

# Coordinate Suppression of *ERBB2* and *ERBB3* by Enforced Expression of Micro-RNA *miR-125a* or *miR-125b*\*

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Deregulation of micro-RNAs (miRNAs) is emerging as a major aspect of cancer etiology because their capacity to direct the translation and stability of targeted transcripts can dramatically influence cellular physiology. To explore the potential of exogenously applied miRNAs to suppress oncogenic proteins, the *ERBB* oncogene family was chosen with a bioinformatics search identifying targeting seed sequences for *miR-125a* and *miR-125b* within the 3'-untranslated regions of both *ERBB2* and *ERBB3*. Using the human breast cancer cell line SKBR3 as a model for *ERBB2* and *ERBB3* dependence, infection of these cells with retroviral constructs expressing either *miR-125a* or *miR-125b* resulted in suppression of *ERBB2* and *ERBB3* at both the transcript and protein level. Luciferase constructs containing the 3' 3'-untranslated regions of *ERBB2* and *ERBB3* demonstrated ~35% less activity in *miR-125a*- and *miR-125b*-expressing cells relative to controls. Additionally, phosphorylation of ERK1/2 and AKT was suppressed in SKBR3 cells overexpressing either *miR-125a* or *miR-125b*. Consistent with overexpression of both *ERBB2* and *ERBB3* signaling, *miR-125a*- or *miR-125b*-overexpressing SKBR3 cells were impaired in their anchorage-dependent growth and exhibited reduced migration and invasion capacities. Parallel studies performed on MCF10A cells demonstrated that *miR-125a* or *miR-125b* overexpression produced only marginal influences on the growth and migration of these non-transformed human mammary epithelial cells. These results illustrate the feasibility of using miRNAs as a therapeutic strategy to suppress oncogene expression and function.

Micro-RNAs (miRNAs)<sup>2</sup> comprise a large class of regulatory noncoding RNAs capable of exerting pronounced influences upon the translation and stability of mRNAs (1–4). Efforts to understand their biogenesis and target specificity have become

active areas of research as their role in such processes as development, differentiation, and apoptosis comes into focus (1–4). Additional impetus to understanding their biology has emerged from growing evidence implicating miRNA deregulation in cancer etiology (5–8).

More than 400 human miRNAs have been identified to date with up to ~1000 miRNAs postulated to exist on the basis of bioinformatics analyses, leading to the speculation that a majority of human mRNAs are subject to miRNA regulation (9–10). miRNAs are initially excised from a primary RNA transcript in the nucleus by the nuclear RNase III endonucleases Drosha working in cooperation with DGCR8 (4, 11). Export to the cytoplasm is facilitated by exportin-5, where the ~70-nt pre-miRNA is further processed by the RNase III endonuclease Dicer into a mature ~22-nt double-stranded miRNA (1–4). Subsequent incorporation of one strand into RISC (RNA-induced silencing complex) establishes the miRNA as competent to target mRNAs for translational arrest and, in some instances, transcript decay (1–3). Although targeting of a mRNA by a miRNA remains a poorly defined process, it appears to be largely mediated by complementarity between nucleotides 2 to 5 (numbered from the miRNA 5' end), occasionally referred to as the “seed sequences,” with a target element in the transcript 3'-UTR (1–3, 9, 12).

Involvement of miRNAs in the oncogenic process has been supported by the observation that the 13q14 deletion characterizing more than half of all chronic lymphocytic leukemias results in loss of *miR-15a* and *miR-16-1* genes (5). Additional studies using microarray profiling have demonstrated aberrant expression of miRNAs in human cancers, with deregulation of specific miRNAs distinguishing different cancer types (7, 8). One breast cancer study concluded that two up-regulated miRNAs (*miR-21*, *miR-155*) might act as oncogenes, whereas three other down-regulated miRNAs (*miR-10b*, *miR-125b*, and *miR-145*) might act as breast cancer suppressor genes (8). Of interest, these same breast cancer suppressing miRNA candidates, *miR-10b*, *miR-125b* (along with its homolog, *miR-125a*), and *miR-145*, were also identified in a recent study as being significantly down-regulated in *ERBB2*-amplified and -overexpressing breast cancers clinically matched against *ERBB2*-negative human breast cancers (13).

Given this involvement of miRNAs with cancer development, the manipulation of cellular miRNA levels has emerged as a potential strategy for therapeutic intervention (14–16). Efforts to predictably alter intracellular transcript profiles by increasing specific miRNA levels either through transfection or viral delivery methods have demonstrated the potential of this

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<sup>2</sup> The abbreviations used are: miRNA, micro-RNA; RISC, RNA-induced silencing complex; nt, nucleotide(s); UTR, untranslated region; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase.

## ERBB2 and ERBB3 Suppression by miR-125a/b Micro-RNA

strategy to modulate cellular physiology (12, 14). Conversely, attempts to reduce miRNA levels using biologically stable antisense moieties such as 2'-O-methyl oligonucleotides or "antagomirs" have also proven capable of altering intracellular transcript profiles (15, 16).

A bioinformatics comparison of the 3'-UTRs of *ERBB2* and *ERBB3* revealed a consensus target element for both *miR-125a* and *miR-125b*; thus, the present study was undertaken to assess *ERBB2* and *ERBB3* suppression after retroviral delivery and overexpression of these miRNAs in an *ERBB2*-amplified and -overexpressing human breast cancer cell line, SKBR3. Overexpression of *miR-125a* or *miR-125b* reduced *ERBB2* and *ERBB3* at both the transcript and protein level in these cells, leading to reduced ERK1/2 and AKT signaling. Functionally, *miR-125a*- or *miR-125b*-overexpressing SKBR3 cells displayed diminished plating and anchorage-dependent growth in addition to markedly reduced cell migration and invasion capacities. Parallel studies performed on MCF10A cells demonstrated that *miR-125a* or *miR-125b* overexpression produced only marginal influences on the anchorage-dependent growth and migration of these non-transformed and *ERBB2*-independent human mammary epithelial cells.

