 0.01; miR-222: 0.0094, 95% CI = 0.0087 to 0.01 **CDKN1B**: miR-206: 0.018, 95% CI = 0.017 to 0.0260; miR-221: 0.0095, 95% CI = 0.0086 to 0.0099; miR-222: 0.009, 95% CI = 0.0087 to 0.0094. **BIM**: miR-206: 0.0015, 95% CI = 0.0012 to 0.00165; miR-221: 0.0008, 95% CI = 0.00076 to 0.0087; miR-222: 0.0007, 95% CI = 0.00067 to 0.00094. **BMP4**: miR-221: 0.0065, 95% CI = 0.0061 to 0.0067; miR-222: 0.007, 95% CI = 0.0067 to 0.0074. **BMP7**: miR-221: 0.0055, 95% CI = 0.0051 to 0.0058. miR-222: 0.0067, 95% CI =

0.0063 to 0.0071. **POLA1**: miR-206: 0.23, 95% CI = 0.18 to 0.26. **MET**: miR-206: 0.00011, 95% CI = 0.00009 to 0.00013. All *P* of difference between samples and the control were less than .05, calculated using the two-sided Student's *t* test. Scr = scrambled sequence miRNA control; ERα = estrogen receptor; PGR = progesterone receptor; CAV1/CAV2 = caveolin 1 and caveolin 2; PTEN = phosphatase and tensin homolog; CDKN1B = cyclin-dependent kinase inhibitor 1B; BIM = BCL2-like 11 apoptosis facilitator; TSC1 = tuberous sclerosis 1; FOXO3 = forkhead box O3; BMP4/BMP7 = bone morphogenetic protein 4 and 7; POLA1: DNA polymerase alpha 1, MET = hepatocyte growth factor receptor.



