Downregulation of microRNA-515-5p by the Estrogen Receptor Modulates Sphingosine Kinase 1 and Breast Cancer Cell Proliferation

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Abstract
Sphingosine kinase 1 (SK1) plays an important role in estrogen-dependent breast tumorigenesis, but its regulation is poorly understood. A subset of microRNAs (miRNA, miR) is regulated by estrogen and contributes to cellular proliferation and cancer progression. Here, we describe that miR-515-5p is transcriptionally repressed by estrogen receptor α (ERα) and functions as a tumor suppressor in breast cancer. Its downregulation enhances cell proliferation and estrogen-dependent SK1 activity, mediated by a reduction of miR-515-5p posttranscriptional repression. Enforced expression of miR-515-5p in breast cancer cells causes a reduction in SK1 activity, reduced cell proliferation, and the induction of caspase-dependent apoptosis. Conversely, opposing effects occur with miR-515-5p inhibition and by SK1 silencing. Notably, we show that estradiol (E2) treatment downregulates miR-515-5p levels, whereas the antiestrogen tamoxifen causes a decrease in SK1, which is rescued by silencing miR-515-5p. Analysis of chromatin immunoprecipitation sequencing (ChIP-Seq) data reveals that miR-515-5p suppression is mediated by a direct interaction of ERα within its promoter. RNA-sequencing (RNA-Seq) analysis of breast cancer cells after overexpressing miR-515-5p indicates that it partly modulates cell proliferation by regulating the Wnt pathway. The clinical implications of this novel regulatory system are shown as miR-515-5p is significantly downregulated in ER-positive (n = 146) compared with ER-negative (n = 98) breast cancers. Overall, we identify a new link between ERα, miR-515-5p, proliferation, and apoptosis in breast cancer tumorigenesis. Cancer Res; 73(19): 5936–48. ©2013 AACR.

Introduction
Breast cancer is the most common cancer in women worldwide. About 75% of breast cancers are estrogen receptor α (ERα)-positive and cell proliferation in these tumors is highly estrogen-dependent (1). ERα-positive breast cancers have been successfully treated with hormonal therapies that either block estrogen production or antagonize its action (2). Among these, tamoxifen (+4-hydroxytamoxifen) blocks estrogen binding to the ERα and is primarily responsible for the dramatic increase in survival of patients with breast cancer over the past 30 years (3).

Sphingosine kinase 1 (SK1) is the key regulator of the sphingolipid rheostat, which helps maintain the balance between cell growth/survival and cell-cycle arrest/apoptosis. It converts the proapoptotic sphingosine into the prosurvival sphingosine 1-phosphate, mediating breast cancer cell survival, proliferation, and migration (4). In ERα-positive breast cancers, SK1 has been shown to mediate estrogen-dependent tumorigenesis (5) and high expression has been correlated clinically with reduced metastasis-free survival (6). Blocking SK1 expression using siRNAs or selective inhibitors abolishes the response of breast cancer tumors in vivo to estrogens and restores the sensitivity of breast cancer cells resistant to antiestrogens and tamoxifen (7). This indicates the requirement of SK1 in the estrogen response mechanism and the importance of this enzyme in breast cancer hormonal chemoresistance (7). Although estrogens have been described to contribute to a higher activation of SIP receptors, by stimulating the sphingosine 1-phosphate export (8), the mechanism underlying the upregulation of SK1 activity by estrogens is still unclear.

MicroRNAs (miRNA, miR) are small noncoding RNAs, which negatively regulate target gene expression. By binding to the 3′-untranslated region (3′–UTR) or open reading frame (ORF) of their gene targets, they induce mRNA
destabilization/degradation and translational repression (9). They are able to act as tumor suppressors or oncogenes, and we and others have shown that a subset of miRNAs is regulated by estrogen (10–12). This indicates that miRNAs may be a new generation of anticancer molecules and miRNA-based therapies could provide an alternative to standard chemotherapeutics (13).

In this study, we aimed to elucidate the mechanism by which estrogen upregulates SK1 levels. Because dysregulation of miRNA expression has been observed in breast cancer cell lines and tumors upon estrogen or tamoxifen treatment (10), we investigated whether miRNAs control the oncogenic role of SK1 in estrogen-dependent breast cancer. We found two miRNAs capable of directly regulating SK1 expression, and we showed that loss of miR-515-5p in particular results in increased oncogenic SK1 activity. Furthermore, miR-515-5p overexpression in breast cancer cells inhibited cell proliferation and induced caspase-dependent apoptosis. Analysis of chromatin immunoprecipitation sequencing (ChIP-Seq) data of ERα revealed that ligand-activated ERα directly suppresses miR-515-5p gene transcription, and accordingly tamoxifen treatment upregulated mature levels and decreased SK1 activity. RNA-sequencing (RNA-Seq) of breast cancer cells after miR-515-5p overexpression revealed several downregulated transcripts involved in apoptosis and the Wnt pathway. Finally, we found that miR-515-5p expression is significantly lower in ERα–positive breast cancers than ERα–negative tumors. Therefore, miR-515-5p replacement strategies may offer a novel therapeutic approach in hormone-resistant ERα–positive breast cancer and as an adjunct to currently available therapies.

