Induction of cell proliferation and survival genes by estradiol-repressed microRNAs in breast cancer cells

Xinfeng Yu1*, Xuemei Zhang2, Ishwori B Dhakal3, Marjorie Beggs3, Susan Kadlubar3 and Dali Luo1

Abstract

Background: In estrogen responsive MCF-7 cells, estradiol (E2) binding to ERα leads to transcriptional regulation of genes involved in the control of cell proliferation and survival. MicroRNAs (miRNAs) have emerged as key post-transcriptional regulators of gene expression. The aim of this study was to explore whether miRNAs were involved in hormonally regulated expression of estrogen responsive genes.

Methods: Western blot and QPCR were used to determine the expression of estrogen responsive genes and miRNAs respectively. Target gene expression regulated by miRNAs was validated by luciferase reporter assays and transfection of miRNA mimics or inhibitors. Cell proliferation was evaluated by MTS assay.

Results: E2 significantly induced bcl-2, cyclin D1 and survivin expression by suppressing the levels of a panel of miRNAs (miR-16, miR-143, miR-203) in MCF-7 cells. MiRNA transfection and luciferase assay confirmed that bcl-2 was regulated by miR-16 and miR-143, cyclinD1 was modulated by miR-16. Importantly, survivin was found to be targeted by miR-16, miR-143, miR-203. The regulatory effect of E2 can be either abrogated by anti-estrogen ICI 182,780 and raloxifene pretreatment, or impaired by ERα siRNA, indicating the regulation is dependent on ERα. In order to investigate the functional significance of these miRNAs in estrogen responsive cells, miRNAs mimics were transfected into MCF-7 cells. It revealed that overexpression of these miRNAs significantly inhibited E2-induced cell proliferation. Further study of the expression of the miRNAs indicated that miR-16, miR-143 and miR-203 were highly expressed in triple positive breast cancer tissues, suggesting a potential tumor suppressing effect of these miRNAs in ER positive breast cancer.

Conclusions: These results demonstrate that E2 induces bcl-2, cyclin D1 and survivin by orchestrating the coordinate downregulation of a panel of miRNAs. In turn, the miRNAs manifest growth suppressive effects and control cell proliferation in response to E2. This sheds a new insight into the integral post-transcriptional regulation of cell proliferation and survival genes by miRNAs, a potential therapeutic option for breast cancer.

Background

17-β-estradiol (E2) regulates genes directly by binding to estrogen receptors (ERs) that are ligand-activated transcription factors and indirectly by activating plasma membrane-associated ERs which, in turn, activates intracellular signaling cascades leading to altered gene expression [1]. Therefore, ERs may participate in both the genomic (transcriptional) and non-genomic actions of E2 [2]. E2-liganded ERs interacts directly with a specific DNA sequence called the estrogen response element (ERE = 5'-AGGTCAnnnTGACCT-3') located in the promoter region of target genes [3]. DNA bound ERs then recruits transcriptional coregulators or interacts with other transcription factors, such as AP-1[4] and Sp-1 [5] to indirectly modulate target gene transcription.

To date, two isoforms of the ERs (α and β) have been identified which are able to bind to DNA as homo- or heterodimers. However, it has been shown that, in MCF-7 cells, ERα represents the predominant form, while ERβ is barely detectable [6]. Most studies so far have focused on E2-ERα mediated transcriptional
regulation of genes involved in the control of cell proliferation and survival. It has been reported that E2 up-regulates the bcl-2 mRNA level in MCF-7 cells via two EREs located within the coding region [7]. The expression of cyclin D1, a gene involved in G1 phase cell cycle progression, is induced by E2 in human breast cancer cells. Further studies have identified multiple enhancer elements involved in this regulation [8-11]. E2 also induces survivin upregulation as shown by a gene expression profiling analysis [12]. In hormone-responsive human breast cancer cells, ligand-activated ERα regulates target gene transcription by binding to their DNA response elements (EREs) or by tethering to other trans-acting factors [13,14]. However, the effect of E2 on gene expression at the post-transcriptional level still needs further investigation.

MicroRNAs (miRNAs) are a class of evolutionarily conserved small, non-coding RNAs that control gene expression at the post-transcriptional level [15]. They regulate gene expression by base pairing to the 3’UTR of target mRNA, resulting in direct cleavage and/or translation inhibition of the target mRNA [16,17]. Several studies on miRNA array analysis in MCF-7 cells have demonstrated that E2 regulates a variety of miRNAs. E2 upregulates 21 miRNAs and downregulated 7 miRNAs in MCF-7 vector control stable cells treated with E2 for 4 h [18]. E2 downregulates the expression of mature miRNAs and pre-miRNAs (miR-195, miR-125a, miR-143, miR-145, miR-16, miR-190), but not pri-miRNAs in both mice and cells [19]. Maillot et al. [20] have shown the expression of a broad set of miRNAs (miR-181a, miR-21, miR-26a, miR-200c, miR-27b, miR-23b) decreases following E2 treatment in an ER-dependent manner. Based on previous microRNA expression profiling, we demonstrated that miR-16, miR-143 and miR-203 were potentially suppressed in response to E2 treatment in MCF-7 cells by QPCR quantification. Recently, estradiol-regulated miRNAs have been reported to control estrogen response and cell growth in breast cancer cells [18,20]. However, whether these estradiol-repressible miRNAs coordinately control cell proliferation and survival by targeting bcl-2, cyclin D1 and survivin at the post-transcriptional level in breast cancer cells is not fully investigated.