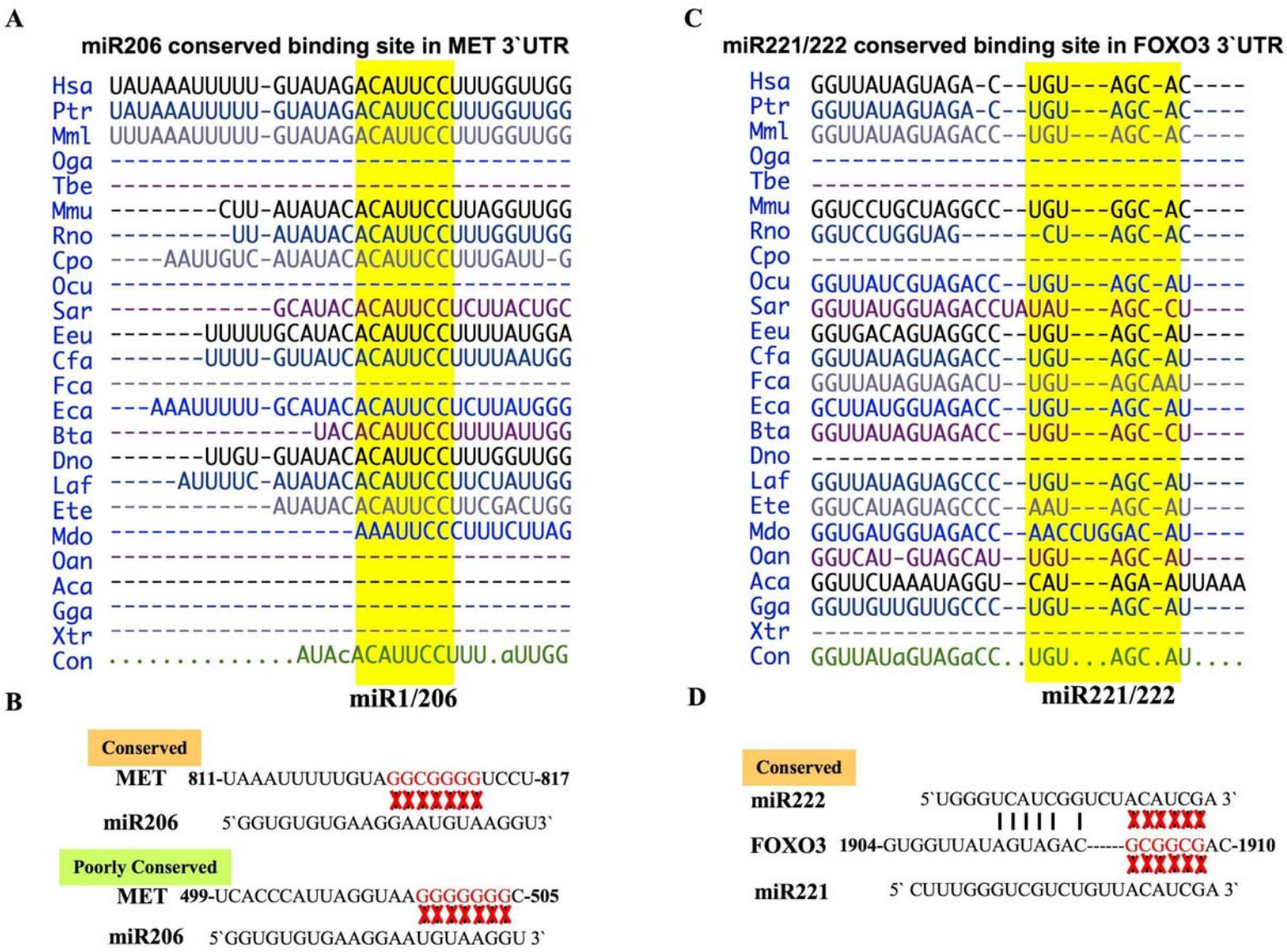
Supplementary Figure 5

Supplementary Figure 6. Modulation of CDKN1B Promoter Activity by miR-206 and miR-221 and 222. A) Schematic representation of wildtype and mutated FOXO3 binding site in CDKN1B promoter. **B)** Luciferase assay of wildtype and mutated CDKN1B promoter in MCF7 cells after overexpression of miR221, 222, and 206; luciferase experiments were performed in quadruplicate and the luciferase activity was read in triplicate. (Mean activity, relative luciferase units: **WT**: Scr: 2.8, 95% CI = 2.89 to 3.0; mir-206: 3.45, 95% CI = 3.33 to 3.6; miR-221: 2.1, 95% CI = 2 to 2.2; miR-222: 1.58, 95% CI = 1.45 to 1.60. **MUTANT**: Scr: 2.8, 95% CI = 2.89 to 3.0; miR-206: 2.6, 95% CI = 2.4 to 2.65; miR-221: 2.7, 95% CI = 2.67 to 2.75; miR-222: 2.7, 95% CI = 2.55 to 2.8. All *P* of difference between samples and control were less than .05). **Error bars** = 95% confidence intervals (CIs). *P* values were calculated by using the two-sided Student's *t* test. Scr = scrambled sequence miRNA control. Scr = scrambled sequence miRNA control. CDKN1B = cyclin-dependent kinase inhibitor 1B; FOXO3 = forkhead box O3.