### EXPERIMENTAL PROCEDURES

**Cell Lines, Reagents, and Supplies**—The *ERBB2*-dependent human breast cancer cell line, SKBR3, and the non-transformed and *ERBB2*-independent human mammary epithelial cell line, MCF10A, were obtained from the American Type Culture Collection (Manassas, VA). SKBR3 cells were maintained in McCoy's media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. MCF10A cells were maintained in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 5% fetal bovine serum, 20 ng/ml recombinant EGF (Invitrogen), 0.5  $\mu$ g/ml hydrocortisone (Sigma), 100 ng/ml cholera toxin (Sigma), and 10  $\mu$ g/ml insulin (Sigma). Antibodies used in this study included the *ERBB2* mouse monoclonal antibody Ab-3 from EMD Biosciences (La Jolla, CA), *ERBB3* mouse monoclonal antibody Ab-6 from NeoMarkers (Fremont, CA), rabbit polyclonal antibodies to total ERK1/2, phospho-Ser-ERK1/2, total AKT and phospho-Ser-AKT from Cell Signaling Technology Inc. (Beverly, MA), and mouse monoclonal antibody to  $\beta$ -actin from Abcam (Cambridge, MA). Invasion and migration assays were performed using 24-well formatted BioCoat Matrigel Invasion Chambers and 24-well formatted 8- $\mu$ m PET track-etched porous membranes, respectively, commercially obtained from BD Biosciences (Bedford, MA).

**Micro-RNA Data Base and Retroviral Expression of miRNAs**—Nucleotides 2–7 from the 5' end of miRNA of the 321 human miRNA listed in the Sanger miRBase 7.0 release were searched for complementarity to the 3'-UTRs of *ERBB2* (Ensemble gene ENSG00000141736) and *ERBB3* (Ensemble gene ENSG00000065361). Full-length human miRNAs *miR-125a* and *miR-125b* together with  $\sim$ 200 bp of flanking sequence were amplified from human genomic DNA and cloned into the pTOPO-D entry vector (Invitrogen). Cloned fragments were verified by sequencing, and recombinase was cloned into the pMSCVpuro expression vector (Clontech).

Primers used for the genomic amplification of *miR-125a* and *miR-125b* were: *miR-125a*, 5'-caccggcttctgtgtctctttcacag-3' (forward) and 5'-gacgggacggcactggccagag-3' (reverse); *miR-125b*, 5'-cacctgtatgtgcgtgattgtatag-3' (forward) and 5'-cctcaggcatcaataacatgca-3' (reverse).

Infectious and replication-incompetent retroviral particles were produced as previously described (17). SKBR3 and MCF10A cells were infected with retroviral particles expressing *miR-125a*, *miR-125b* or only the puromycin resistance gene (*puro* control). After retroviral infection and primary puromycin selection at 0.5  $\mu$ g/ml, cell pools of SKBR3-*125a*, SKBR3-*125b*, and SKBR3-*puro* together with MCF10A-*125a*, MCF10A-*125b*, and MCF10A-*puro* were maintained for multiple culture passages under lower puromycin concentration (0.25  $\mu$ g/ml).

**3'-UTR-luciferase Reporter Constructs and SKBR3 Transient Transfection Assays**—EST clones containing the 3'-UTR sequences from *ERBB2* and *ERBB3* cDNA were commercially obtained (Invitrogen) and used as templates for PCR amplification of the full-length or deleted (nt 1–44 and 1–26 for deleted *ERBB2* and *ERBB3* UTR constructs, respectively) 3'-UTR products. PCR products were cloned into the XbaI site of the luciferase reporter pGL3 (Promega, Madison, WI) to yield p*ERBB2*wtUTR, p*ERBB2* $\Delta$ UTR, p*ERBB3*wtUTR, and p*ERBB3* $\Delta$ UTR containing the wild type and deleted 3'-UTRs from *ERBB2* and *ERBB3*, respectively. The 3'-UTR inserts from all constructs were sequence-verified. Transient transfection assays were performed in 12-well plates seeded with  $\sim$ 1.5  $\times$  10<sup>5</sup> SKBR3-*125a*, SKBR3-*125b*, or SKBR3-*puro* cells per well using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. For each transfection experiment, equal volumes of a given DNA-lipid complex formulation were distributed in duplicate to each of the SKBR3-*125a*, SKBR3-*125b*, and SKBR3-*puro* cultures with at least three separate transfection experiments performed for each 3'-UTR construct. The  $\beta$ -galactosidase expression vector pCH110 (Amersham Biosciences) was co-transfected with each experiment for normalization of transfection efficiency. Because both the pCH110 and pGL3 co-transfected constructs use the SV40 promoter, potential promoter differences resulting from *miR-125a* or *miR-125b* overexpression were presumed nullified.

**Northern and Western Blotting**—Total RNA extracted using Trizol (Invitrogen) from retrovirally infected SKBR3 and MCF10A cells was analyzed by Northern blotting. Methods and probes described previously (18) were used to detect *ERBB2* (4.6 kilobases) and *ERBB3* (4.9 kilobases) transcripts relative to control glyceraldehyde-3-phosphate dehydrogenase transcripts. Levels of enforced and endogenous expression of *miR-125a* and *miR-125b* were detected using 15% Tris borate-EDTA urea polyacrylamide gels (Invitrogen), with RNA transferred onto Hybond Plus membranes (Amersham Biosciences) as previously described (18) and probed with antisense *miR-125a* or *miR-125b* DNA oligonucleotides that were <sup>32</sup>P end-labeled. Loading for small RNA species was controlled by probing with a <sup>32</sup>P end-labeled antisense snU6 DNA oligonucleotide (5'-ttgcgtgtcatccttgccgag-3'). Total protein extracted from retrovirally infected SKBR3 sublines was analyzed by Western blotting using methods and antibodies

described previously (19) to detect endogenous *ERBB2* and *ERBB3* receptor levels relative to  $\beta$ -actin and phospho-Ser-ERK1/2 and phospho-Ser-AKT relative to total ERK1/2 (42/44 kDa) and AKT.

