

microRNA-205 Regulates HER3 in Human Breast Cancer

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Abstract

An increasing amount of experimental evidence shows that microRNAs can have a causal role in breast cancer tumorigenesis as a novel class of oncogenes or tumor suppressor genes, depending on the targets they regulate. HER2 overexpression is a hallmark of a particularly aggressive subset of breast tumors, and its activation is strictly dependent on the *trans*-interaction with other members of HER family; in particular, the activation of the PI3K/Akt survival pathway, so critically important in tumorigenesis, is predominantly driven through phosphorylation of the kinase-inactive member HER3. Here, we show that miR-205, down-modulated in breast tumors compared with normal breast tissue, directly targets HER3 receptor, and inhibits the activation of the downstream mediator Akt. The reintroduction of miR-205 in SKBr3 cells inhibits their clonogenic potential and increases the responsiveness to tyrosine-kinase inhibitors Gefitinib and Lapatinib, abrogating the HER3-mediated resistance and restoring a potent proapoptotic activity. Our data describe miR-205 as a new oncosuppressor gene in breast cancer, able to interfere with the proliferative pathway mediated by HER receptor family. Our study also provides experimental evidence suggesting that miR-205 can improve the responsiveness to specific anticancer therapies. [Cancer Res 2009; 69(6):2195–200]

Introduction

In the last few years, an increasing amount of experimental evidence has strongly supported the involvement of microRNAs, highly conserved small noncoding RNAs able to regulate gene expression at posttranscriptional level, in the biology of human cancer. Several studies have indeed described the aberrant expression of microRNAs in human tumors (1) and have investigated their potential role as oncogenes or tumor suppressor genes, depending on the targets they regulate (2–4).

In the genome-wide miR expression profile of human breast cancer we published in 2005 (5), we identified a number of microRNAs that have been later confirmed to play a crucial role in the biology of human breast cancer (6): one of the first microRNAs

identified as putative oncogene, miR-21, overexpressed in breast cancer, promotes cell proliferation and survival targeting TPM1 and PDCD4 (7, 8); miR-10b is associated with metastatic potential (9); miR-125a and miR-125b, down-regulated in breast cancer, are able to target ERBB2 and ERBB3 receptors (10).

MiR-205, which we initially found associated with absence of vascular invasion in breast cancer (5), was then investigated by *in situ* hybridization by Sempere and colleagues (11), who described that miR-205 expression is restricted to normal myoepithelial cells, whereas its expression is reduced or completely eliminated in matching tumor specimens. More recently, miR-205 and miR-200 family have been shown to regulate epithelial to mesenchymal transition (12).

Searching for putative targets of miR-205 by *in silico* analysis, we found HER3 tyrosine-protein kinase receptor. Members of human epidermal growth factor receptor (EGFR; HER) family are involved in tumorigenesis when their signaling functions are deregulated. In particular, it is known that in breast cancer, HER2 overexpression is a hallmark of a particularly aggressive subset of tumors (13, 14). Lacking a ligand-binding activity, HER2 activation is strictly dependent on the *trans*-interaction with other members of the HER family; in particular, the activation of the PI3K/Akt survival pathway, so critically important in tumorigenesis, is for the most part driven through phosphorylation of the kinase-inactive member HER3. HER3 plays an important and necessary function in HER2-mediated tumorigenesis and coexpression of the two members of the family is a poor prognostic factor.

Thus, given the importance of HER3 receptor in survival and proliferation of breast cancer cells, and the evidence suggesting the involvement of miR-205 in breast cancer, we investigated a possible regulation on this protein by miR-205.

Indeed, here, we show that miR-205, negatively regulating HER3, is able to inhibit breast cancer cell proliferation and improves the response to specific targeted therapies.

Materials and Methods

Human samples, cell lines, and transfections. Primary human breast cancers and normal samples were collected at the Istituto Nazionale Tumori. SKBr3, MCF7, and HEK293 cells were obtained from the American Type Tissue Culture Collection and maintained in 10% fetal bovine serum RPMI.

For transient transfection assays, SKBr3 cells were transfected with SiPort Neo-FX (Ambion) according to the manufacturer's instructions. Either pre-miR-205 or pre-miR negative control (Ambion), and siRNA-HER3 or a scrambled siRNA (Dharmacon), were transfected at a final concentration of 100 nmol/L.