Materials and Methods

Cell lines

Human breast cancer cell lines (MCF7, ZR-75-1, and MDA-MB-231) were obtained from American Type Culture Collection and maintained in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS; Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin (pen/strep) at 37°C with 5% CO₂. Before treatment with ethanol (used as negative control), estradiol (17β-estradiol, E2; Sigma-Aldrich Ltd.), or tamoxifen (Sigma), cells were maintained for 3 days in DMEM without phenol red (Gibco) supplemented with 10% double charcoal–stripped FCS (Gibco) and pen/strep at 37°C with 5% CO₂. On the day of treatment, media was changed to DMEM without phenol red (Gibco) supplemented with 10% FCS (Gibco) and 100 U/mL penicillin and 100 mg/mL streptomycin.

Tumor tissues

Analysis of miRNAs in formalin-fixed paraffin-embedded (FFPE) tissues was approved by a U.K. National Research Ethics Committee (London; 07/QH040/20) and by Imperial College Healthcare NHS Trust (London, United Kingdom). Three sections of 5 μm sections were obtained from FFPE tumor samples (n = 34) and macrodissected. Clinicopathologic information about the patients is provided in Supplementary Table S1.

Luciferase assays

Amplification of the 3’-UTR region of SK1 was achieved by PCR using genomic DNA and the primers were described in the Supplementary Table S2. The DNA fragment obtained was then cloned into the HindIII and Spel sites of the multiple cloning sites (MCS) of pMIR-REPORT Firefly Luciferase vector (Applied Biosystems). After selecting the positive clones, sequencing confirmed the presence of the 3’-UTR region of SK1 in the plasmid. Site-directed mutagenesis of the SK1 3’-UTR was conducted by using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Agilent Technologies) according to the manufacturer’s instructions, and the specific primer sets are described in the Supplementary Table S2. The mutation was confirmed by sequencing. Cells were plated in 24-well plates the day before transfection. Cells were then cotransfected with 100 ng of pMIR-3’-UTR SK1 or pMIR-3’-UTRmut SK1 (Firefly), 50 ng of pRLTK (Renilla; Applied Biosystems) and 50 nmol/L of precursor miRNA mimics (pre-miRs; Applied Biosystems) using 1.3 μL of Lipofectamine 2000 (Invitrogen). After 24 hours, both Firefly and Renilla luciferase activities were quantified using the Dual-Glo Luciferase Assay System (Promega). Firefly luciferase activity was normalized to the luminescence obtained for Renilla luciferase activity. Cloning of the miR-515-5p promoter region into the pGII-basic plasmid (Promega) was carried out by Eurogentec (Supplementary Table S3). Cells were plated in 24-well plates. After starving for 3 days, cells were treated with 10 nmol/L E2 and transfected with 200 ng of miR-515-5p promoter pGII-basic and 100 ng of pRLTK (Applied Biosystems) using 1.5 μL of Fugene reagent (Roche). As previously mentioned, after 24 hours, both Firefly and Renilla luciferase activities were quantified using the Dual-Glo Luciferase Assay System and Firefly luciferase was normalized to Renilla luciferase.

miRNA and siRNA transfection

Cells were plated the day before transfection. Cells were cotransfected with 50 nmol/L of pre-miR, 200 nmol/L of anti-miR, or 20 nmol/L of siRNA (Applied Biosystems) using the HiPerFect Transfection Reagent (Qiagen) according to the manufacturer’s instructions.

SK1 activity assay

MCF7 and MDA-MB-231 cell pellets were resuspended in SK1 buffer (20 mmol/L Tris pH 7.4, 20% glycerol, 1 mmol/L β-mercaptoethanol, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 40 mmol/L β-glycerophosphate, 15 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin, 10 mg/mL aprotinin, 10 mg/mL soybean trypsin inhibitor, and 0.5 mmol/L 4-deoxyzytidylozone). The SK1 activity assay was conducted as previously described (14).

Cell proliferation assays

To analyze cell viability and proliferation, MTT (Sigma; ref. 15) and Trypan blue (16) assays were conducted as previously described.