In the present study, we demonstrated that E2 significantly induced bcl-2, cyclin D1 and survivin expression by suppressing the expression of a set of miRNAs in ERα dependent manner in MCF-7 cells. The downregulated miRNAs exhibited the growth suppressive effect in response to E2 and were highly expressed in triple positive breast cancer tissues. The study revealed the post-transcriptional regulation of estradiol-induced cell proliferation and survival genes by coordinately suppressing a panel of miRNAs, which may serve as therapeutic options in breast cancer treatment.

Methods

Cells and treatment

17β-estradiol, raloxifene, fulvestrant (ICI 182, 780) were purchased from Sigma (Sigma, St. Louis, MO). The breast cancer cells MCF-7, MDA-MB-231 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were maintained at 37°C under 5% CO2. MCF-7 cells were maintained in IMEM medium supplemented with 10% fetal bovine serum (FBS) and 0.01 mg/ml bovine insulin (Invitrogen, Carlsbad, CA). MDA-MB-231 were maintained in DMEM medium with 10% FBS. Prior to ligand treatment, cells were incubated with phenol red-free IMEM supplemented with 5% charcoal stripped FBS for 48 h (serum-starved). Then cells were treated with ethanol (vehicle control, 0.01% final volume), 10 nM E2 for each time course or pretreated with 1 μM ICI 182, 780 or raloxifene respectively for 6 h, then treated in combination with 10 nM E2 for 48 h.

Western blot

Cells were harvested and whole cell extracts were prepared in modified RIPA buffer and separated by 4-12% NuPAGE Bis-Tris gel electrophoresis (Invitrogen, Carlsbad, CA). Proteins were transferred onto PVDF membrane and probed with anti-cyclin D1, anti-bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-survivin (R&D systems, Minneapolis, MN) antibodies at 4°C overnight, then the membrane was incubated with secondary antibody for 1 h before chemiluminescence detection using Pierce ECL Western Blotting Substrate (Pierce, Rockford, IL). β-actin was also detected as a loading control using mouse monoclonal antibody (Sigma, St. Louis, MO).

Transfection of siRNA and miRNA

MiR-16, miR-143, miR-203 mimics and inhibitors were purchased from Ambion and transfected into cells with lipofectamine 2000 (Invitrogen, Carlsbad, CA). The final concentration of miR-16, miR-143, miR-203 mimics and inhibitors was 40 nM. After 48 h, cells were harvested and bcl-2, cyclin D1 and survivin expression were measured. A nonspecific miRNA mimic or inhibitor was used as negative control. ERα siRNA was purchased from Dharmacon SMARTpool siRNA, MCF-7 cells were transfected with 100 nM ERα siRNA for 24 h using lipofectamine 2000, then stimulated with 10 nM E2 for 24 h and 48 h.

Real-time quantitative PCR (QPCR)

For detection of miRNA expression, mirScript Reverse Transcription Kit (Qiagen, Valencia, CA) was used for
cDNA synthesis. miScript SYBR Green PCR Kit (Qiagen, Valencia, CA), in combination with a pair of miRNA specific primers were used for mature miRNA detection. RNU6B was used as an internal control. Taqman probes and gene specific primers for FAM-labeled ERα and VIC-labeled β-actin were obtained from Applied Biosystem (Foster City, CA) and PCR condition for determination of ERα mRNA level was described previously [21]. Relative gene expression was determined using a previously described method [22].

Plasmid construction and luciferase assay
To make luciferase constructs containing 3’ UTR of survivin, 3’UTR was amplified using a pair of primers whose sequences were 5’-gcTCTAGActgcctggtccacagagt-3’and 5’-gcTCTAGAataaaccatagacctattgg-3’. PCR was performed with genomic DNA and digested using XbaI and ligated into pGL3-control vector (Promega Corporation, Madison, WI). The constructs were sequenced to ascertain the right orientation and authenticity in the vector. The 3’ UTR of bcl-2 and cyclin D1 cloned into XbaI site of pGL-3 promoter were kindly provided by Dr. Ruggero De Maria (Mediterranean Institute of Oncology, Catania, Italy).

Cells were plated in a 96-well plate and grown to 80-90% confluence. The firefly luciferase constructs (100 ng) were cotransfected with 40 nM miRNA mimics into MCF-7 cells using lipofectamine 2000 reagent. To monitor transfection efficiency, cells were cotransfected with 10 ng of the pRL-SV40 plasmid which encodes Renilla luciferase. Luminescence was measured 24 h after transfection using a dual-luciferase reporter assay system (Promega Corporation, Madison, WI). All transfections were performed in triplicate, and data were analyzed by normalizing firefly luciferase activity to Renilla luciferase activity for each sample. Each construct was tested in three independent transfections.

MTS Cell proliferation assay
MCF-7 cells were incubated in 5% charcoal stripped FBS for 24 h, then transfected with 40 nM negative control or miR-143, miR-16 and miR-203 mimics. After 24 h, cells were trypsinized into 96-well plates. Cells were treated with 10 nM E2 or ethanol (vehicle control) for 5 days, CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) (Promega Corporation, Madison, WI) was performed to detect cell proliferation.