RNA extraction and Northern blot. Total RNA was extracted from cell lines and human samples with Trizol (Invitrogen) according to the manufacturer's instructions, and Northern blot analysis was performed as previously described (15). The oligonucleotide used as probe was

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

C.M. Croce and E. Tagliabue contributed equally to this work.

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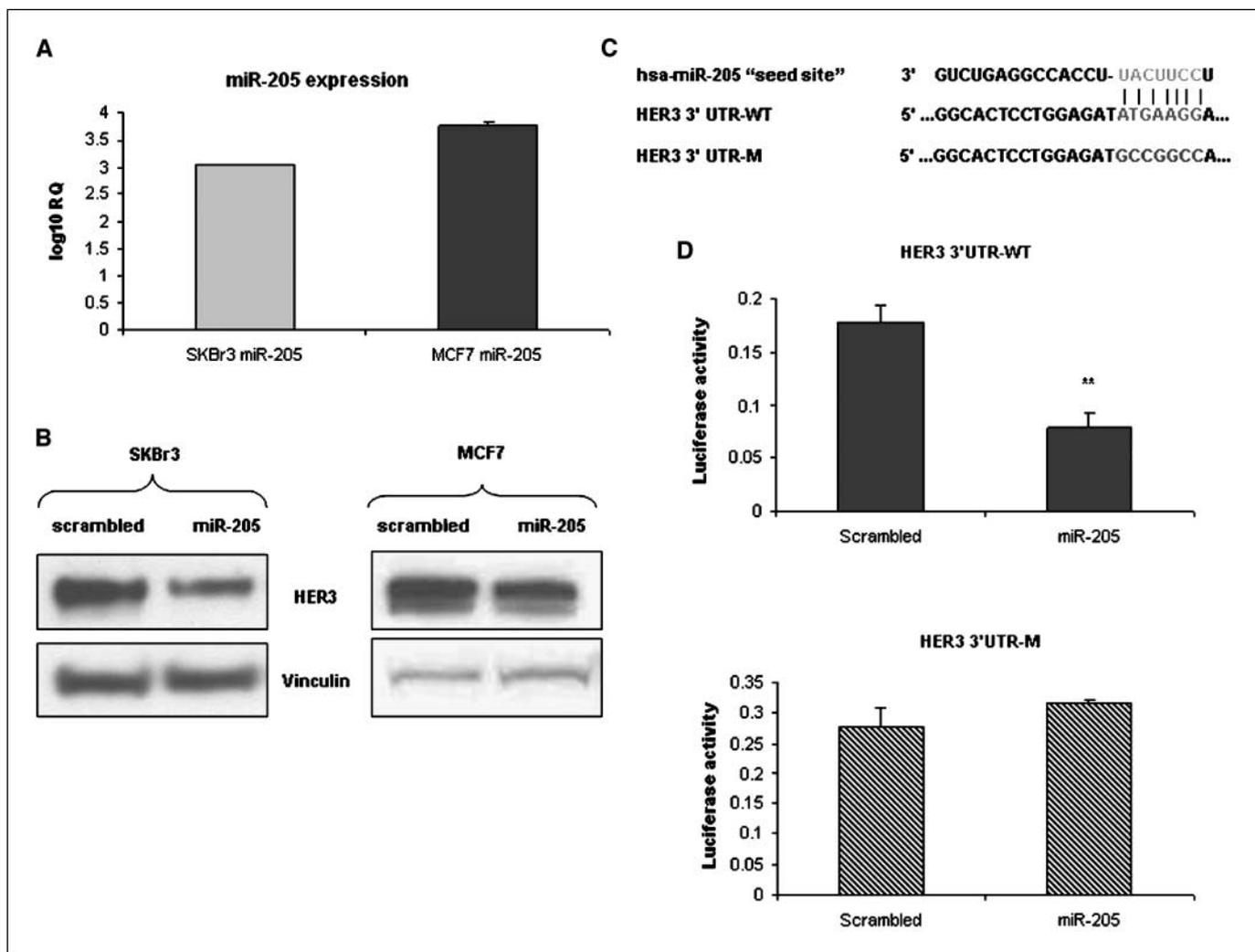