Supplementary Figure 6

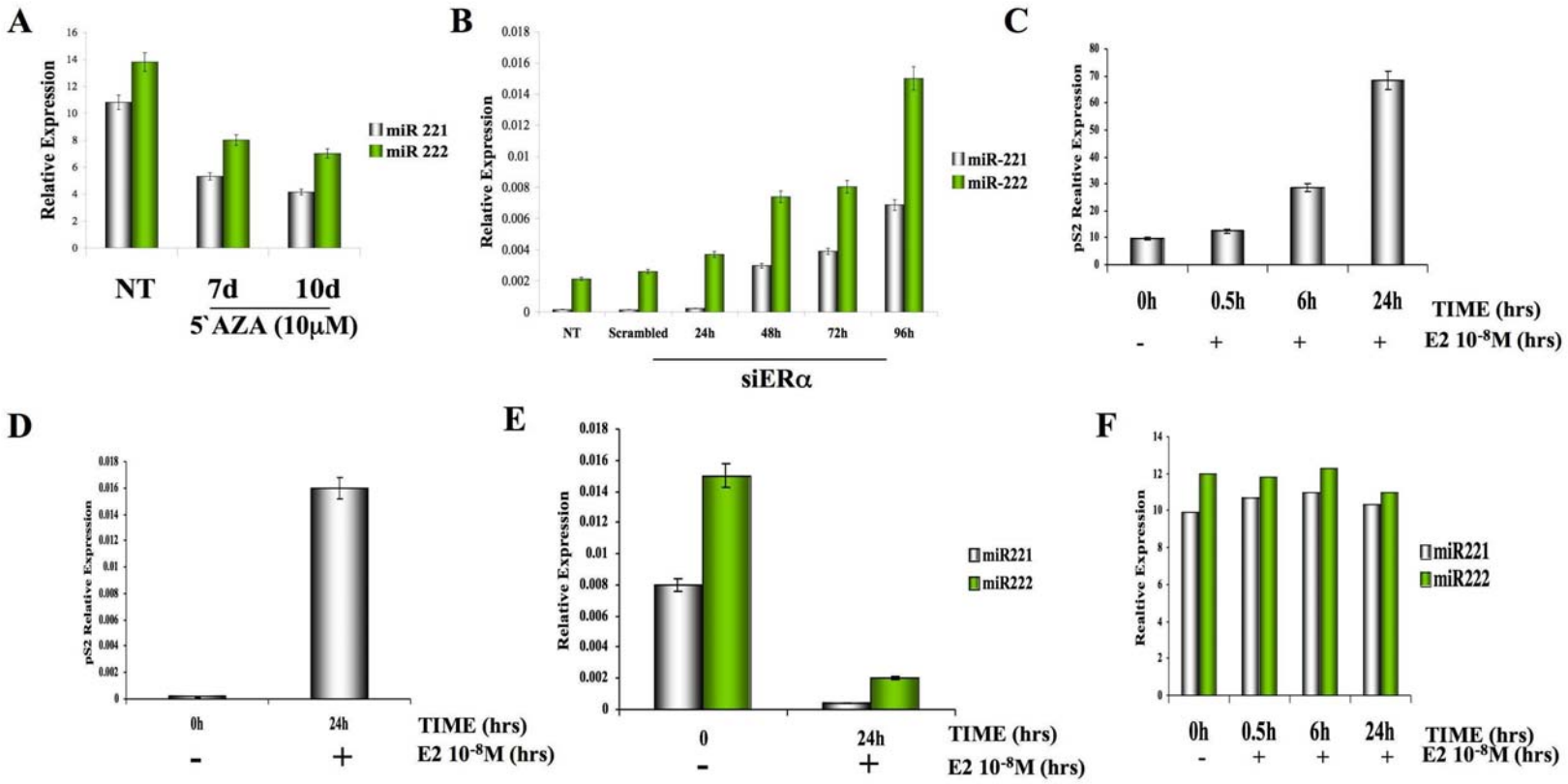
**Supplementary Figure 7. miR-206 and miR-221-222 Binding Sites in the 3`UTR of c-MET and FOXO3. A, C,** conservation of miR-206 and miR-221-222 binding site in c-MET (binding site 811-817) (**A**) and FOXO3 (**C**). 3`UTR is shown across several species. Because of the perfect homology between the seed regions of miR-221 and 222, both microRNAs are able to target the same sequence in the 3`UTR. **B**, **D**, schematic representations showing the mutations introduced into the binding site and the resulting disruption of homology (**red crosses**) against miR-206 (**B**) and miR-221-222 (**D**). 3`UTR = 3` untranslated region; MET = hepatocyte growth factor receptor; FOXO3 = forkhead box O3.



Supplementary Figure 7

Supplementary Figure 8. Suppression of miR-221-222 expression by ERα. A) MDA-MB-231 cells were treated in triplicate with 5-aza-2`-deoxycytidine (5`AZA) at a concentration of 10 μM for 10 days. 5'AZA-treated cells were subjected to qRT-PCR analysis for the detection of miR221-222 expression (Mean expression levels, relative units: **miR-221**(10 d): 4.13, 95% CI = 3.93 to 4.3; $P_{221(10d)\text{ vs NT}}$ <.001. **miR-222** (10d): 7.1, 95% CI = 6.5 to 7.2; $P_{222(10d)\text{ vs NT}}$ = .0098, All *P* values were determined using the two-sided Student's *t* test **[Au: correct?]**). **B)** ER+ T47D cells were transfected with siRNA against ERα or control siRNA and harvested at different time points (24, 48, 72, and 96 h). First, ERα knockdown was checked by western blot (data not shown); then, total RNAs were extracted and subjected to qRT-PCR analysis to determine the expression level of mature miR221-222. Data are means of three experiments (Mean expression levels, relative units: **miR-221**(96h): 0.0069, 95% CI = 0.0060 to 0.0075. **miR-222** (96h): 0.015, 95% CI = 0.013 to 0.02. All *P* of difference between samples and the control were less than .05, determined using the two-sided Student's *t* test). **C**, qRT-PCR for detection of TFF1/pS2 transcript after E2 stimulation in MCF7 cells. **D**, **E,** T47D cells were hormone-starved for 5 days and were then stimulated by adding estradiol (E2) at a concentration of 10 nM. The experiment was performed in triplicate. Total RNAs from E2-treated cells were subjected to qRT-PCR for detection of TFF1 transcipt (**D**) and mature miR-221-222 (Mean expression levels, relative units: **miR-221**: 0 h: 0.008, 95% CI = 0.0065 to 0.0092; 24h: 0.00039, 95% CI = 0.00035 to 0.00045; **miR-222**: 0h: 0.015, 95% CI = 0.012 to 0.018; 24h: 0.002, 95% CI = 0.0018 to 0.0027. All *P* of difference between samples and the control were less than .05, determined using the two-sided Student's *t* test) **(E)**. (**F)** MDA-MB-231 were hormone-starved for 5 days and were then stimulated by adding E2 at a concentration of 10 nM.