RNA-Seq and ChIP-Seq analysis

Two micrograms of total RNA from each sample was used to produce cDNA libraries from polyA enriched RNA using the
True-Seq RNA Preparation Kit (Illumina Inc.) according to the manufacturer’s instructions. Paired end sequences (reads) 100 nt in length were then generated using a HiSeq 2000 instrument (Illumina). FASTQ files containing the sequenced reads, obtained at the end of the sequencing, were mapped to the University of California at Santa Cruz (UCSC; Santa Cruz, CA) human genome (hg19 assembly) with TopHat version 1.4.1 (http://tophat.cbcb.umd.edu), using default settings. The mapped bam files obtained at the end of the runs were then analyzed using Cufflinks version 2.1.0 http://cufflinks.cbcb.umd.edu for RNA quantification and analysis. To evaluate whether ERα interacts with the miR-515-5p promoter, we reanalyzed ChIP-Seq published data. We downloaded the samples GSM614606, GSM614607, GSM614610, and GSM614611 from the samples at the end of the sequencing, were then analyzed using Cufflinks version 2.1.0 http://cufflinks.cbcb.umd.edu for RNA quantification and analysis. To find significant peaks of ERα interaction. We used UCSC genome browser (http://genome.ucsc.edu) to map the obtained BED files, containing the significant peaks, onto the HG19 human assembly and zoomed on the locus containing miR-515-5p.

Cell apoptosis assay
Cell apoptosis was determined by measuring the activity of caspase-3 and -7 (Caspase-Glo 3/7 Assay; Promega). The culture media from each well was collected; cells were trypsinized and then washed with the respective culture media. The resulting solution was then mixed and 50 μL was transferred to a white 96-well plate; 100 μL of Caspase-Glo reagent was added and incubated in the dark at room temperature for 1 hour. The luminescence was then measured and normalized to the total protein, quantified using BCA Protein Assay Kit (Pierce).

Immunoprecipitation assay
Cell pellets were collected, resuspended in lysis buffer (20 mmol/L Tris–HCl pH 7.5, 150 mmol/L KCl, 0.5% NP-40, 2 mmol/L EDTA, and 1 mmol/L NaF), and passed three times through a 25 G needle and once through a 27 G needle. The lysate was then preclared by being rotated with the beads (Protein G Sepharose Fast Flow; Sigma) for 2 hours at 4°C. In parallel, the beads were washed three times (washing buffer: 50 mmol/L Tris–HCl pH 7.5, 300 mmol/L NaCl, 5 mmol/L MgCl2, and NP-40 0.05%) and then conjugated with 10 μg of each antibody [monoclonal AGO2 antibody produced in rat (Sigma); immunoglobulin G (IgG) antibody from rat serum (Sigma)] and heparin (final concentration of 1 mg/mL; AppliChem GmbH) by rotation for 2 hours at 4°C. Next, the preclared lysate was divided into the appropriate antibodies-beads samples and rotated for 4 hours at 4°C. The beads were then washed once with lysis buffer and three times with washing buffer. Next, the beads were reconstituted by adding proteinase K (New England Biolabs) and DNA was degraded by adding DNase (Promega). The RNA fraction was then isolated by phenol–chloroform (Sigma) extraction and the RNA was precipitated by adding 100% ethanol and leaving the samples at −20°C for at least 20 minutes. The RNA pellet was washed with 70% ethanol and finally resuspended in RNase-free water.

miRNA expression
To analyze miRNA expression, TaqMan miRNA Assays (Applied Biosystems) were used and stem-loop quantitative reverse transcription (qRT)-PCR was conducted as previously described (17). Each sample was quantified in quadruplicate and using the ΔΔCt method, miRNA expression was normalized to U6 small nuclear RNA expression.

SK1 mRNA expression
To measure SK1 mRNA expression, qRT-PCR was conducted following the manufacturer’s protocol for the PrimerDesign Gene Detection Kit (PrimerDesign Ltd.). Primer sequences used are provided in Supplementary Table S4. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Protein expression
Protein from each sample (30 μg) was electrophoresed in a 10% acrylamide gel and then transferred to a polyvinylidene difluoride membrane. The membranes were then incubated with the primary (phospho-ERK1/2 and GADPH) and secondary antibodies (mouse IgG). Details of the antibodies are provided in Supplementary Table S5. The protein bands were finally detected using Chemiluminescent horseradish peroxidase substrate (Immobilon Western, Millipore Ltd.).

Analysis of GEO2R and human protein atlas datasets
Using GEO2R (18) on the Gene Expression Omnibus (GEO) database website, we examined a recently published dataset by Buffa and colleagues (GSE22220; ref. 19). This dataset contains Illumina miRNA expression profiling for a large cohort of ERα-negative (n = 82) and ERα-positive (n = 128) breast cancers. Using Human Protein Atlas (http://www.proteinatlas.org/), we analyzed ERα and SK1 protein expression in ERα-negative (n = 31) and ERα-positive (n = 26) breast cancer cell lines. Data were analyzed using GraphPad Prism 5.0 (GraphPad Software Inc.).

Statistical analysis
All experiments were carried out independently at least three times. Data (mean ± SEM) were analyzed by Student t test (unpaired, two-tailed) and correlations were analyzed by Spearman rank correlation test. All P values less than 0.05 were considered statistically significant.