Figure 1. miR-205 directly regulates HER3. SKBr3 and MCF7 cells were transfected with 100 nmol/L pre-miR-205 or a scrambled oligonucleotide, RNA, and proteins collected at 72 h. **A**, miR-205 expression was evaluated by real-time PCR. **B**, HER3, and Vinculin as loading control, were quantified by Western blot. **C**, schematic representation of the interaction between miR-205 and the binding site on the wild-type HER3-3'UTR, and the 7 bp modified in the mutated control (HER3 3'UTR-M). **D**, relative luciferase activity in HEK293 cells. **, *P* = 0.001. The results are representative of three independent experiments.

antisense to the sequence of the mature miR-205 (miR Registry)³; 5'-CAGACTCCGGTGAATGAAGGA-3' 5S RNA or EtBr gel staining were used to normalize.

Real-time PCR. TaqMan MicroRNA Reverse Transcription kit and TaqMan MicroRNA Assay were used to detect and quantify mature microRNA-205 in accordance with manufacturer's instructions (Applied Biosystems). Normalization was performed with U6 RNA.

Protein extraction, Western blotting, and antibodies. Protein extraction and Western blot were performed as previously described (16). The antibodies used in this work were as follows: anti-HER3 (sc-285), anti-Vinculin (sc-7649; SantaCruz Biotechnology, Inc.), anti-P-HER3, anti-P-mitogen-activated protein kinase (MAPK), anti-total MAPK, anti-P-AKT (Ser 473), anti-total AKT (Cell Signaling), anti-HER2 (Calbiochem).

DNA constructs. The expression plasmids for miR-205 (Vec-miR-205) and the corresponding empty vector (miR-Vec) were kindly provided by Dr. Reuven Agami (The Netherlands Cancer Institute, Amsterdam, the Netherlands; ref. 4). The plasmid encoding HER3-shRNA (sh-HER3) and the corresponding empty vector (sh-CTR) were purchased from Origene. For

luciferase reporter experiments, a region of 1251 bp of the HER3 3' untranslated region (UTR) including the binding site for miR-205 was amplified from HEK293 cells by using the following primers:

ERBB3-L: 5'-AATTCTAGAGTAACTCCTGCTCCCTGTGG-3'
ERBB3-R: 5'-AATTCTAGATGAATTTGCCCTCGGATAAG-3'

The PCR product was then digested with *Xba*I and cloned into the reporter plasmid pGL3 (Promega) downstream of the luciferase gene.

Mutations into the miR-205 binding site of the HER3 3'UTR were introduced using Quik-Change Site-Directed Mutagenesis kit (Stratagene). The primers used for the miR binding site mutagenesis were as follows:

fw 5'-aggcactcctggagatgccggccactctccatattcc-3'
rw 5'-ggatatggagagtaatggccggcatctccaggagtgcct-3'

Transfection efficiency was corrected using a Renilla luciferase vector (pRL-cytomegalovirus; Promega).

Luciferase target assay. HEK293 cells were cotransfected (as previously described in ref. 3) in 12-well plates with the modified firefly luciferase vectors described above, the Renilla luciferase reporter plasmid, and the

³ <http://www.sanger.ac.uk/Software/Rfam/mirna/>

RNA oligonucleotides. Firefly and Renilla luciferase activities were measured 48 h after transfection with the Dual-Luciferase Reporter Assay System (Promega). Firefly activity was normalized to Renilla activity to control the transfection efficiency.

Stable transfections and cell colony-forming assay. SKBr3 cells were plated in 100-mm dishes and transfected with 8 μg of Vec-miR-205 (miR-Vec as control) or sh-HER3 (sh-CTR as control) using Lipofectamine 2000 (Invitrogen). After 48 h, Blasticidin (InvivoGen) or Puromycin (Sigma) was added at the final concentration of 5 and 0.5 $\mu\text{g}/\text{mL}$, respectively. Three weeks after the onset of drug selection, the cells were fixed and stained with Toluidine Blue. Colonies were counted using the QuantityOne software.

Drugs treatment and apoptosis assay. After 72 h of transfection with miR-205 or siRNA-HER3, compared with the corresponding scrambled oligonucleotides, SKBr3, cells were treated with 0.5 $\mu\text{mol}/\text{L}$ Gefitinib (ZD1839, IRESSA; kindly provided by Astra-Zeneca) or 0.25 $\mu\text{mol}/\text{L}$ Lapatinib (Glaxo Smith-Kline Research). At 48 h of treatment, cells were collected and processed to evaluate the expression of the molecules of interest by Western blotting.