Triple positive and negative breast cancer tissues
Fourteen triple positive and 36 triple negative formalin-fixed paraffin-embedded (FFPE) breast cancer tissue specimens were obtained from Bioserve Global Biorepository (Beltsville, MD). RNA was isolated from these FFPE tissue specimens using miRNeasy FFPE kit (Qiagen, Valencia, CA) for detection of miR-16, miR-143 and miR-203 expression in triple positive and triple negative breast samples. MiR-631, a non-estradiol inducible miRNA was used as control. These studies were approved by the Institutional Review Board at University of Arkansas for Medical Sciences.

Statistical analysis
The differential analysis per miRNA expression was performed using a 2-sample (treated vs. control and triple positive vs. negative samples) Student’s t-test. Micro-RNAs expression was normalized with respect to RNU6B as an internal control. All statistical analyses were performed using the SAS software (version 9.1; SAS Institute, Inc., Cary, NC). A P value of less than 0.05 (2-sided) was considered to be statistically significant.

Results
Identification of estradiol induced cell proliferation and survival genes in MCF-7 cells
ER-positive MCF-7 cells have been extensively used as a model of hormone-dependent breast cancer. In response to E2 stimulation, MCF-7 cells exhibit cell proliferation and growth response in an E2 dependent manner. Bcl-2, cyclin D1 and survivin have been shown to be implicated in the process of cell proliferation and survival. In previous studies, bcl-2 [7,23,24] and cyclin D1[9,10,25] mRNA levels have been found to be upregulated by E2. Yet very few studies on the regulation of survivin by E2 [12]. Consistently, we found that E2 significantly induced cyclin D1 expression at 6 h and 24 h, it also dramatically enhanced bcl-2 and survivin expression at 24 h and 48 h (Figure 1). The upregulation of bcl-2, cyclin D1 and survivin induced by E2 was in a time-dependent manner.

Downregulation of a panel of miRNAs induced by E2
ERx-mediated transcriptional regulation is one of the mechanisms of gene upregulation induced by E2. MiRNAs have recently been shown to be another important post-transcriptional regulation of genes involved in cell growth and estradiol response in MCF-7 cells [18,26,27]. In order to explore whether miRNAs participate in the upregulation of bcl-2, cyclin D1 and survivin induced by E2, we focused on the downregulated miRNAs that could play a role in the modulation of cell proliferation and survival genes at the post-transcriptional level. Based on previous miRNA array profilings [18,20], QPCR was performed to examine the expression of miRNAs and it showed that mir-143, miR-16 and miR-203 were robustly repressed at 6, 24 and 48 h upon E2 treatment (Figure 2) miR-631, a non-estriadol inducible miRNA, was used as a control in response to E2 treatment.

T47D is another estrogen responsive ERα positive breast cancer cell line which is used to examine the
expression of bcl-2, cyclinD1 and survivin in response to E2 treatment. Consistently, E2 enhanced the expression of these genes but moderately reduced the expression of miR-16, miR-143 and miR-203 as shown in Additional file 1: Figure S1. Therefore, T47D cells displayed a similar effect in response to E2 stimulation, although the effect was weaker than that of MCF-7 cells.

MiRNAs regulate bcl-2, cyclin D1 and survivin at the post-transcriptional level

E2 treatment leads to an increase in cell proliferation and survival genes bcl-2, cyclin D1 and survivin, it can also reduce the expression of several miRNAs. So we hypothesize that E2 upregulates target genes associated with cell proliferation and survival by coordinately suppressing the expression of miR-143, miR-16 and miR-203. It has been clearly demonstrated that miR-15 and miR-16 cluster targets bcl-2 and cyclin D1 [28,29] and miR-143 targets bcl-2 [30]. Our results were in accordance with previous studies as shown in Figure 3A and 3B. These miRNAs were able to significantly downregulate endogenous target genes bcl-2 and cyclin D1 expression at the protein level. QPCR was used to examine the level of the miRNAs and it indicated that transfection of miRNA mimics remarkably increased the level of miRNAs (Figure 3C). Furthermore, TargetScan and miRbase programs predict conserved binding sites of miR-16, miR-143 and miR-203 in the 3’-UTR of survivin. We further confirmed that these miRNAs target survivin at the post-transcriptional level by transfection of miRNA inhibitors and subsequent luciferase assay. It has been shown that transfection of miRNA inhibitors can potentially interfere with the expression of endogenous miRNAs and therefore enhance the expression of target gene survivin (Figure 3D and 3E).