Results
miR-515-5p directly regulates SK1 levels
Because SK1 has a short 3'-UTR, similar in length to housekeeping genes, we first verified whether SK1 mRNA was regulated by any miRNAs in breast cancer cell lines. To this end, we conducted an immunoprecipitation in MCF7 cells using AGO2 and IgG (negative control) antibodies, and analyzed SK1 mRNA expression in the subsequent RNA isolated from both immunoprecipitation fractions. We found a significant enrichment of SK1 mRNA levels (P < 0.05; Fig. 1A) in the AGO2 fraction compared with the IgG fraction. AGO2 is part of...
the RNA-induced silencing complex (RISC) through which miRNAs are able to regulate gene expression. Therefore, the presence of SK1 mRNA in the AGO2 fraction suggests the existence of endogenous miRNAs that can interact directly with the SK1 3′-UTR and regulate their levels. Next, we used TargetScan Web-based software to determine which human miRNAs could potentially target SK1 (Supplementary Fig. S1A). Because SK1 is oncogenic in breast cancer, among the miRNAs that potentially interact with its 3′-UTR, we selected only the ones that are known to act as tumor suppressors or known to be downregulated in human cancers for further investigation (Supplementary Table S6). To validate whether these miRNAs directly target SK1, we cloned the SK1 3′-UTR region into the pMIR-REPORT vector. Among the nine selected miRNAs, miR-206, miR-515-5p, and miR-766 significantly reduced luciferase activity (P < 0.01; P < 0.001, and P < 0.05, respectively), proving for the first time that these miRNAs directly interact with the SK1 3′-UTR (Fig. 1B). As miR-766 showed the least reduction in luciferase activity it was not considered further.

To validate SK1 as a direct target of miR-206 and miR-515-5p, we next measured SK1 activity levels in MCF7 cells after overexpression of each miRNA (Fig. 1C). miR-206 and miR-515-5p induced a reduction in SK1 activity of 75% (P < 0.001) and 45% (P < 0.001), respectively. For miR-515-5p, the decrease in SK1 activity was also accompanied by a minor, but significant reduction in SK1 mRNA levels as expected (P < 0.001; Supplementary Fig. S1B), without any change in activated (phospho-) extracellular signal–regulated kinase (ERK) expression (Supplementary Fig. S1C).

We then mutated the miR-515-5p seed region in the SK1 3′-UTR (at the point detected by the TargetScan analysis) located in the luciferase plasmid, to evaluate whether miR-515-5p regulates SK1 by directly binding to this region. miR-515-5p overexpression significantly increased the luciferase activity of the samples transfected with the plasmid containing the mutated sequence (pMIR-SK1 3′-UTRmut) in comparison with the cells transfected with the plasmid containing the wild-type (wt) sequence, proving that miR-515-5p directly targets SK1 by directly interacting with its 3′-UTR (Supplementary Fig. S1D).

miR-515-5p inhibits breast cancer cell proliferation and induces apoptosis

Because SK1 mediates cell proliferation in cancer cells (20), we used MTT assays to determine the effect of miR-206 and
miR-515-5p overexpression on breast cancer cell viability and proliferation (Fig. 2A). miR-206 overexpression has been previously associated with decreased MCF7 cell viability (21), and therefore miR-206 was not considered further. miR-515-5p induced a 70% decrease in MCF7 cell viability \( (P < 0.001; \text{Fig. 2A}) \), which was accompanied by a significant decrease in the expression of the antiapoptotic Bcl-2 protein \( (P < 0.001; \text{Fig. 2B}) \). Therefore, to clarify the cause behind this considerable decrease in breast cancer cell growth, we conducted Trypan blue (Fig. 2C and E) and caspase-3/7 activity (Fig. 2D and F) assays to further evaluate the effect of miR-515-5p on cell viability and apoptosis, respectively. In two breast cancer cell lines, proliferation was dramatically inhibited by miR-515-5p (70% of viable MCF7, \( P < 0.001; \) 42% of viable ZR-75-1, \( P < 0.001; \text{Fig. 2C and E, respectively} \)). In addition, the activities of caspase-3 and -7 were significantly increased after 48 hours of treatment, indicating that apoptosis had been activated by miR-515-5p (in both MCF7 and ZR-75-1 cells; \( P < 0.001; \text{Fig. 2D and F, respectively} \)).

Silencing SK1 partially rescues the effect of miR-515-5p inhibition on breast cancer cell proliferation

Next, we investigated the effect of miR-515-5p by loss-of-function on cell proliferation (Fig. 3A) and apoptosis (Fig. 3C). We observed that inhibition of miR-515-5p in MCF7 cells caused increased cell proliferation \( (P < 0.001; \text{Fig. 3A}) \) and a decrease in apoptosis activation \( (P < 0.001; \text{Fig. 3C}) \).