At 72 h, cells were fluorescence-activated cell sorting (FACScalibur; BD Biosciences) analyzed to evaluate the percentage of apoptotic cells, identified, and quantified by double staining with Annexin V and propidium iodide (BD Pharmingen).

Results and Discussion

miR-205 directly targets HER3 in breast cancer cells.

According to our breast cancer miR signature (5) and recent studies published by other groups, miR-205 seemed to be an interesting candidate for its involvement in breast cancer. To validate the down-modulation of miR-205 in breast tumors compared with normal breast tissue, we first performed a Northern blot on a panel of breast tumor specimens (Supplementary Fig. S1).

Among the potential mRNAs targeted by miR-205, identified using the miRGen database⁴ (17), we selected HER3 receptor, known to play a crucial role in breast cancer development. HER3 is indeed frequently up-modulated in breast cancer with EGFR or HER2 overexpression, and it has been shown that it synergistically increases the transforming potency of HER2 (18). Evaluating miR-205 and HER3 expression in a panel of breast carcinoma specimens, we observed an inversed correlation (Supplementary Fig. S2), suggestive of a possible regulation of HER3 exerted by miR-205. To experimentally validate this hypothesis, we first investigated the modulation of HER3 protein levels in SKBr3 and MCF7 breast cancer cells transfected with miR-205 precursor molecule or a scrambled oligonucleotide by Western blot. Overexpression of miR-205 (verified by Northern blot in Supplementary Fig. S3 and real-time PCR in Fig. 1A) decreased HER3 protein expression of $\sim 44\%$ and 30% in SKBr3 and MCF7, respectively, as evaluated by densitometric analysis (Fig. 1B).

Considering that miRs are thought to control gene expression by base pairing with specific recognizing elements in their target messenger, we performed a luciferase reporter assay to verify that a direct interaction between miR-205 and HER3-3'UTR was responsible for the decreased expression of HER3 protein. 1251 bp of HER3 3'UTR, including the binding site for miR-205 (Fig. 1C), were inserted downstream of the luciferase open reading frame in the reporter plasmid. The reporter vector was cotransfected in HEK293 cells with miR-205 precursor molecule or a scrambled oligonucle-

otide as negative control, and the luciferase activity was markedly decreased (57% reduction) after miR-205 overexpression ($P = 0.001$; Fig. 1D). Moreover, the mutation of the miR-205 binding site in HER3-3'UTR prevented the down-regulation of luciferase expression, supporting the evidence that the effect of the microRNA is exerted through direct interaction with the mRNA target, and that the "seed site" at the 5' end of the microRNA is necessary for the binding.

miR-205 interferes with the survival pathway downstream of HER3. The primary oncogenic signaling induced by the heterodimer HER2-HER3, and directly mediated through HER3, is the activation of PI3K/Akt survival pathway. Because HER3 was directly targeted by miR-205, we investigated whether it was able to interfere with the proliferation/survival pathways activated downstream of HER receptor activation. As reported in Fig. 2, transfection of miR-205 molecule in SKBr3 cells clearly decreased the levels of phosphorylated AKT compared with a scrambled oligonucleotide. Instead, we observed only a slight reduction in phospho-MAPK levels.

miR-205 inhibits breast cancer cell growth. Because miR-205 was able to impair the activation of the Akt-mediated survival and proliferation pathway, it was of high interest and priority to assess whether this microRNA had a functional effect on breast cancer cell growth. To address this, we performed a colony forming assay on SKBr3 breast cancer cells transfected with the plasmid encoding miR-205 (Vec-miR-205) or the corresponding empty vector (miR-Vec). As parallel control, we directly knocked down HER3 transfecting a plasmid encoding a specific shRNA (shRNA-HER3) or the corresponding empty vector (sh-RNA-CTR; Western blot