**Cell Proliferation, Migration, and Invasion Assays**—All cell proliferations assays were replicated in three independent experiments using the retrovirally infected cell pools (SKBR3-125a, SKBR3-125b, SKBR3-puro, MCF10A-125a, MCF10A-125b, and MCF10A-puro). In each experiment, cells were trypsinized, counted, and plated into duplicate wells of 6-well plates at  $2 \times 10^5$  cells per well. Total cell counts were obtained and averaged on days 2, 4, and 6 after plating. All cell migration and invasion assays were similarly replicated three times using independently infected cell pools. Cells were trypsinized, pelleted, and resuspended in media without serum or growth factors. After counting,  $1 \times 10^4$  SKBR3 cells in 0.5 ml of medium were added to either 8- $\mu$ m porous chamber inserts or 8- $\mu$ m porous BioCoat Matrigel chamber inserts (BD Biosciences) and placed in wells filled with 0.75 ml of medium supplemented with 10% fetal calf serum as chemoattractant for both migration and invasion assays. MCF10A cells are capable of migration but not Matrigel invasion; after counting,  $3 \times 10^4$  MCF10A cells in 0.5 ml of medium were added to 8- $\mu$ m porous chamber inserts placed in wells filled with 0.75 ml of medium supplemented with 5% fetal calf serum and growth factors (see above) as chemoattractant. After 20 h of incubation, cells remaining on the insert top layers were removed with cotton swab scrubbing as recommended by the manufacture. Cells on the membrane lower surfaces were fixed in 100% methanol for 2 min followed by staining (2 min) in a 1% toluidine blue solution prepared in phosphate-buffered saline. Inserts were washed several times in distilled water before air drying; membranes were photographed using an Olympus I  $\times$  70 microscope at 70 $\times$ , and cell counts were determined after totaling five random fields (each field capturing  $\sim$ 6% of total membrane area) and expressed as a percentage of the control cell number.

## RESULTS

**Conserved miR-125 Targeting Element Identified within the 3'-UTRs of ERBB2 and ERBB3 and Retroviral Overexpression of miR-125 Species in Human Mammary Epithelial Cells**—To explore the strategy of externally delivered miRNAs to promote the down-regulation of proteins with known oncogenic properties, the *ERBB* family, consisting of *ERBB1/EGFR* (epidermal growth factor receptor), *ERBB2*, *ERBB3*, and *ERBB4* family members, was chosen for miRNA targeting. A bioinformatics search of *ERBB* family 3'-UTRs for complementarity to miRNA seed sequences (nt 2–7 from the 5' end), as listed in the Sanger miRBase data base (Version 7.0 release), focused attention on *miR-125a* and *miR-125b*. The most proximal 3'-UTR regions of both *ERBB2* and *ERBB3* were found to exhibit perfect complementarity with the 5' nucleotides, nt 1–8, of *miR-125a* and *miR-125b*. Interest in the *miR-125* family was also supported by its evolutionary link to *lin-4*, the first identified miRNA species functionally known to regulate *Caenorhabditis elegans* cellular proliferation (21, 22) as well as recent identification of this miRNA family as candidate breast cancer suppressors (8). As shown in Fig. 1A, the maximum free energy

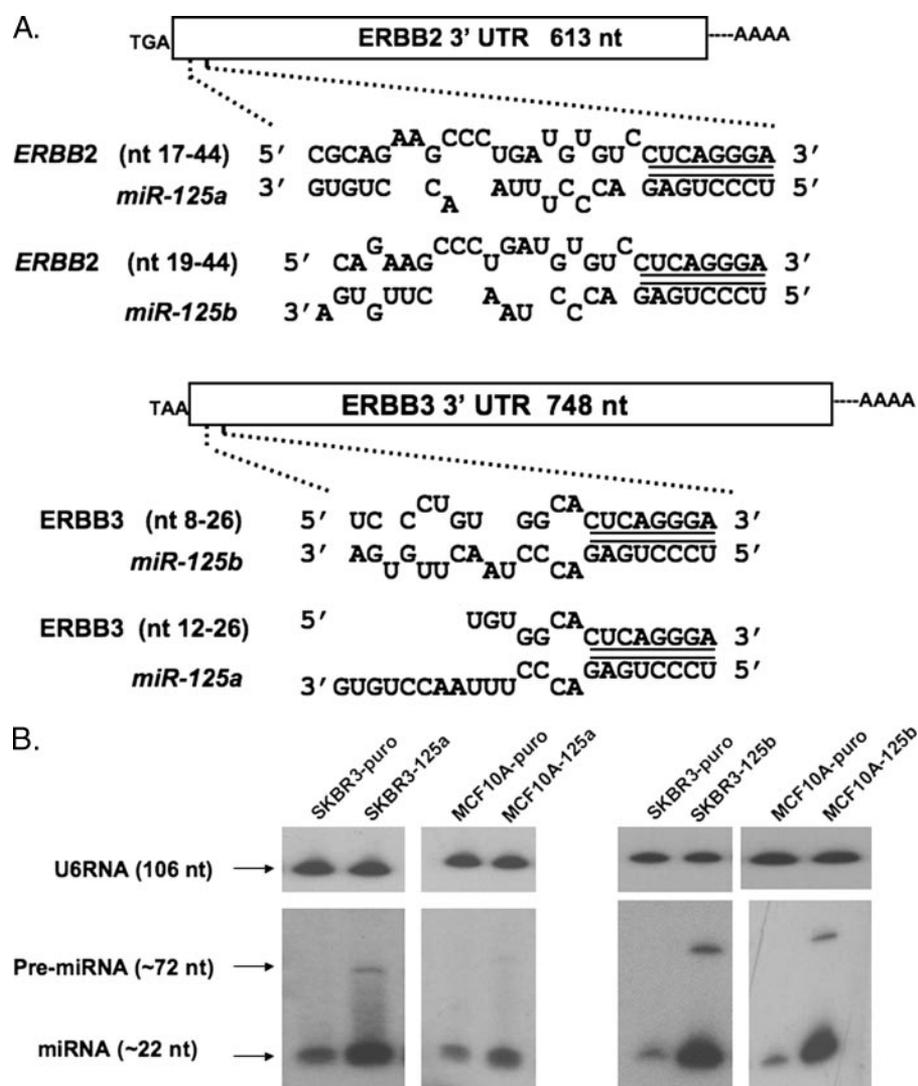
predicted hybridization configurations between the putative 3'-UTR *miR-125* target elements in *ERBB2* and *ERBB3* and the seed sequences in *miR-125a* or *miR-125b* all exhibit similar stabilities at  $\Delta G \sim -14$  kcal/mol, determined by mFold analysis and consistent with authentic miRNA targeting (20).