To investigate whether an increase in SK1 expression was mediating this increase in cell proliferation (Fig. 3B) and inhibition of cell apoptosis (Fig. 3D) caused by anti-miR-515-5p treatment, we silenced SK1 using a siRNA (siSK1) together with miR-515-5p inhibition in MCF7 cells. SK1 silencing induced a decrease in viability of cells cotransfected with anti-miR-515-5p compared with siRNA negative control (siNC; Fig. 3B). However, silencing SK1 was not enough to rescue the total effect of anti-miR-515-5p (neither the decrease in cell proliferation \( (P < 0.01; \text{Fig. 3B}) \), nor the increase in caspase-7 activity \( (P < 0.01; \text{Fig. 3D}) \)).
miR-515-5p targets many oncogenes with an enrichment of genes belonging to the Wnt pathway

Our data so far indicated that apart from SK1, it is likely that other oncogenic targets are involved in the tumor suppressor role of miR-515-5p. To confirm this hypothesis, we overexpressed miR-515-5p continuously in breast cancer cells for 6 days and conducted RNA-Seq analysis first to identify other targets of this miRNA and second, to evaluate whether any of these regulated transcripts are involved in cell proliferation (Supplementary Table S7). Notably, SylArray analysis (http://www.ebi.ac.uk/enright-srv/sylarray/) indicated that the downregulated transcripts are significantly enriched with miR-515-5p "seeds" (Supplementary Fig. S3), indicating that there were direct targets among the downregulated genes. To identify the high-confidence direct targets among these, we considered transcripts that were commonly downregulated (≥1.2-fold) between two replicates and that were predicted as potential targets by TargetScan. This analysis identified 212 high-confidence miR-515-5p directly regulated transcripts (Supplementary Table S7). Interestingly, Gene Ontology (GO) terms and pathway enrichment analysis identified apoptosis and Wnt pathways as significantly regulated by this miRNA (Supplementary Table S7).

Figure 3. SK1 silencing partially rescues the enhanced cell proliferation after miR-515-5p inhibition. Cells were transfected with 200 nmol/L of anti-miRNAs and/or 20 nmol/L of siRNAs (Ambion). Viable cells were counted using Trypan blue (A and B) and caspase-3/7 activities were measured over time (C and D). Data are presented as the mean ± SEM (**, *P < 0.01; ***P < 0.001). E. FGFR2, PI3K, and TCF7L1 mRNA levels are downregulated by miR-515-5p (RNA-Seq validation). MCF7 cells were transfected twice with 50 nmol/L of miR-515-5p over 6 days. FGFR2, PI3K, and TCF7L1 mRNA levels were then measured by TaqMan qRT-PCR. Data are presented as the mean ± SEM (**, *P < 0.05).
Interestingly, among the genes downregulated by miR-515-5p that also belong to the Wnt signaling pathway, all are known to positively regulate cell proliferation. BCL9 improves β-catenin–mediated transcription (22); DIKC1 targets p21 and cyclin D1 through the activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway (23); FRAT2 activates β-catenin–TCF signaling pathway (24); FZD4 is a mediator of the ERG oncogene–induced Wnt signaling (25); and TCF7L1 is a transcription factor activated by β-catenin that mediates Wnt signaling (26). In addition, we identified important genes that have been shown to promote breast cancer, such as FGFR2 and PIK3C2B (27, 28), and these seem to be directly regulated by miR-515-5p. We further validated miR-515-5p–mediated regulation of a few of these genes (TCF7L1, FGFR2, and PIK3C2B) by using qRT-PCR (Fig. 3E). Thus, we show here that although SK1 plays an important role in miR-515-5p cell proliferation regulation, it is not the only gene regulated by miR-515-5p that is responsible for its effects on cancer cell proliferation.

**ERα and SK1 expression is positively correlated**

To evaluate whether ERα and SK1 expression is correlated, we analyzed two publically available datasets, one from the GEO database (miRNA levels) and one from THPA (The Human Protein Atlas) immunohistochemistry database (protein levels). A significant, but weak, positive correlation was observed between ERα and SK1 mRNA expression in ERα–positive breast cancers ($R = 0.181; P = 0.0233$; Fig. 4A). In support of this finding, we also observed a significant and stronger positive correlation between ERα and SK1 protein expression in ERα–positive breast cancer cell lines ($R = 0.352; P = 0.0389$; Fig. 4B). This observation has reinforced our hypothesis that the two genes are correlated through miR-515-5p regulation. Furthermore, and also consistent with this finding, we found that miR-515-5p had a stronger effect inhibiting protein translation, compared with mRNA destabilization in our *in vitro* assays; it reduces SK1 mRNA up to approximately 15% (Supplementary Fig. S1B) and SK1 activity expression to up to approximately 60% (Fig. 1C).

Interestingly, and in line with our hypothesis, ERα and SK1 mRNA and protein expression were not significantly correlated in ERα–negative breast cancers and ERα–negative cell lines (Fig. 4A and B).