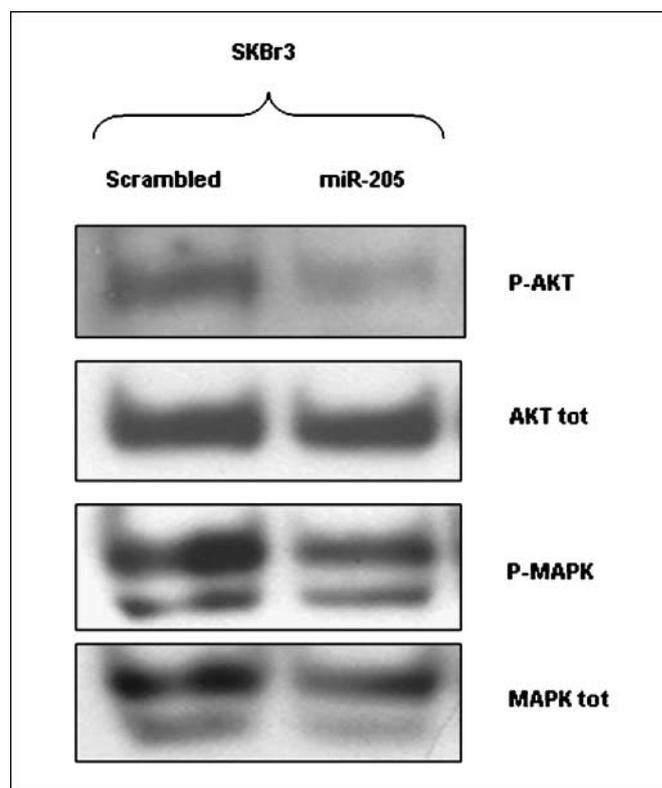


Figure 2. miR-205 interferes with the survival pathway downstream HER3. Western blot showing p-AKT and p-MAPK levels after miR-205 transfection.

⁴ <http://www.diana.pcbi.upenn.edu/miRGen.html>

showing HER3 silencing in Supplementary Fig. S4). After 3 weeks of antibiotic selection, we stained the culture dishes and evaluated the number of colonies; as shown in Fig. 3, transfection with miR-205 (Fig. 3A) consistently decreased the ability to form colonies, revealing an inhibitory effect even greater than what was obtained with the shRNA (Fig. 3B; ~55% inhibition versus 36%).

At a molecular level, miR-205 overexpression, verified by real-time PCR (Fig. 3C), decreased the quantity of total HER3 protein, causing a reduction also in P-HER3 and P-AKT levels (Fig. 3D). The fact that we did not detect any modification in HER2 protein level supports the hypothesis that the observed cell growth inhibition mediated by miR-205 is exerted through specific HER3 repression.

miR-205 enforced expression increases the responsiveness to Gefitinib and Lapatinib. The control exerted by miR-205 on HER3-mediated survival pathway also raised the intriguing possibility of a role of this microRNA in the responsiveness to tyrosine kinase inhibitor (TKI) therapies in breast cancer. Recent findings have indeed unraveled a critical role exerted by the

inactive protein kinase HER3 in the development of resistance to HER TKIs such as Gefitinib, an EGFR inhibitor, and the EGFR/HER2-selective inhibitor Lapatinib. It has been postulated that the persistent activation of AKT pathway, only transiently suppressed by TKIs, could contribute to mediating this effect (19). Recent studies have also shown that siRNA-mediated HER3 inhibition can impair the reactivation of Akt-mediated pathway, thus averting the escape from TKIs therapy (20). Therefore, HER3 is a much more suitable biomarker to guide TKI treatments efficacy, but, at the same time, it also represents a challenging target for pharmaceuticals.

To determine whether miR-205, targeting HER3 protein and able to inhibit AKT phosphorylation, could contribute to TKI responsiveness, we transiently transfected SKBr3 cells for 72 hours with 100 nmol/L pre-miR-205, compared with a negative control, and then treated with 0.5 μ mol/L Gefitinib or 0.25 μ mol/L Lapatinib for an additional 72 hours, evaluating then the percentage of apoptotic cells. As a parallel control, we directly silenced HER3 transfecting a specific siRNA. As shown in Fig. 4, forced expression of miR-205 and siRNA-mediated HER3 silencing induced, respectively, ~57%