The use of viral constructs offers the possibility of rapidly producing high intracellular levels of mature miRNA via the endogenous miRNA processing pathway. To this end,  $\sim$ 300-bp fragments containing genomic *miR-125a* and *miR-125b* sequences were obtained by PCR amplification from human DNA and cloned into pMSCV, a retrovirus producing high transcript levels driven by its long terminal repeat. A human breast cancer cell line, SKBR3, dependent upon endogenous *ERBB2* amplification and receptor overexpression for its growth and survival, and the non-transformed and *ERBB2*-independent human mammary epithelial cell line, MCF10A, were retrovirally infected and selected for viral integration by puromycin selection. As shown in the Northern blots of Fig. 1B, enforced expression of mature *miR-125a* and *miR-125b* was achieved in both cell lines at  $\sim$ 7–10-fold increases above their endogenous miRNA levels.

**SKBR3 Overexpression of miR-125a or miR-125b Suppresses ERBB2 and ERBB3 Transcript and Protein Expression and Inhibits Signaling by ERK1/2 and AKT Pathways**—After retroviral infection and  $\sim$ 10 days of puromycin selection, total RNA and protein extracts were prepared from pooled SKBR3 cells showing either *miR-125a* or *miR-125b* overexpression and from control cells similarly infected but with the pMSCV empty vector and puromycin selected. As shown in the Northern blots of Fig. 2A, *ERBB2* transcript levels in the SKBR3-125a and SKBR3-125b cells were 52 and 80%, respectively, of SKBR3-puro control levels after loading normalization by glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels. Likewise, *ERBB3* mRNA levels in SKBR3-125a and SKBR3-125b cells were 67 and 90%, respectively, that of SKBR3-puro control levels after normalization. As shown in the Western blots of Fig. 2B, *ERBB2* protein levels were reduced to 40 and 65% that of control (SKBR3-puro) levels in SKBR3-125a and SKBR3-125b cells, respectively, whereas *ERBB3* protein levels were reduced to 60 and 80% that of control levels, respectively, after loading normalization by  $\beta$ -actin levels.

The downstream signaling response of *ERBB2* and *ERBB3* receptor activation and heterodimerization includes serine phosphorylation of ERK1/2 and AKT pathway intermediates. Thus, the basal activation status of these two signal transduction pathways were assessed by Western blotting protein extracts isolated from SKBR3-125a and SKBR3-125b cells relative to those from control SKBR3-puro cells. As shown in the Western blots of Fig. 2B, phospho-ERK1/2 (*pERK1/2*) and phospho-AKT (*pAKT*) levels relative to total ERK1/2 and total AKT levels were significantly reduced in the *miR-125a*- and *miR-125b*-overexpressing SKBR3 cells, with the former showing greater suppression than the latter, consistent with the more pronounced *ERBB2* and *ERBB3* protein suppression observed in the SKBR3-125a cells compared with the SKBR3-125b cells.

## ERBB2 and ERBB3 Suppression by miR-125a/b Micro-RNA



**FIGURE 1. Minimum free energy states of hybridization between miR-125a or miR-125b with conserved ERBB2 and ERBB3 3'-UTR target elements and retroviral overexpression of miR-125a and miR-125b in SKBR3 and MCF10A breast epithelial cells.** A, predicted hybridization of miR-125a and miR-125b with the conserved ERBB2 and ERBB3 target element CUCAGGGA (underlined), as determined by the mFold Server. Non-bonding bases protrude above and below the duplexed UTR and miRNA bases, respectively, with mapping of the target element into the ERBB2 and ERBB3 3'-UTRs as indicated. B, Northern analyses of total RNA prepared from SKBR3 and MCF10A cells after infection and puromycin selection using retroviral constructs expressing either miR-125a (SKBR3-125a, MCF10A-125a), miR-125b (SKBR3-125b, MCF10A-125b), or empty vector controls (SKBR3-puro, MCF10A-puro). Northern blots in the left two panels were probed for miR-125a, whereas Northern blots in the right two panels were probed for miR-125b. Mature and pre-miRNA species are noted, and equal RNA loading was confirmed by probing for the small RNA species, U6 RNA.