Figure 1 E2 induced bcl-2, cyclin D1 and survivin expression in MCF-7 cells. (A) MCF-7 cells were incubated with phenol red-free IMEM supplemented with 5% charcoal stripped FBS for 48 h (serum-starved). Then cells were treated with vehicle control or 10 nM E2 for 6, 24 and 48 h, total protein was extracted to detect the expression of bcl-2, cyclin D1 and survivin by Western blot. (B) The densitometry of each gene vs. β-actin was indicated and the statistical analysis was shown. * denotes P < 0.05 compared with vehicle control.
The biological activity of miRNAs is primarily mediated by interaction with binding sites in the 3'-UTR of target genes and translational inhibition. Finally, luciferase assay was performed to confirm the binding of the miRNAs to the 3'-UTR of target genes. MCF-7 cells were co-transfected with miR-16, miR-143 or miR-203 mimics with bcl-2, cyclin D1, survivin 3'-UTR luciferase constructs containing the miRNAs binding sites respectively. As expected, miR-16 significantly suppressed the luciferase activity of bcl-2, cyclin D1, and survivin whereas miR-143 moderately inhibited luciferase activity of bcl-2 and survivin. Noticeably, miR-203 impaired luciferase activity of survivin. Therefore, the luciferase assay revealed that these miRNAs directly regulate the expression of the target genes bcl-2, cyclin D1, and survivin by binding to the 3'UTR of these genes (Figure 3F). Based on our previous results, it is likely that E2-mediated reduction in miRNAs expression resulted in lower amounts of miRNAs available to bind to their recognition sequences of bcl-2, cyclin D1 and survivin and thus increasing the luciferase activity of the reporter transcripts. This is possibly one mechanism of E2 induced upregulation of genes involved in cell proliferation.

**Regulation of miRNAs and the expression of target genes are ERα-dependent**

To investigate whether E2-mediated miRNA reduction and upregulation of target genes can be abrogated by anti-estrogens, we used ICI 182, 780 and raloxifene as antagonists of E2. ICI 182, 780, a pure antiestrogen, is a competitive antagonist of E2 and blocks the transcriptional activation properties of ERs [31,32]. Raloxifene belongs to the family of selective estrogen receptor modulators (SERMs) that display agonistic or antagonistic activity in a tissue-dependent manner [33]. As we have shown, both ICI 182, 780 and raloxifene can attenuate the reduction of the miRNAs and consequently the induction of bcl-2, cyclin D1 and survivin expression by E2 (Figure 4A, B).

E2 binds predominantly to ERα which leads to transcriptional regulation of genes involved in the control of cell growth and survival. To explore whether regulation of miRNA and the target genes is ERα-dependent, siRNA was used to interfere with the expression of ERα. It was found that knockdown of ERα remarkably impaired the induction of bcl-2, cyclin D1 and survivin by E2 (Figure 4C, D). In addition, E2-regulated cell proliferation and survival genes were examined in ER-negative MDA-MB-231 cells compared with those of MCF-7 cells. In contrast, E2 could not enhance bcl-2, cyclinD1 and survivin expression in MDA-MB-231 cells (Figure 4E). Further studies on miRNA expression indicated that miR-16, miR-143 and miR-203 were not significantly affected by E2 in MDA-MB-231 cells (Figure 4F), indicating the potential modulation of cell growth and survival genes by miRNAs in response to E2 was mediated by ERα.
E2-responsive miRNAs downregulated endogenous bcl-2, cyclin D1 and survivin expression. (A) MCF-7 cells were transfected with 40 nM miR-16, miR-143 and miR-203 mimics for 48 h. Western blot was performed to detect endogenous bcl-2, cyclin D1 and survivin expression. (B) The densitometry of each gene vs. β-actin was indicated and statistically analyzed. * denotes $P < 0.05$ compared with negative control miRNA. (C) RNA was extracted from MCF-7 transfected with miRNA mimics and RT-QPCR was performed to confirm the overexpression of these miRNAs. (D, E) MCF-7 cells were transfected with 40 nM miR-16, miR-143 and miR-203 inhibitors. After 48 h, Western blot was performed to examine endogenous survivin expression. Meanwhile, RT-QPCR was performed to examine the miRNAs expression. (F) MCF-7 cells were co-transfected with 40 nM miR-16, miR-143 and miR-203 mimics together with 100 ng bcl-2, cyclin D1 and survivin 3'UTR luciferase constructs and 10 ng pRL-SV40. After 24 h, luciferase activity assay was determined. * denotes $P < 0.05$ compared with negative control.
MiR-16, miR-143 and miR-203 suppress E2-dependent cell proliferation

E2 functions as a mitogen to stimulate cell proliferation and cell cycle transition. In response to E2 treatment, miR-143, miR-16 and miR-203 were repressed and their target genes bcl-2, cyclin D1 and survivin were upregulated. We therefore investigated whether overexpression of miR-143, miR-16 and miR-203 interfered with E2-induced cell proliferation in MCF-7 cells. The MTS assay showed that transfection of miR-143, miR-16 and miR-203 significantly inhibited E2-induced cell proliferation (Figure 5A). Since miRNAs regulated bcl-2, cyclin D1 and survivin at the endogenous level as shown in Figure 3A, we further explored whether transfection of these miRNAs impaired E2-induced upregulation of bcl-2, cyclin D1 and survivin that control cell proliferation and cell cycle transition. As shown in Figure 5B and 5C, Western blot and densitometry were performed to determine the protein levels of these genes. In negative control miRNA group, E2 significantly induced upregulation of bcl-2, cyclin D1 and survivin compared with vehicle control. Western blot was used to detect the expression of bcl-2, cyclin D1 and survivin. QPCR was used to examine the level of ERα mRNA. (E, F) MDA-MB-231 cells were treated with 10 nM E2 for 24 and 48 h, miRNAs and target genes were examined. MCF-7 cells were used as ER-positive control.
with negative control miRNA group. But the miRNAs have little inhibitory effects on E2-induced bcl-2 and no inhibitory effects on E2-induced survivin at all when compared with negative control miRNA group (Figure 5D). This probably was due to the robust interference of endogenous genes by the miRNAs in each group, which was in agreement with the result of Figure 3A. More importantly, these miRNAs impaired E2-induced gene upregulation when compared with E2-induced negative control miRNA group. The data indicated that miR-143, miR-16 and miR-203 not only inhibited endogenous expression of bcl-2, cyclin D1 and survivin, but also disturbed E2-induced upregulation of cyclin D1, an important cell cycle regulator, thereby, accounting for the impairment of cell proliferation.