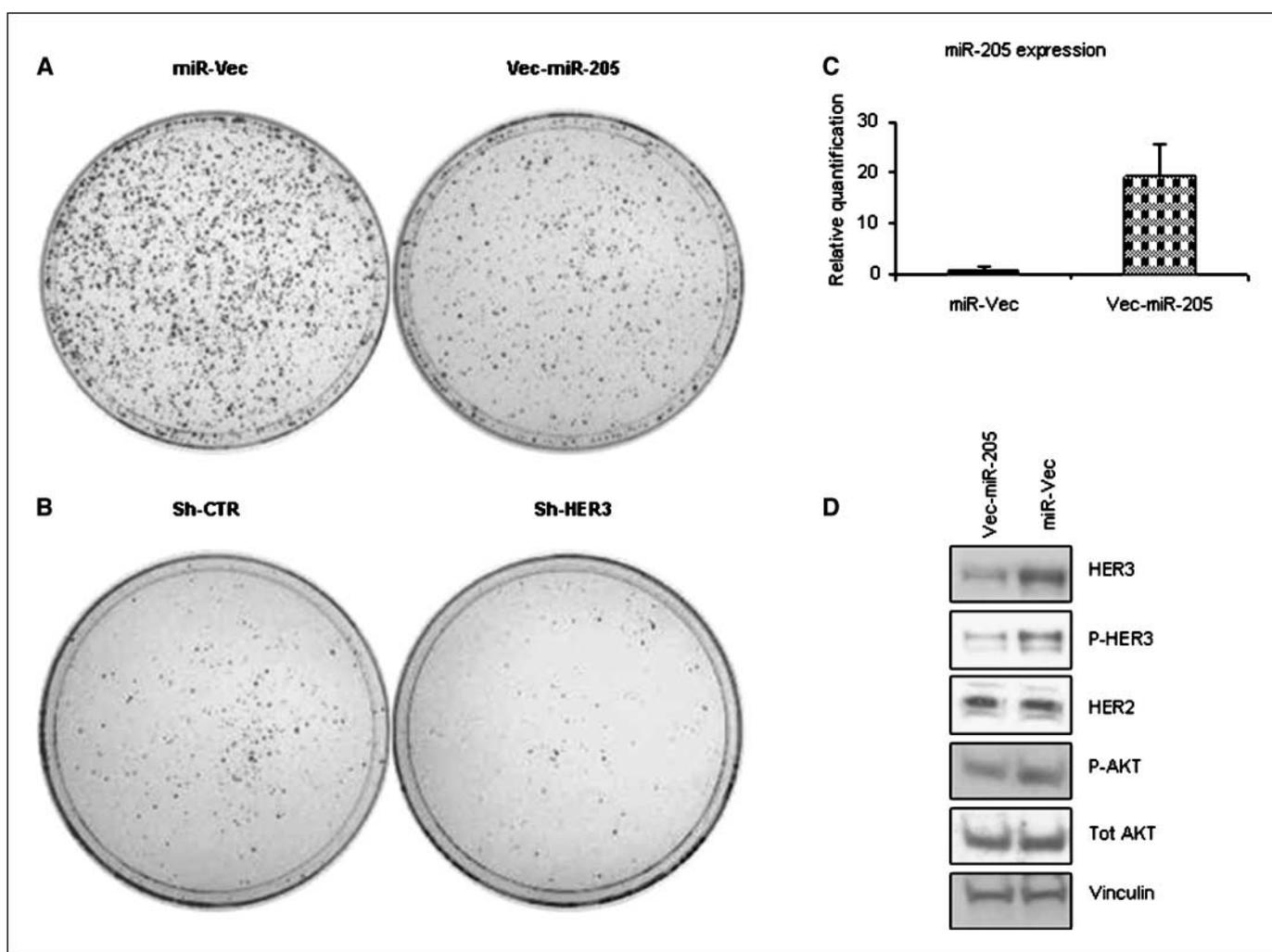
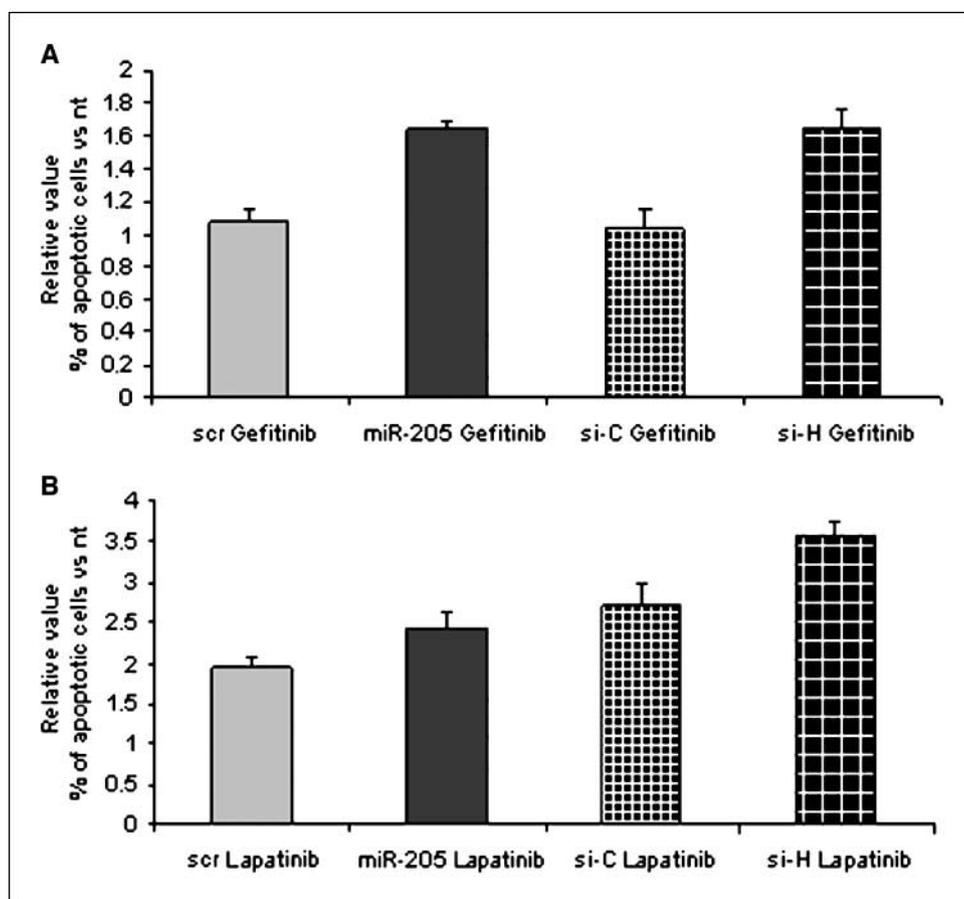