**Analysis of ERBB2 and ERBB3 3'-UTRs for miR-125 Responsiveness**—To examine the 3'-UTRs from ERBB2 and ERBB3 for responsiveness to miR-125a or miR-125b overexpression, the full-length 3'-UTR sequences from ERBB2 and ERBB3 cDNAs were cloned into the luciferase expressing pGL3-promoter vector using a unique XbaI site just downstream of the luciferase stop codon to create pERBB2wtUTR and pERBB3wtUTR expression constructs, respectively, for transient transfection into SKBR3-125a, SKBR3-125b, and SKBR3-puro cells. As shown in Fig. 3A, luciferase activities expressed off the pERBB2wtUTR or pERBB3wtUTR constructs were suppressed by 60–72% in the miR-125a- and miR-125b-expressing SKBR3 cells relative to luciferase activity expressed in the SKBR3-puro control cells. To confirm the involvement of

miR-125a and miR-125b in this reporter gene suppression, 3'-UTR deletion constructs ( $\Delta$ UTR) were made that eliminated the conserved CUCAGGGA-targeting elements from the ERBB2 and ERBB3 UTR reporters; bases 1–44 were deleted to create pERBB2 $\Delta$ UTR, whereas bases 1–26 were deleted to create pERBB3 $\Delta$ UTR. In experiments where the full-length and deleted UTR constructs for ERBB2 and ERBB3 were each transiently transfected into SKBR3-125a and SKBR3-125b cells, luciferase activity from pERBB2 $\Delta$ UTR was ~1.4-fold greater than that from pERBB2wtUTR, whereas the luciferase activity from pERBB3 $\Delta$ UTR was ~1.7-fold greater than activity from pERBB3wtUTR, as shown in Fig. 3B. Also seen in Fig. 3B is the much lower but still slightly increased (1.1-fold) luciferase activity resulting from the UTR deletion constructs (relative to their respective wild type UTR constructs) in the SKBR3-puro control cells, possibly reflecting the endogenous levels of miR-125a and miR-125b in these cells (as shown in Fig. 1B).

**Overexpression of miR-125a or miR-125b Impairs Anchorage-dependent Growth of SKBR3**—The anchorage-dependent growth consequences of enforced miR-125a or miR-125b overexpression were compared in the ERBB2-dependent SKBR3 and ERBB2-independent MCF10A cell lines. All experiments were performed on pooled puromycin-selected cells within 2–3 weeks after their retroviral infection and initial selection and while under

puromycin selection pressure. As shown in Fig. 4A, within 2 days of plating an equal number of viable SKBR3-125a, SKBR3-125b, and SKBR3-puro cells, only 50 and 75% of the miR-125a- and miR-125b-overexpressing cells, respectively, remained viably attached and growing relative to the SKBR3-puro control cells. After this initial 2-day interval, growth rates estimated by linear extrapolation out to day 6 indicated an apparent 60% slower growth rate by the SKBR3-125a and SKBR3-125b cells relative to the SKBR3-puro cells. In contrast to the impaired anchorage-dependent growth associated with miR-125a or miR-125b overexpression in the SKBR3 cells, Fig. 4B shows that the similarly produced and miRNA-overexpressing MCF10A-125a and MCF10A-125b cells exhibited little if any impaired plating and growth within the first 2 days relative to control

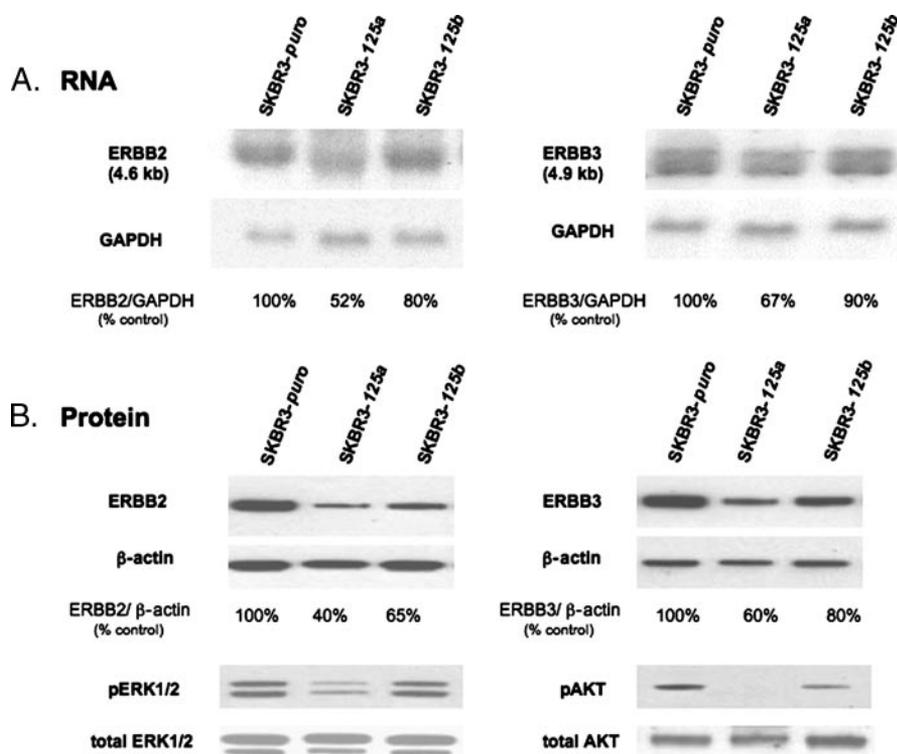


FIGURE 2. Northern and Western analyses of SKBR3 cells overexpressing miR-125a or miR-125b. A, Northern blots using total RNA isolated 2 weeks after retroviral infection and puromycin selection from control (SKBR3-puro) or miRNA-overexpressing cell pools (SKBR3-125a, SKBR3-125b) probed for ERBB2 or ERBB3 transcripts. ERBB2 and ERBB3 mRNA band intensities normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA are shown below the lanes as percent of the respective control value (100%). B, Western blots of total protein extracted from the same SKBR3 cell pools as above were probed for ERBB2 or ERBB3 receptor content relative to  $\beta$ -actin protein and phospho (p)-Ser-ERK1/2 or phospho-Ser-AKT relative to total ERK1/2 and AKT protein content. ERBB2 and ERBB3 band intensities normalized by  $\beta$ -actin are shown below the lanes as percent of the respective control value (100%).