**MiR-16, miR-143 and miR-203 are highly expressed in ER positive breast tumors**

Triple-negative and triple positive breast cancers are defined by the status of estrogen receptor (ER), progesterone receptor (PR) and HER-2 expression. Because the triple-negative phenotype is more aggressive and not amenable to any form of endocrine therapy and has a high incidence of metastasis, the patients have a worse prognosis than patients with the triple positive phenotype [8]. Since miR-16, miR-143 and miR-203 target...
genes control cell proliferation and the miRNAs exhibit a growth suppressive effect in response to E2, we speculated that these miRNAs may be differentially expressed in ER positive and negative breast tumors, acting as a potential causal link with tumor suppressive effects in breast cancer progression. Notably, as shown in Figure 6, miR-16, miR-143 and miR-203 were highly expressed in ER positive breast tumor, in contrast, miR-631 was not differentially expressed between ER positive and negative breast cancer, suggesting these anti-onco miRNAs may play an important role in breast cancer progression and response to chemotherapy.

Discussion

The discovery of miRNAs as a novel class of gene expression regulators has provided new strategies for disease diagnostics and therapeutics. Cell cycle, cell proliferation, cell survival and tumorigenesis are all regulated by miRNAs. Altered abundance of cell survival and cell cycle regulation proteins and aberrant expression of miRNAs frequently coexist in human breast cancers [34]. miRNAs are aberrantly expressed or mutated in cancer, acting as a novel class of oncogenes or tumor suppressor genes [35]. In this study, we demonstrated that a set of E2-repressible microRNAs in breast cancer cell lines was associated with altered cell cycle progression and cell proliferation, which could play a causal role for miRNAs in controlling breast tumor growth.

In breast cancer, abnormalities of the cell cycle are frequently observed in response to E2 treatment. Cyclin D1 encodes a key regulator of the cell cycle transition from G1 to S phase and is overexpressed in more than 50% of breast cancers, functioning as a rate-limiting factor for human breast cancer cell proliferation in vivo and in vitro [36,37]. The bcl-2 protein is associated with the inner mitochondrial membrane and functions to inhibit apoptosis and promote survival [38,39]. Survivin, a member of the inhibitor of apoptosis (IAP) family of antiapoptotic proteins, regulates the G1 checkpoint and G2/M phase of the cell cycle by associating with mitotic spindle microtubules. Survivin directly inhibits caspase-3 and caspase-7 activity, is inversely correlated with apoptosis and is positively correlated with cell proliferation [40,41]. We demonstrated that E2 induced upregulation of cyclin D1, bcl-2 and survivin in MCF-7 cells, which played an important role in E2 stimulated cell proliferation and cell cycle transition. This result was consistent with previous studies [7,9,10,23-25]. Similar results were also observed in another estrogen-responsive breast cancer cells T47D, E2 dramatically induced the expression of cyclin D1, bcl-2 and survivin as shown in Additional file 1: Figure S1.

MiRNAs have emerged as a novel regulator of gene expression at the post-transcriptional level by base-pairing interactions between miRNAs and the 3’-UTR of their target mRNAs [16]. In order to explore whether miRNAs participate in the upregulation of bcl-2, cyclin D1 and survivin by E2, we focused on the E2-repressible miRNAs that may play a role in the modulation of cell proliferation and survival genes. Several studies have demonstrated that E2 upregulates or downregulates a variety of miRNAs by miRNA expression profilings in MCF-7 cells [18-20]. The difference of the results may be due to the cell status, treatment and stimulation time. Based on previous studies, we demonstrated miR-16, miR-143 and miR-203 were coordinately suppressed in response to E2 treatment using QPCR quantification. Therefore, we proposed that these miRNAs might be involved in the regulation of cell proliferation and survival by targeting bcl-2, cyclin D1 and survivin at the post-transcriptional level.

Some publications have provided support for our hypothesis, Cimmino et al. [28] have demonstrated that miR-15a and miR-16 expression is inversely correlated with bcl-2 expression in Chronic lymphocytic leukemia and that both microRNAs negatively regulate bcl-2 at a post-transcriptional level. Bonci et al. [29] have demonstrated that the miR-15a-miR-16 cluster targets CCND1 (encoding cyclin D1), acting as tumor suppressor genes in prostate cancer by the control of cell survival, proliferation and invasion. miR-143 has been validated to target the oncogene KRAS [42] and may also modulate extracellular-regulated protein kinase 5 (ERK5) [43,44] and bcl-2 [30]. In this study, we confirmed the previous studies that miR-16 targets bcl-2 and cyclin D1 and miR-143 targets bcl-2. However, we can also see the impairment of endogenous cyclin D1 by miR-143 as shown in Figure 3A, which may result from the interference of ERK5 expression by miR-143 [30], since ERK5 has been shown to regulate cyclin D1[45,46]. TargetScan and miRbase programs predict conserved binding sites of miR-16, miR-143 and miR-203 in 3’-UTR of survivin. We further elucidated that miR-16, miR-143 and miR-203 target survivin at the post-transcriptional level by transfection of miRNA mimics and inhibitors. Luciferase activity assay indicated these miRNA directly regulated the expression of survivin by binding to the 3’-UTR of survivin.