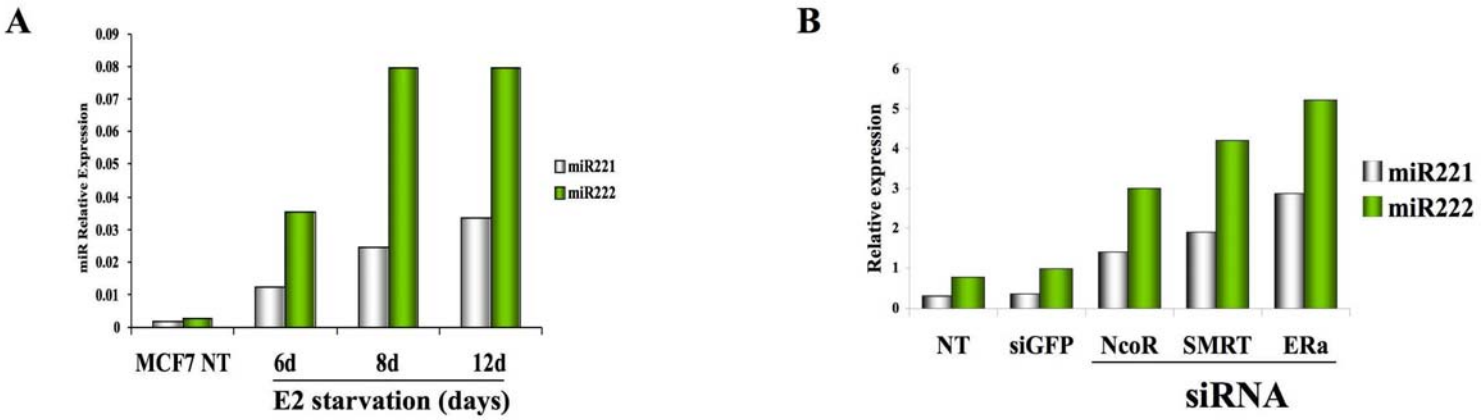
Total RNAs from E2-treated cells were subjected to qRT-PCR for detection of mature miR221-222**.** The experiment was performed twice with similar results. (Mean expression levels, relative units: **miR-221**: 0 h: 9.88; 24 h: 10.3. **miR-222**: 0 h: 12; 24 h: 11). E2 = estradiol; ERα = estrogen receptor; TFF1/pS2 = trefoil factor 1.



Supplementary Figure 8

Supplementary Figure 9: **Acetylation status of Histones H3 and H4 in ERα-mediated miR221-222 suppression. A)** MCF7 cells were hormone-starved in duplicate for 12 days and collected after 6, 8, and 12 days. Total RNAs were subjected to qRT-PCR for detection of mature miR221&222. Data are the means of two experiments. (Mean expression levels, relative units: **miR-221**: NT: 0.00162; 6 d: 0.0122; 8 d: 0.025; 12 d: 0.0336. **miR-222**: NT: 0.0026; 6 d: 0.035; 8 d: 0.079; 12 d: 0.0794). **B)** ER+ MCF7 cells were transfected with siRNA against ERα, NcoR, SMRT or control siRNA and harvested 72 h after transfection. Total RNAs were extracted and subjected to qRT-PCR analysis to determine the expression level of mature miR-221-222. Data are the means of two experiments (Mean expression levels, relative units: **miR-221**: siGFP: 0.35, siNcoR: 1.4, siSMRT: 1.9, siER: 2.87. **miR-222**: siGFP: 0.98, siNcoR: 3, siSMRT: 4.2, siER: 5.2). ERα = estrogen receptor; NCoR = nuclear receptor corepressor; SMRT =

silencing mediator of retinoic acid and thyroid. hormone receptor.



Supplementary Figure 9