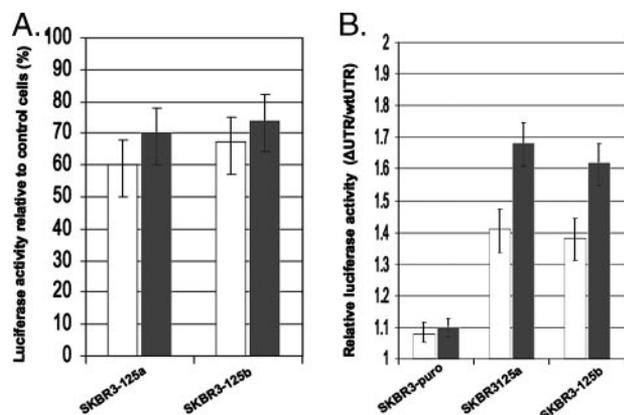


FIGURE 3. Influence of miR-125a and miR-125b overexpression on ERBB2- and ERBB3 3'-UTR-regulated reporters in SKBR3 cells. A, SKBR3 cells overexpressing miR-125a (SKBR3-125a) or miR-125b (SKBR3-125b) and control SKBR3 cells (SKBR3-puro) were transiently transfected with luciferase reporters containing either the wild type ERBB2 3'-UTR (pERBB2wtUTR) or wild type ERBB3 3'-UTR (pERBB3wtUTR). Luciferase activities of pERBB2wtUTR and pERBB3wtUTR in SKBR3-125a and SKBR3-125b cells were normalized by their respective activities in control cells (SKBR3-puro) and plotted as percent activity relative to control cells. Open bars display ERBB2 3'-UTR activity, whereas solid bars display ERBB3 3'-UTR activity. B, SKBR3-125a, SKBR3-125b, and SKBR3-puro cells were transiently transfected with luciferase reporters containing either wild type ERBB2 or ERBB3 3'-UTR sequences as in panel A or UTR reporters deleted of their respective 3'-UTR miR-125 target elements (pERBB2 $\Delta$ UTR, pERBB3 $\Delta$ UTR), as described under "Experimental Procedures." Open bars display relative ERBB2 3'-UTR luciferase activity (pERBB2 $\Delta$ UTR/pERBB2wtUTR), whereas solid bars display relative ERBB3 3'-UTR luciferase activity (pERBB3 $\Delta$ UTR/pERBB3wtUTR).

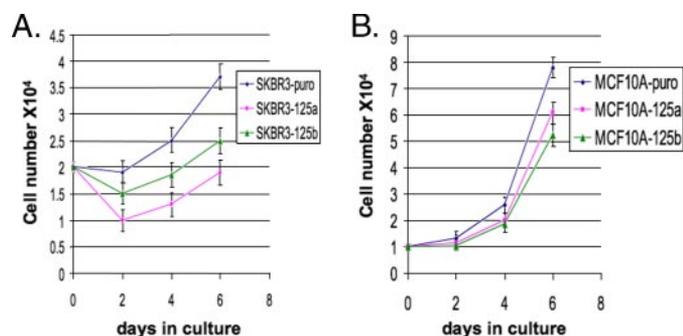
MCF10A-puro cells. Extrapolating their growth rates out to day 6 indicated an apparent 25% slower growth rate by the MCF10A-125a and MCF10A-125b cells relative to the control MCF10A-puro cells, suggesting a mild negative influence by these miRNAs on cellular proliferation mechanisms independent of ERBB2 status and possibly related to the reported tumor suppressing function of miR-125a and miR-125b (8).

Overexpression of miR-125a or miR-125b Impairs Migration and Invasiveness of SKBR3—Because previous studies have linked ERBB2 receptor activation and ERBB3 heterodimerization with cellular motility and invasiveness (23, 24), a Boyden chamber assay format was used to measure the effects of miR-125a and miR-125b overexpression on cellular migration and invasion. As shown by the graph and images in Fig. 5A, SKBR3-125a and SKBR3-125b cells exhibited a greater than 50% reduction in their motility relative to control SKBR3-puro cells. In contrast and as shown in Fig. 5B, MCF10A-125a and MCF10A-125b cells exhibited less than a 10%

reduction in their motility relative to control MCF10A-puro cells. Invasiveness, requiring both cell motility and proteolytic properties enabling advancement through a solid and proteinaceous extracellular matrix like Matrigel, is thought to more directly assess cell metastatic potential. Although immortalized and capable of motility comparable with SKBR3 cells, the non-transformed MCF10A cells are incapable of significant invasiveness through an extracellular matrix. Although the invasiveness of SKBR3 cells may be lower than that of other much more aggressive breast cancer cell lines (e.g. MDA-231), it is easily measurable as are the effects of miR-125a or miR-125b overexpression on SKBR3 invasiveness. As shown in Fig. 5C, SKBR3-125a and SKBR3-125b cells displayed pronounced impairments in their invasive potentials, with the former showing 75% reduction and the latter showing 67% reduction in their proportions of invading/migrating cells relative to SKBR3-puro controls.