**miR-515-5p expression is downregulated by E2**

Previous studies have reported that SK1 mediates the estrogen-dependent tumorigenesis of MCF7 cells (29). However, the molecular processes underlying the upregulation of SK1 expression by E2 are still unclear. We hypothesized that miR-515-5p expression could be downregulated by an E2–associated mechanism, and therefore E2 may upregulate SK1.

To test this hypothesis, we measured miR-515-5p expression and SK1 activity levels in MCF7 cells treated with 10 nmol/L of E2. Remarkably, we found that E2 significantly downregulated miR-515-5p levels in a time course experiment (up to 60% reduction at 48 hours; $P<0.001$; Fig. 4C) and induced a biphasic upregulation of SK1 activity: an early 4-fold upregulation occurred after 6 hours of E2 treatment ($P<0.05$), followed by a later 2-fold upregulation after 48 hours of E2 treatment ($P<0.05$; Fig. 4D; Supplementary Fig. S2A). We also observed a reduction in miR-515-5p levels after E2 treatment in another ERα–positive breast cancer cell line (ZR-75-1; Supplementary Fig. S2B). Because the upregulation of SK1 after 6 hours of E2 treatment (Fig. 4D) could not be explained by miR-515-5p–mediated regulation, as no significant change in miR-515-5p expression was observed until 12 hours from the E2 treatment (Fig. 4C), we next verified whether SK1 activation by E2, at 48 hours, was caused by the downregulation of miR-515-5p. To do this, we measured SK1 activity levels in MCF7 cells transfected with miR-515-5p precursor (overexpression), together with E2 treatment (Fig. 4E). When miR-515-5p is overexpressed, SK1 activity levels are not altered by E2, thus proving that the increase in SK1 activity after E2 treatment is caused by loss of miR-515-5p (Fig. 4E).

**SK1 activity downregulation by tamoxifen requires miR-515-5p**

To further validate our hypothesis that miR-515-5p is required for E2–dependent SK1 expression, we measured SK1 activity and miR-515-5p levels in MCF7 cells treated with 100 nmol/L tamoxifen. Notably, miR-515-5p expression was significantly upregulated by tamoxifen (2.8-fold at 24 hours; $P < 0.01$; Fig. 5A). This indicated that ERα is involved in the downregulation of miR-515-5p by E2 treatment. In addition to the upregulation of miR-515-5p, we observed a significant reduction of SK1 activity in MCF7 cells treated with tamoxifen ($P < 0.001$; Fig. 5B and Supplementary Fig. S2C and S2D), but not in MCF7 cells that were treated with tamoxifen and anti-miR-515-5p (Fig. 5B). The absence of a response in SK1 activity to tamoxifen in the presence of a miR-515-5p inhibitor suggests that this miRNA is required for the downregulation in SK1 activity caused by the antiestrogen tamoxifen (Fig. 5B).

To further investigate the effect of tamoxifen on miR-515-5p levels, we next conducted an immunoprecipitation of AGO2 in MCF7 cells treated with tamoxifen and extracted total RNA from the immunoprecipitation (Fig. 5C). qRT-PCR showed that miR-515-5p levels in the RNA bound to AGO2 were significantly higher in the MCF7 cells treated with tamoxifen, compared with the levels measured in the cells treated with ethanol (4-fold; $P < 0.05$; Fig. 5C). Therefore, not only were miR-515-5p levels upregulated after the tamoxifen treatment, but miR-515-5p was also recruited to the RISC when AGO2 is known to be responsible for the silencing of sequence-specific transcripts.

**miR-515-5p levels are not altered in ERα–negative breast cancer cells after E2 and tamoxifen treatment**

Next, we analyzed the effect of E2 and tamoxifen on miR-515-5p expression and SK1 activity in MDA-MB-231 breast cancer cells, which unlike MCF7 cells, do not express the ERα. This showed that miR-515-5p and SK1 activity levels were unaltered after both treatments (Figs. 4F and G and 5D and E).