A miRNA regulates a variety of target genes, and a gene is modulated by many miRNAs. Therefore, in response to E2 stimulation, several miRNAs are coordinately suppressed to upregulate the target genes which are involved in cell proliferation and survival. It provides a novel mechanism for regulation of genes containing ERE in the promoters. Previous studies have shown the ERα-mediated transcriptional regulation of bcl-2, cyclin D1 mRNAs by binding to the ERE of target genes. We cannot conclude that the increase of bcl-2, cyclin D1...
and survivin is due solely to E₂-mediated reduction of miR-16, miR-143 and miR-203, but miRNAs do play a crucial role in the post-transcriptional regulation because when actinomycin D was used to inhibit de novo RNA synthesis, E₂ can still enhance the expression of bcl-2, cyclinD1 and survivin, though at a low level (Additional file 2: Figure S2). Further studies will be needed to dissect the relative contributions of ERα-mediated multiple pathways controlling bcl-2, cyclin D1 and survivin expression.

Figure 6 miRNAs were highly expressed in triple positive breast cancer tissues. (A-D) Total RNA was extracted from 14 triple negative and 36 triple positive breast cancer tissues to detect miRNAs expression by qPCR. MicroRNAs expression was normalized with respect to RNU6B as an internal control. T-test was used to indicate the difference. P values are shown in the figures. TP denotes triple positive breast samples, TN means triple negative breast samples.
ERα is essential for E2-dependent growth, and its level of expression is a crucial determinant of response to endocrine therapy and prognosis in ERα-positive breast cancer. Our data indicated that ICI 182, 780 and Raloxifene can abrogate E2 repressed miRNA levels and therefore attenuate the expression of target genes. It is reported that ICI 182, 780 and Raloxifene can locally alter the ERα ligand binding structure via specific hydrophobic residues and decrease its transcriptional activity [47]. In addition, knockdown of the ERα protein also impaired E2 induced upregulation of bcl-2, cyclin D1 and survivin. In ERα negative MDA-MB-231 cells, E2 has no effect on the regulation of miRNAs and target genes as shown in Figure 4E and 4F. In ERα negative non-cancer cells MCF-10A, we also observed similar effect (data not shown). Thus, the regulation is mainly dependent on ERα protein expression and transactivation in both breast cancer cells and in a normal breast cells.

Suzuki et al. [48] have recently shown that a central tumor suppressor, p53, enhances the post-transcriptional maturation of several miRNAs with growth-suppressive function, including miR-16, miR-143, miR-145 and miR-203 in response to DNA damage. P53 interacts with the Drosha processing complex through the association with the DEAD-box RNA helicase p68 and facilitates the processing of primary miRNAs to precursor miRNAs. In our study, we used MCF-7 cells carrying wild type p53 genes as a model to investigate the E2-repressible miRNAs target genes involved in cell proliferation. However, in p53 mutant ERα-positive T47D cells, we observed similar regulation yet to a weaker extent. Previous studies have elucidated the interaction of ERα and p53 [49,50]. It is likely that both ERα and p53 participated in the regulation of E2-repressible miRNAs. Further studies are needed to elucidate whether ERα-mediated induction of target genes by E2 via p53-regulated miRNA maturation.

The downregulated miRNA exhibits the growth suppressive effect in response to E2. miR-16 and miR-15 act as tumor suppressors and control cell cycle transition by targeting cyclin D1 and cyclin E [29,51]. MiR-143 has been claimed to be a anti-oncomir in human colorectal cancers, which may open new avenues for therapeutic intervention in breast cancer treatment.

Conclusions
It is our novel discovery that estradiol suppressed a panel of miRNAs, involving in the coordinated modulation of target genes that control cell proliferation and survival in breast cancer progression. Importantly, these miRNAs manifest tumor suppressive effects in response to estradiol stimulation and could possibly be biomarkers in triple positive breast tumors. In addition they may be potential biomarkers of breast cancer subtypes. Uncovering the critical role of these miRNAs in tumor suppression will contribute to the efficacy of breast cancer therapy.

Acknowledgements
We thank Ruggero De Maria (Mediterranean Institute of Oncology, Catania, Italy) for the generous gift of pGL3-bcl-2 and pGL3-cyclinD1 plasmids. We thank Dr. Zhihua Liu (Chinese Academy of Medical Sciences) for the technical support. This work was supported by the funding of Susan G. Komen for the Cure BCTR0707584 and the National Natural Science Foundation (30973537).

Additional material

Additional file 1: Figure S1. E2 induced the upregulation of bcl-2, cyclinD1 and survivin and moderately suppressed the level of the miRNAs in T47D cells. (A) T47D cells were incubated with phenol red-free IMEM supplemented with 5% charcoal stripped FBS for 48 h. Then cells were treated with vehicle control or 10 nM E2 for 24 and 48 h, total protein was extracted to detect the expression of bcl-2, cyclin D1 and survivin by Western blot. (B) RNA was extracted from the cells and RT-QPCR was used to examine the level of the miRNAs.

