

Regulation of Epidermal Growth Factor Receptor Signaling in Human Cancer Cells by MicroRNA-7*[§]

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The epidermal growth factor receptor (EGFR) is frequently overexpressed in cancer and is an important therapeutic target. Aberrant expression and function of microRNAs have been associated with tumorigenesis. Bioinformatic predictions suggest that the human *EGFR* mRNA 3'-untranslated region contains three microRNA-7 (miR-7) target sites, which are not conserved across mammals. We found that miR-7 down-regulates *EGFR* mRNA and protein expression in cancer cell lines (lung, breast, and glioblastoma) via two of the three sites, inducing cell cycle arrest and cell death. Because miR-7 was shown to decrease *EGFR* mRNA expression, we used microarray analysis to identify additional mRNA targets of miR-7. These included *Raf1* and multiple other genes involved in EGFR signaling and tumorigenesis. Furthermore, miR-7 attenuated activation of protein kinase B (Akt) and extracellular signal-regulated kinase 1/2, two critical effectors of EGFR signaling, in different cancer cell lines. These data establish an important role for miR-7 in controlling mRNA expression and indicate that miR-7 has the ability to coordinately regulate EGFR signaling in multiple human cancer cell types.

The epidermal growth factor receptor (EGFR)⁴, a member of the erbB receptor family, is widely expressed in human tissues

and regulates important cellular processes, including proliferation, differentiation, and development (1). EGFR overexpression occurs in a range of solid tumors and is associated with disease progression, resistance to chemotherapy and radiation therapy, and poor prognosis (2). Consequently, the EGFR and its downstream signaling effectors are major targets for new therapeutics such as monoclonal antibodies and tyrosine kinase inhibitors (3). However, the clinical responses of tumors to existing anti-EGFR agents are often limited, and thus a major research focus is the development of novel approaches to block EGFR expression and signaling (4).

MicroRNAs (miRNAs) are short, endogenous, non-coding RNA molecules that bind with imperfect complementarity to the 3'-untranslated regions (3'-UTRs) of target mRNAs, causing translational repression of the target gene or degradation of the target mRNA (5–7). miRNAs are involved in a range of processes that includes development, differentiation (8), proliferation, and apoptosis (9) and have been implicated in cancer (10). Many miRNA genes are located at sites in the human genome that are frequently amplified, deleted, or rearranged in cancer (11), suggesting that some miRNAs may act as oncogenes (“oncomirs” 12) or tumor suppressors (10). For instance, reduced expression of the *let-7* family of miRNAs is associated with increased Ras oncogene expression and reduced survival in patients with non-small cell lung cancer (NSCLC) (13, 14). In contrast, increased expression of miR-21 in gliomas (15), and breast, colon, lung, pancreas, prostate, and stomach cancers (16) is associated with apoptosis resistance, reduced chemosensitivity, and increased tumor growth (17).

Computational approaches to miRNA target prediction have used criteria such as sequence complementarity between target mRNAs and a “seed” region within the miRNA, and conservation of predicted miRNA-binding sites across 3'-UTRs from multiple species (reviewed in Refs. 18, 19). Recently, additional features that determine target site functionality have been identified (20). Nevertheless, the imperfect complementarity of miRNA and target sequences means that identification and functional validation of authentic miRNA targets remains a major challenge. It has been suggested that miRNAs may have the ability to regulate hundreds or even thousands of target mRNAs (21) and that much of this regulation could occur at the level of mRNA decay (22). Because specific miRNAs have the potential to regulate the expression of several members of a signaling pathway or cellular process (23), we hypothesized a

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⁴ The abbreviations used are: EGFR, epidermal growth factor receptor; KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA, microRNA; NSCLC, non-small cell lung cancer; RT, reverse transcription; qRT-PCR, quantitative RT-PCR; UTR, untranslated region; nt, nucleotide(s); WT, wild type; MT, mutant; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activate protein kinase; MEK, MAPK/ERK kinase; IRS2, insulin receptor substrate 2; PI3K, phosphatidylinositol 3-kinase; ARF4, ADP-ribosylation factor 4; PAK1, p21/Cdc42/Rac1-activated kinase 1; PBS, phosphate-buffered saline.

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role for miRNAs in aberrant EGFR expression and signaling in human cancers. In this study, we identify *EGFR* and *Raf1* as direct targets of miR-7 in cancer cells, with miR-7 blocking EGFR and Raf1 expression by inducing mRNA decay. Furthermore, we demonstrate the potential for miR-7 to act as a tumor suppressor by coordinately regulating the EGFR signaling pathway at multiple levels to repress protein kinase B (Akt) and extracellular signal-regulated kinase 1/2 (ERK1/2) activity in human cancer cell lines.

EXPERIMENTAL PROCEDURES

Cell Culture, miRNA Precursors, and Normal Tissue RNA—A549, MDA-MB-468, U87, DU145, and U373 cell lines were obtained from the American Type Culture Collection (ATCC) and cultured at 37 °C in 5% CO₂ with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Synthetic miRNA precursor molecules corresponding to human miR-7 (Pre-miR miRNA Precursor Product ID: PM10047) and a negative control miRNA (miR-NC; Pre-miR miRNA Precursor Negative Control #1, Product ID: AM17110) were obtained from Ambion. Total RNA (First-Choice) from normal brain, normal lung, and normal breast tissue was purchased from Ambion.