**ERα mediates miR-515-5p downregulation by E2 by directly binding to its promoter region**

To determine the mechanism by which ERα regulates miR-515-5p expression levels (Figs. 4 and 5), we reanalyzed the
Figure 4. miR-515-5p downregulation mediates the increase in SK1 activity caused by E2. A and B, miR-515-5p levels are upregulated in ERα-positive breast tumors compared with ERα-negative tumors. A positive and significant correlation between ERα and SK1 expression was observed using two public mRNA and protein datasets (GEO2Rand THPA). A and B, the mRNA analysis (A) was conducted in RNA isolated from 216 breast tumors (ERα-negative, n = 82; ERα-positive, n = 128) and the protein analysis (B) was conducted in protein isolated from 57 breast cancer cell lines (ERα-negative, n = 31; ERα-positive, n = 26). C and D, after 72 hours of starvation, MCF7 and MDA-MB-231 were treated with 10 nmol/L E2 and ethanol (negative control). miR-515-5p expression was measured by qRT-PCR (C), and SK1 activity levels (D) were measured in MCF7 treated with E2 after 3, 6, 12, 24, and 48 hours. For each time point, miR-515-5p/SK1 activity levels represent the ratio between the values for E2 and ethanol. Data presented are the mean ± SEM (*, P < 0.05; **, P < 0.01; ***, P < 0.001). E, the combined effect of E2 treatment and miR-515-5p overexpression on SK1 activity. SK1 activity assay was conducted in MCF7 cells treated with E2. Before E2 treatment and under starvation, MCF7 cells were transiently transfected with 50 nmo/L of miRNAs. Data presented are the mean ± SEM (*, P < 0.05; **, P < 0.01). F and G, miR-515-5p expression and SK1 activity levels in MDA-MB-231 treated with E2. After 24 and 48 hours of treatment, miR-515-5p levels were quantified by qRT-PCR (F) and SK1 activity assays (G) were conducted. For each time point, miR-515-5p/SK1 activity levels represent the ratio between the values obtained for E2 and ethanol. Data presented are the mean ± SEM.
published ChIP-Seq data from MCF7 cells (sequencing data at ArrayExpress accession number E-TABM-828 or GEO accession number GSE25021; ref. 30). E2 treatment induced 116.77-fold enrichment of ERα (Bonferroni-corrected $P = 0.00$) close to the transcription start site (TSS) of miR-515-5p (Fig. 6A). Accordingly, the level of ERα on the same site was substantially reduced after vehicle treatment (23.78-fold; Bonferroni-corrected $P = 0.24$; Fig. 6A), indicating that E2 induces the binding of ERα on the miR-515-5p promoter. Therefore, to confirm the importance of ERα in the downregulation of miR-515-5p, we silenced ERα and measured miR-515-5p levels. Consistent with our proposed mechanism, this led to a dose response increase of miR-515-5p levels (Fig. 6B and C), providing further evidence that the binding of ERα to the miR-515-5p gene promoter downregulates mature miR-515-5p expression. Importantly, E2 treatment induced a significant decrease in the luciferase activity of a reporter construct transfected into MCF-7 cells, which contained the miR-515-5p promoter sequence, indicating that the binding of ERα to this region actually results in downregulation of upstream genes. This finding reinforces our hypothesis that ERα reduces miR-515-5p levels by directly binding to its promoter (Fig. 6D).

**Ex vivo analysis reveals downregulated miR-515-5p levels in ERα-positive breast tumors compared with ERα-negative breast tumors**

To prove the clinical relevance of our findings, we correlated miR-515-5p expression with the presence of ERα in breast tumors (Fig. 7A). In accordance with our in vitro findings (i.e., miR-515-5p downregulation is mediated by E2 via ERα), miR-515-5p levels were found to be significantly lower in ERα-positive breast tumors compared with ERα-negative lesions ($P < 0.01$; Fig. 7A). In addition, we used a publically available dataset (19) to assess the relationship between miR-515-5p and ERα status. Consistent with our in vitro and ex vivo data, we found that in a large cohort of breast cancer patients, miR-515-5p...
5p expression was significantly lower in ERα–positive tumors, whereas there was no association between miR-515-3p (guide strand) levels and ERα status (Supplementary Fig. S5).

Discussion

By screening miRNAs that are predicted to regulate SK1, we identified that miR-515-5p directly interacted with SK1 3′-UTR and regulated its expression (Fig. 1). We found that miR-515-5p expression was downregulated by estrogen in two different breast cancer cell lines (MCF7 and ZR75-1) with a concomitant biphasic activation of SK1 (Fig. 4 and Supplementary Fig. S2B). Transient transfection with precursor miR-515-5p abrogated the later stimulation of SK1 activity (48 hours) by estrogen. This highlighted miR-515-5p as a potential, previously unidentified link, between estrogen and SK1-mediated oncogenesis. Conversely, tamoxifen, which antagonizes estrogen action, upregulated miR-515-5p expression, and SK1 activity was subsequently reduced. Following silencing of miR-515-5p in ERα–positive MCF7 cells treated with tamoxifen, we did not observe any change in SK1 activity levels (Fig. 5), suggesting that reduced levels of miR-515-5p are required for the upregulation of SK1 after estrogen stimulation. Because tamoxifen has been described to have an antagonistic effect on the activation of
several ERs (31), we hypothesized that ERα mediates the estrogen-induced downregulation of miR-515-5p. In MDA-MB-231 cells, miR-515-5p expression is not altered upon estrogen or tamoxifen treatment (Figs. 4 and 5). The absence of the effect of both treatments in these cells, which express ERβ, but not ERα, indicated that ERα was the mediator of the downregulation and upregulation of miR-515-5p induced by estrogens and tamoxifen, respectively (32). In accordance with our experimental findings, our patient data also showed that miR-515-5p expression is significantly higher in ERα–negative breast cancers, compared with ERα–positive breast cancers (Fig. 7). This indicated again the importance of ERα expression in regulating miR-515-5p levels and also the potential clinical relevance of this study.