Additional file 2: Figure S2. E2 induced upregulation of bcl-2, cyclin D1 and survivin at both transcriptional and the post-transcriptional level. (A) MCF-7 cells were pretreated or not with 2 μg/ml actinomycin D for 1 h and then stimulated with 10nM E2 for 12 h. Total protein was extracted to determine the expression of bcl-2, cyclin D1 and survivin. (B) The densitometry of each gene vs. β-actin was indicated and statistical analysis was shown. * denotes P < 0.05 compared with control (the first group).

Abbreviations
E2: 17B-estradiol; ER: Estrogen receptor; PR: Progesterone receptor; ERE: Estrogen response element; miRNA: MicroRNA; QPCR: Real-time quantitative PCR; UTR: Untranslated region; DMEM: Dulbecco’s modified eagle’s medium; iMEC: Improved minimum essential medium; FBS: Fetal bovine serum; ECL: Enhanced chemiluminescence; FFPE: Formalin-fixed paraffin-embedded; qRT-PCR: Reverse transcriptase PCR; RT: Enhanced real-time quantitative PCR; UTR: Untranslated region; DMEM: Dulbecco’s modified eagle’s medium; iMEC: Improved minimum essential medium; FBS: Fetal bovine serum; ECL: Enhanced chemiluminescence; FFPE: Formalin-fixed paraffin-embedded; qRT-PCR: Reverse transcriptase PCR.

Author details
1Department of Pharmacology, School of Chemical Biology & Pharmaceutical Sciences, Capital Medical University, 100069, Beijing, China. 2Institute of Molecular Genetics, College of life science, Hebei United University, TangShan, 063000, China. 3Department of Medical Genetics, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, 72205 USA.
7. Perillo B, Sasso A, Abbondanza C, Palumbo G:

5. Safe S:

3. Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoute J, Brodsky AS, Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V:

8. Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V:

13. McKenna NJ, Oxy et al

16. Lai EC:

15. Hammond SM:

12. Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR, Katzenellenbogen BS:

17. Bartel DP:

18. Bhat-Nakshatri P, Wang G, Collins NR, Thomson MJ, Geistlinger TR, Aqeilan RI, Zupo S, Dono M, et al. MicroRNA-143 reduces viability and increases sensitivity to 5-fluorouracil in HCT116 human colorectal cancer cells. FEBS J 2009, 276(22):6689-6700.

Robertson JF. Estrogen induction of cyclin D1 promoter: involvement of a CAMP response-like element. Proc Natl Acad Sci USA 1996, 93(20):11127-11122.

Cicatiello L, Addo R, Sacco A, Attucci L, Petritelli V, Borgho R, Cancemi M, Caporal S, Caristi S, Scafoglio C, et al. Estrogens and progesterone promote persistent CCND1 gene activation during G1 by inducing transcriptional derepression via Jun-C-Fos/estrogen receptor (progesterone receptor) complex assembly to a distal regulatory element and recruitment of cyclin D1 to its own gene promoter. Mol Cell Biol 2004, 24(16):7260-7274.

Fraser J, Danes JM, Kornm B, Chang KC, Lyttle CR, Katzenellenbogen BS: Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 2003, 144(10):4562-4574.

Sabbah M, Courtois D, Mester J, Redeuilh J, Estrogen induction of the cyclin D1 promoter: involvement of a CAMP response-like element. Proc Natl Acad Sci USA 1996, 93(20):11127-11122.

Cicatiello L, Addo R, Sacco A, Attucci L, Petritelli V, Borgho R, Cancemi M, Caporal S, Caristi S, Scafoglio C, et al. Estrogens and progesterone promote persistent CCND1 gene activation during G1 by inducing transcriptional derepression via Jun-C-Fos/estrogen receptor (progesterone receptor) complex assembly to a distal regulatory element and recruitment of cyclin D1 to its own gene promoter. Mol Cell Biol 2004, 24(16):7260-7274.

12. Frasor J, Danes JM, Kornm B, Chang KC, Lyttle CR, Katzenellenbogen BS: Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 2003, 144(10):4562-4574.

13. McKenna NJ, O’Malley BW: Minireview: nuclear receptor coactivators—an update. Endocrinology 2002, 143(7):2461-2465.

14. Glass CK, Rosenfeld MG: The coregulator exchange in transcriptional up- and down-regulation. Cell 2000, 106(37):402-408.

15. Caroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoute J, Brodsky AS, Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V:

16. Lai EC:

17. Bartel DP:

18. Bhat-Nakshatri P, Wang G, Collins NR, Thomson MJ, Geistlinger TR, Aqeilan RI, Zupo S, Dono M, et al. MicroRNA-143 reduces viability and increases sensitivity to 5-fluorouracil in HCT116 human colorectal cancer cells. FEBS J 2009, 276(22):6689-6700.

Robertson JF. Estrogen induction of cyclin D1 promoter: involvement of a CAMP response-like element. Proc Natl Acad Sci USA 1996, 93(20):11127-11122.