### DISCUSSION

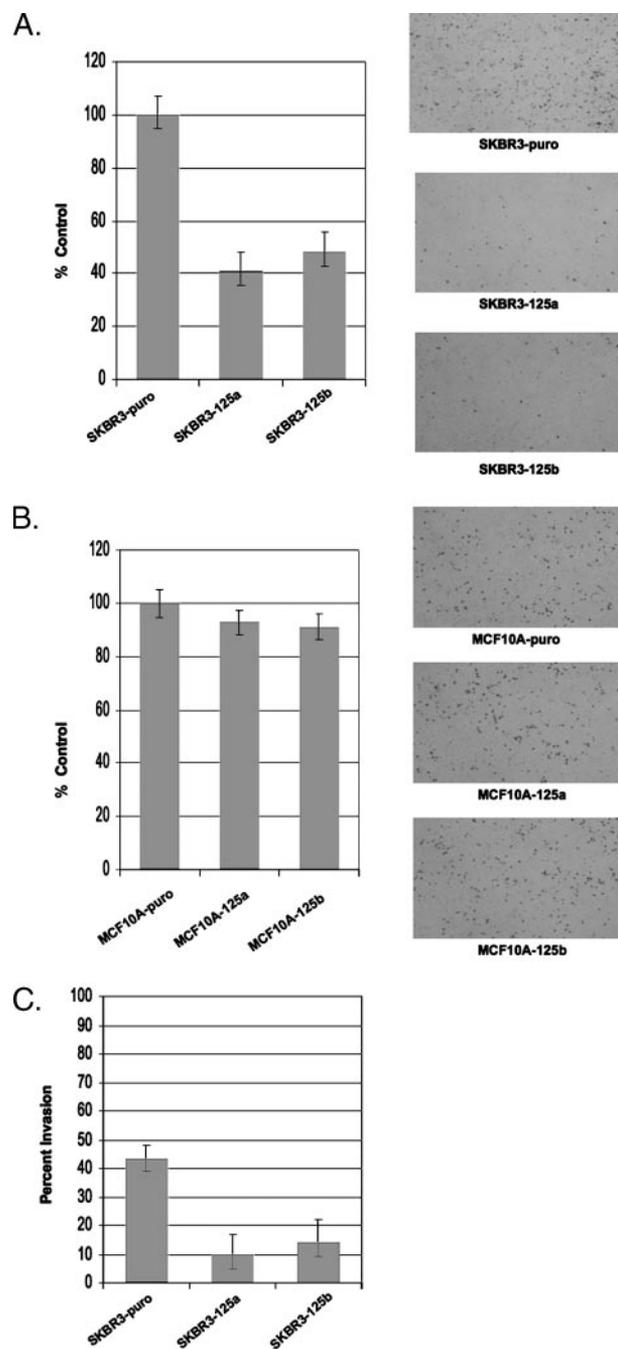
As presented here, retroviral overexpression of miR-125a or miR-125b in the ERBB2-dependent human breast cancer cell line, SKBR3, produced suppression of its anchorage-dependent growth potential and even greater inhibition of its motility and invasive capabilities. Similar retroviral overexpression of these miRNAs in non-transformed and ERBB2-independent MCF10A cells produced marginal effects on its anchorage-de-



**FIGURE 4. Comparative effects of miR-125a or miR-125b overexpression on the anchorage-dependent growth of SKBR3 and MCF10A cell lines.** Retrovirally infected SKBR3-125a, SKBR3-125b and SKBR3-puro control cells (panel A) as well as MCF10A-125a, MCF10A-125b, and MCF10A-puro control cells (panel B) were seeded into replicate wells of 6-well culture plates on day 0 ( $2 \times 10^5$  cells/well). Adherent cells were counted on days 2, 4, and 6, and mean values are plotted as shown; error bars represent the S.D. of mean values from three independent experiments.

pendent growth and no significant impact on the motility of these non-invasive human breast epithelial cells. Such phenotypic alterations seen in the aneuploid and *ERBB2*-amplified SKBR3 breast cancer model and not observed in the diploid MCF10A breast epithelial model are consistent with inhibition of the overexpressed *ERBB2* and *ERBB3* transcript and protein levels in SKBR3, caused by enforced expression of either *miR-125a* or *miR-125b*, two closely related miRNAs that appear capable of targeting the cognate miR-125 target element, CUCAGGGA, found conserved within the proximal 3'-UTR sequences of human *ERBB2* and *ERBB3*. The observed reduction in *ERBB2* and *ERBB3* protein levels may be reasonably attributed to miRNA effects on *ERBB2* and *ERBB3* transcript stability and translation, as a previous report indicates that *miR-125a* and *miR-125b* can bifunctionally mediate both transcript decay and translational inhibition (22). Although the molecular mechanisms explaining MCF10A cell immortalization remain unclear, these motile and proliferating cells express little or no *ERBB2* or *ERBB3* and do not respond to any *ERBB*-targeted therapeutics. The possibility that *miR-125a* and *miR-125b* target other growth regulating mechanisms independent of *ERBB2* and *ERBB3* may be surmised by the slight growth inhibitory response (25% reduction) observed in MCF10A-125a and MCF10A-125b cells relative to control MCF10A-puro cells and also the purported tumor suppressing function of *miR-125a* and *miR-125b* in human breast cancers independent of *ERBB2* status (8).

Although only a single *ERBB* homologue exists in invertebrates such as *C. elegans* and *Drosophila*, there are four *ERBB* paralogs expressed in higher organisms encoding distinct transmembrane receptors capable of homo- or heterodimerization, activated by numerous epithelial cell growth factor ligands and coordinately transducing a complex network of intracellular signaling pathways used for epithelial cell development, proliferation, and survival (25). Dysregulation of three of these four receptor-encoding paralogs (*ERBB1/EGFR*, *ERBB2*, *ERBB3*) singly or in combination can induce malignant transformation. About 25% of all human breast cancers are associated with amplification and overexpression of *ERBB2*, and the aggressive tumorigenic properties of such *ERBB2*-positive



**FIGURE 5. Inhibitory effects of miR-125a or miR-125b overexpression on migration and invasion of SKBR3 cells.** A, migration of SKBR3-125a and SKBR3-125b cells across 8- $\mu$ m porous membranes relative to control SKBR3-puro cells. Error bars represent the S.D. of mean values (expressed as percent of control) from three independent experiments. Photomicrographs on the right represent 70 $\times$  images ( $\sim$ 6% of total membrane area) of toluidine blue-stained membranes used to obtain cell migration counts for each of the SKBR3 cell pools. B, migration of MCF10A-125a and MCF10A-125b cells across 8- $\mu$ m porous membranes relative to control MCF10A-puro cells. Error bars represent the S.D. of mean values (expressed as percent of control) from three independent experiments. Photomicrographs on the right represent 70 $\times$  images of toluidine blue-stained membranes used to obtain cell migration counts for each of the MCF10A cell pools. C, percentage of SKBR3-puro, SKBR3-125a, and SKBR3-125b cells invading through BioCoat Matrigel inserts and across 8- $\mu$ m porous membranes relative to the number of cells from each cell pool migrating across the porous membranes without Matrigel inserts. Error bars represent the S.D. of mean values (expressed as percent of control) from three independent experiments.