Figure 3. miR-205 effects on cell growth. *A*, colony assay showing the inhibition of colonies formation obtained transfecting SKBr3 cells with a vector encoding miR-205 (*Vec-miR-205*) compared with the corresponding empty vector (*miR-Vec*). Dishes were stained with Toluidine Blue after 3 wk of antibiotic selection. The figure is representative of three different experiments. *B*, parallel control showing the inhibition of colony formation obtained by transfecting SKBr3 cells with a vector encoding a shRNA silencing HER3 (*sh-HER3*) compared with the corresponding empty vector (*sh-CTR*). *C*, real-time PCR validating miR-205 overexpression after SKBr3 transfection with *Vec-miR-205* and (*D*) analysis by Western blot of HER3, P-HER3, HER2, Akt, P-Akt, and Vinculin proteins levels.

Figure 4. miR-205 effect on the responsiveness to anticancer drugs Lapatinib and Gefitinib. SKBr3 cells transiently transfected for 72 h with 100 nmol/L pre-miR-205 or siRNA-HER3 compared with the corresponding negative controls, and then treated with 0.5 μ mol/L Gefitinib or 0.25 μ mol/L Lapatinib for an additional 72 h, were evaluated for the percentage of apoptotic cells by Annexin/propidium iodide assay (A and B).



and 60% increase in sensitivity to 0.5 μ mol/L Gefitinib (Fig. 4A), and 48% and 87% to 0.25 μ mol/L Lapatinib (Fig. 4B).

To support the hypothesis that the increased responsiveness to TKIs treatment was due to the ability of miR-205 to repress the HER3-mediated pathway, counteracting the reactivation of Akt signaling, we analyzed HER3, p-HER3, p-AKT, and total AKT levels at 48 hours of drugs treatment; as supposed, in miR-205 or siRNA-HER3-transfected cells, total HER3, P-HER3, and P-Akt levels are lower than the corresponding control cells (Supplementary Fig. S5).

In summary, in this work, we describe miR-205 as a new oncosuppressor gene in breast cancer, able to interfere with the proliferative pathway mediated by the HER receptor family. Our study also provides experimental evidence that miR-205 can improve the responsiveness to TKI treatment, raising the intriguing possibility of using this microRNA as a new biomarker

for response to specific therapies and as a tool of an innovative and promising therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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