Cicatiello L, Addo R, Sacco A, Attucci L, Petritelli V, Borgho R, Cancemi M, Caporal S, Caristi S, Scafoglio C, et al. Estrogens and progesterone promote persistent CCND1 gene activation during G1 by inducing transcriptional derepression via Jun-C-Fos/estrogen receptor (progesterone receptor) complex assembly to a distal regulatory element and recruitment of cyclin D1 to its own gene promoter. Mol Cell Biol 2004, 24(16):7260-7274.

12. Frasor J, Danes JM, Kornm B, Chang KC, Lyttle CR, Katzenellenbogen BS: Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 2003, 144(10):4562-4574.

13. McKenna NJ, O’Malley BW: Minireview: nuclear receptor coactivators—an update. Endocrinology 2002, 143(7):2461-2465.

14. Glass CK, Rosenfeld MG: The coregulator exchange in transcriptional up- and down-regulation. Cell 2000, 106(37):402-408.
41. Altieri DC: Survivin, versatile modulation of cell division and apoptosis in cancer. Oncogene 2003, 22(53):8581-8589.

42. Chen X, Guo X, Zhang H, Xiang Y, Chen J, Yin Y, Cai X, Wang K, Wang G, Ba Y, et al: Role of miR-143 targeting KRAS in colorectal tumorigenesis. Oncogene 2009, 28(10):1385-1392.

43. Akao Y, Nakagawa Y, Naoe T: MicroRNAs 143 and 145 are possible common onco-microRNAs in human cancers. Oncol Rep 2006, 16(4):845-850.

44. Esau C, Kang X, Peralta E, Hanson E, Marcusson EG, Ravichandran LV, Sun Y, Koo S, Perera RJ, Jain R, et al: MicroRNA-143 regulates adipocyte differentiation. J Biol Chem 2004, 279(50):52361-52365.

45. Borracho PM, Simes AE, Gomes SE, Lima RT, Carvalho TM, Ferreira DM, Vasconcelos MH, Castro RE, Rodrigues CM: miR-143 overexpression impairs growth of human colon carcinoma xenografts in mice with induction of apoptosis and inhibition of proliferation. PLoS One 2011, 6(8):e25787.

46. Wang X, Tourrier C: Regulation of cellular functions by the ERK5 signalling pathway. Cell Signal 2006, 18(6):753-766.

47. Lupien M, Jeyakumar M, Hebert E, Hilmi K, Côtier-White D, Lorgeri A, Dayan G, Pinard GA, Wurtz JM, et al: Raloxifene and ICI 182, 780 increase estrogen receptor-alpha association with a nuclear compartment via overlapping sets of hydrophobic amino acids in activation function 2 helix 12. Mol Endocrinol 2007, 21(4):797-816.

48. Suzuki HI, Yamagata K, Sugimoto K, Iwamoto T, Kato S, Miyazono K: Modulation of microRNA processing by p53. Nature 2009, 460(7254):529-533.

49. Liu W, Ip MM, Poddarsak MB, Das GM: Disruption of estrogen receptor alpha-p53 interaction in breast tumors: a novel mechanism underlying the anti-tumor effect of radiation therapy. Breast Cancer Res Treat 2009, 113(1):43-50.

50. Menendez D, Inga A, Resnick MA: Estrogen receptor acting in cis enhances WT and mutant p53 transactivation at canonical and noncanonical p53 target sequences. Proc Natl Acad Sci USA 2010, 107(4):1500-1505.

51. Ofir M, Hacohen D, Ginsberg D: miR-15 and miR-16 are direct transcriptional targets of E2F1 that limit E2F-induced proliferation by targeting cyclin E. Mol Cancer Res 2011, 9(4):440-447.

52. Akao Y, Nakagawa Y, Hirata I, Ito A, Itoch T, Kojima K, Nakashima R, Kitade Y, Naoe T: Role of anti-oncomirs miR-143 and -145 in human colorectal tumors. Cancer Gene Ther 2010, 17(6):398-408.

53. Xu B, Niu X, Zhang X, Tao J, Wu D, Wang Z, Li P, Zhang W, Wu H, Feng N, et al: miR-143 decreases prostate cancer cells proliferation and migration and enhances their sensitivity to docetaxel through suppression of KRAS. Mol Cell Biochem 2011, 350(1-2):207-213.

54. Noguchi S, Mori T, Hoshino Y, Maruo K, Yamada N, Kitade Y, Naoe T, Akao Y: MicroRNA-143 functions as a tumor suppressor in human bladder cancer T24 cells. Cancer Lett 2011, 307(2):211-220.

55. Viticchie G, Lena AM, Latina A, Formosa A, Gaggero LH, Lund AH, Bernardini S, Mauriello A, Miano R, Spagnoli LG, et al: MiR-203 controls proliferation, migration and invasive potential of prostate cancer cell lines. Cell Cycle 2011, 10(7):1121-1131.

56. Saini S, Majid S, Yamamura S, Tabatabai ZL, Suh SD, Shahnazari V, Chen Y, Deng G, Tanaka Y, Daiyi R: Regulatory role of miR-203 in prostate cancer progression and metastasis. Clin Cancer Res 2011, 17(16):5287-5298.

57. Li J, Chen Y, Zhao J, Kong F, Zhang Y: miR-203 reverses chemoresistance in p53-mutated colon cancer cells through downregulation of Akt2 expression. Cancer Lett 2011, 304(1):52-59.