breast cancers are dependent on both homodimerization of *ERBB2* receptors as well as their heterodimerization with either *ERBB1/EGFR* or *ERBB3* receptors. In particular, overexpressed *ERBB2-ERBB3* heterodimers are thought to be most critical for the aggressive growth and metastatic potential of *ERBB2*-positive human breast cancers, since it is the transphosphorylated *ERBB3* partner of *ERBB2* that can recruit up to six individual p85 subunits and constitutively activate intracellular phosphatidylinositol 3-kinase, induce serine phosphorylation of AKT, and thereby drive cell survival, proliferation, motility, and invasion mechanisms characteristic of this aggressive form of human breast cancer (23, 24, 26). Consequently, as seen here, the coordinated suppression of both *ERBB2* and *ERBB3* expression in an *ERBB2*-positive breast cancer cell line would be expected to most significantly inhibit phospho-AKT levels and impair not only its proliferative potential but also its motility and invasive capabilities.

Recognizing the mechanistic importance of *ERBB2* heterodimer formation in human breast tumorigenesis, pharmaceutical companies are now developing second-generation kinase inhibitors that simultaneously target multiple members of the *ERBB* receptor family (Ref. 27; e.g. lapatinib/Tykerb<sup>®</sup>, an inhibitor of *ERBB2* and *ERBB1/EGFR* kinases (GlaxoSmithKline) and canertinib/CI-1033, an inhibitor of *ERBB2*, *ERBB1/EGFR*, and *ERBB4* kinases, Pfizer). Although there now exist promising clinical candidates capable of targeting the kinase functions of both *ERBB1/EGFR* and *ERBB2*, there are as yet no specific inhibitors of the docking or signal-initiating functions of *ERBB3*. It was, therefore, of considerable interest when a computational search among *ERBB* family members identified a conserved 8-nt *miR-125* binding element, CUCAGGGA, encoded within the proximal 3'-UTRs of both *ERBB2* and *ERBB3*. Free energy determinations for the predicted hybridization configurations between the putative 3'-UTR *miR-125* target elements in *ERBB2* and *ERBB3* and the seed sequences in *miR-125a* or *miR-125b* are consistent with authentic miRNA targeting (20). Aside from identifying a conserved *miR-125* targeting element in both *ERBB2* and *ERBB3*, BLAST analysis also identified homologous U-rich regions in the 3'-UTRs of *ERBB2* and *ERBB3*. Such U-rich 3'-UTR regions have been reported in other genes to be essential for the mediation of miRNA-induced transcript destabilization (28). Luciferase constructs containing the 3'-UTRs of *ERBB2* and *ERBB3* demonstrated significantly less activity in *miR-125a*- and *miR-125b*-overexpressing SKBR3 cells relative to controls. To confirm that the 3'-UTR elements containing the candidate *miR-125a* and *miR-125b* targeting elements actually mediated this reporter gene repression, deletion constructs missing these *miR-125* targeting elements were generated and similarly transfected into SKBR3-*125a*, SKBR3-*125b*, and control SKBR3-*puro* cells. These deletion experiments demonstrated reversal of the reporter gene repression caused by the intact (wild type) *ERBB2* and *ERBB3* UTRs but only in the context of *miR-125a* or *miR-125b* overexpression. Thus, whereas not explicitly identifying the 8-nt *miR-125* binding element as responsible for this repressive influence, these deletion constructs localized *miR-125* responsiveness to very small regions within the proximal

3'-UTRs of *ERBB2* and *ERBB3* containing the conserved CUCAGGGA targeting elements.

Targeting oncogenic *ERBB2* and *ERBB3* overexpression in a model breast cancer cell line like SKBR3 by enforced expression of *miR-125a* and *miR-125b* demonstrates the potential utility of rapid and efficient miRNA gene delivery by retroviruses to interrogate cancer cell phenotypes. However, as a therapeutic tool, delivery of miRNAs by viral or other means must be balanced against their potential to impair normal cellular functions by competing for easily saturated miRNA processing steps. Two such limiting steps appear to involve nuclear export by exportin-5 and the cytoplasmic Dicer partner and RISC component, TRBP. Sequestration of TRBP by transfected RNA has been shown to attenuate overall Dicer-RISC activity (29). Likewise, *in vivo* delivery and sustained intrahepatic viral expression of short hairpin RNAs has produced dose-dependent liver injury in mice, associated with down-regulation of liver-derived miRNAs that also compete for exportin-5 (30).

Commensurate with the successful down-regulation of *ERBB2* and *ERBB3* proteins levels mediated by overexpression of *miR-125a* and *miR-125b*, influences on such critical features of the malignant cell phenotype as proliferative growth, motility, and invasiveness were also observed. Investigational agents have now been identified that alter critical cancer gene programs by rapidly modulating intracellular miRNA profiles (18). Although delivery of a single miRNA can clearly produce a phenotypic disturbance, it has been suggested that miRNA regulation of a given transcript is most efficient when multiple miRNAs work in concert (1–3). With such recent technological advancements as the delivery of multiple miRNAs from a single polycistronic construct (31), future efforts directed toward delivery of multiple and coordinately targeted miRNAs are anticipated to not only enhance the utility of miRNAs as research tools but also provide further rationale for the design of new therapies that modulate miRNA expression.

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