miR-515-5p belongs to the C19MC cluster (chromosome 19 miRNA cluster), which is the largest miRNA cluster discovered in the human genome so far. This cluster is an imprinted primate-specific chromosomal domain, which means that only one of the allelic copies is expressed (33). A deregulation of an imprinted domain has severe repercussions in many diseases, for example NOEY2 is an imprinted gene whose loss of expression is linked to an increased risk of ovarian and breast cancer (34). In MCF7 cells, treatment with the DNA methyltransferase inhibitor 5-azacytidine (5-aza) reactivates the expression of 20 of 576 (~3%) human miRNAs, 14 of which are located in the C19MC cluster (35). Therefore, DNA methylation might be the epigenetic process by which estrogen/tamoxifen regulate miR-515-5p expression in breast cancer, but this would require further experimental demonstration.

By reanalyzing published ChIP-Seq data (30), we found that one of the ERα–enriched zones in the MCF7 genome (examined after E2 treatment) corresponds to the C19MC cluster promoter, indicating that this particular region is responsible for the miR-515-5p transcription downregulation after E2 treatment. Therefore, the upregulation of miR-515-5p expression when ERα is silenced (Fig. 6), together with our findings in ERα–negative MDA-MB-231 cells (Figs. 4 and 5) and the clinical data (Fig. 7), points to ERα as the mediator for miR-515-5p transcriptional repression upon E2 treatment. Because we showed that ERα specifically interacts upstream of the TSS of a cluster of miRNAs, which are probably derived from the processing of a unique primary transcript, then the other miRNAs belonging to this cluster may also participate in the ERα–mediated cell proliferation mechanism. However, this also would require further investigation.

Figure 7. miR-515-5p levels are upregulated in ERα–positive breast tumors compared with ERα–negative tumors. A, ex vivo analysis reveals miR-515-5p levels are downregulated in ERα–positive breast tumors compared with ERα–negative breast tumors. RNA was isolated from 34 breast cancer tumor tissues (ER-negative, n = 16; ERα-positive, n = 18), miR-515-5p levels were quantified by TaqMan qRT-PCR and analyzed according to ERα status (**, P < 0.01). B, scheme of the proposed mechanism: after its activation by E2, ERα negatively regulates miR-515-5p gene transcription. The loss of mature miR-515-5p leads to a derepression and increase of SK1 activity, which then promotes cell viability by inhibiting apoptosis.
Although we showed a positive correlation between ERα and SK1, which could be mediated by miR-515-5p (Fig. 4), the negative effect of miR-515-5p on cell proliferation could not be explained only due to the negative regulation of SK1 by miR-515-5p. In accordance with this hypothesis, we showed that silencing SK1 only partially rescues the effect of miR-515-5p inhibition on breast cancer cell proliferation (Fig. 3). These findings strongly indicate that miR-515-5p also regulates other genes involved in cell proliferation regulation in addition to SK1. RNA-Seq assessment of the transcriptome after miR-515-5p overexpression revealed that genes that positively regulate Wnt signaling and also contain the seed region for miR-515-5p interaction in their 3'-UTRs were significantly downregulated (Supplementary Table S7). In addition, other important oncogenes that contain a miR-515-5p seed region and were downregulated by this miRNA, include FGFR2, which promotes breast tumorigenicity through maintenance of breast tumor-initiating cells (27), and interleukin-6 receptor (IL-6R), which induces STAT-3 activation and the production of the antiapoptotic proteins bel-2 and BCL2 (36).

Apart from having a critical role in breast cancer tumor growth and formation, estrogens and the ER have been described to be responsible for breast cancer chemoresistance. Because ERα-positive tumors represent the majority of all breast cancers, understanding the mechanisms involved in ERα-mediated breast cancer chemoresistance is essential to finding novel treatments of resistant-tumors. Cell apoptosis is one of the pathways whose dysregulation has been associated with breast cancer chemoresistance (37). Here, we have elucidated one of the mechanisms by which estrogen downregulates cell apoptosis and induces cell proliferation. By downregulating miR-515-5p, estrogen upregulates SK1 activity and the expression of many other oncogenes, which then stimulate cell survival by inhibiting cell apoptosis (Figs. 2 and 3).

We have conducted the first functional studies to evaluate the role of miR-515-5p in cancer and highlighted its therapeutic potential for the treatment of breast cancer. Reexpression of miR-515-5p was found to dramatically inhibit breast cancer cell proliferation and induce caspase-dependent apoptosis (Fig. 2). Therefore, in ERα-positive breast cancers, miR-515-5p replacement could stop the stimulation of cell growth by estrogen and may be an adjunct to current antiestrogen therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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