Luciferase Plasmid Construction—pGL3-miR-7-target was generated by ligating annealed DNA oligonucleotides corresponding to the perfect hsa-miR-7 target site (forward: 5'-CAA CAA AAT CAC TAG TCT TCC A-3' and 5'-TGG AAG ACT AGT GAT TTT GTT G-3') to unique SpeI and ApaI sites that were inserted 3' of the luciferase open reading frame of pGL3-control (Promega) firefly luciferase reporter vector (designated pGL3-control-MCS (24)). Full-length *EGFR* 3'-UTR reporter plasmid was synthesized by GenScript Corp. using the entire *EGFR* 3'-UTR (nt 3879–5616 of GenBankTM accession number NM_005228) and the pMIR-REPORT luciferase plasmid backbone (Ambion). Wild-type (WT) *EGFR* target reporter plasmids pGL3-EGFR-A, -B, and -C were generated by cloning annealed oligonucleotides corresponding to nt 4214–4260, nt 4302–4348, and nt 4585–4631, respectively, of *EGFR* (GenBankTM accession number NM_005228) mRNA 3'-UTR into SpeI and ApaI sites in pGL3-control-MCS. Plasmid pGL3-EGFR-D contained a PCR-generated *EGFR* 3'-UTR sequence that spanned the predicted miR-7 target sites B and C. Mutant (MT) reporters were also generated that included three nucleotide substitutions to impair binding of the miR-7 seed sequence to its target. Plasmids pGL3-RAF1-WT and pGL3-RAF1-MT were constructed by cloning annealed DNA oligonucleotides corresponding to nt 2965–3030 of the *Raf1* mRNA 3'-UTR (GenBankTM accession number NM_002880), into the SpeI and ApaI sites in pGL3-control-MCS. The sequence of all plasmids was confirmed by sequencing.

Transfections and Luciferase Assays—Cells were seeded 24 h prior to transfection in 6-well plates or 10-cm dishes and transfected using Lipofectamine 2000 (Invitrogen) with miRNA precursor molecules at final concentrations ranging from 0.1 to 30 nM. Cells were harvested at 12–24 h (for RNA extraction) or 3 days (for protein extraction). For reporter gene assays, cells were seeded in 24-well plates and co-transfected using Lipofectamine 2000 (Invitrogen) with 100 ng of firefly luciferase

reporter DNA and 5 ng of pRL-CMV *Renilla* luciferase reporter DNA as a transfection control. Lysates were assayed for firefly and *Renilla* luciferase activities 24 h after transfection using the Dual Luciferase Report Assay System (Promega) and a Fluostar OPTIMA microplate reader (BMG Labtech). Expression values were normalized to *Renilla* luciferase.

RT-PCR—Total RNA was extracted from cell lines with TRIzol reagent (Invitrogen) and RNeasy columns (Qiagen) and treated with DNase I (Promega) to eliminate contaminating genomic DNA. For qRT-PCR analysis of *EGFR* and *Raf1* mRNA expression, 1 µg of total RNA was reverse transcribed to cDNA with random hexamers and Thermoscript (Invitrogen). Real-time PCR for *Raf1* and *GAPDH* was performed using a Corbett 3000 RotorGene instrument (Corbett Research) with QuantiTect SYBR Green PCR mixture (Qiagen) with primers from Primer Bank (25) (*EGFR-F*, 5'-GCG TTC GGC ACG GTG TAT AA-3'; *EGFR-R*, 5'-GGC TTT CGG AGA TGT TGC TTC-3'; *RAF1-F*, 5'-GCA CTG TAG CAC CAA AGT ACC-3'; *RAF1-R*, 5'-CTG GGA CTC CAC TAT CAC CAA TA-3'; *GAPDH-F*, 5'-ATG GGG AAG GTG AAG GTC G-3'; *GAPDH-R*, 5'-GGG GTC ATT GAT GGC AAC AAT A-3'). Expression of *EGFR* and *Raf1* mRNA relative to *GAPDH* mRNA was determined using the 2^{-ΔΔC_T} method (26).

For analysis of miR-7 expression by qRT-PCR, reverse transcription and PCR were carried out using TaqMan miRNA assay kits (Applied Biosystems) for hsa-miR-7 (Part #4373014) and U44 snRNA (Part #4373384) with a Corbett 3000 RotorGene thermocycler (Corbett Research) according to the manufacturer's instructions. Statistical analyses of qRT-PCR data were performed using GenEx software (MultiD).

Western Blotting—Cytoplasmic protein extracts were prepared as described (24), resolved on NuPAGE 4–12% Bis-Tris gels (Invitrogen), and transferred to polyvinylidene difluoride membranes (Roche Applied Science). Membranes were probed with anti-EGFR mouse monoclonal antibody (1:1,000, Neomarkers, catalog #MS-400-P1), anti-Raf1 mouse monoclonal antibody (1:1,000, Santa Cruz Biotechnology, sc-7267), anti-IRS2 rabbit monoclonal antibody (1:500, Cell Signaling Technology, #4502), anti-Akt rabbit monoclonal antibody (1:1,000, Cell Signaling Technology, #9272), anti-phospho-Akt (Ser-473) rabbit monoclonal antibody (1:500, Cell Signaling Technology, #4060), anti-p44/42 (ERK1/2) MAPK mouse monoclonal antibody (1:750, Cell Signaling Technology, #4696), anti-phospho-p44/42 (P-ERK1/2) MAPK (Thr-202/Tyr-204) rabbit monoclonal antibody (1:750, Cell Signaling Technology, #9101), or anti-β-actin mouse monoclonal antibody (1:15,000, Abcam ab6276–100), prior to detection with ECL Plus detection reagent (GE Healthcare) and ECL-Hyperfilm (GE Healthcare).

Cell Cycle Analysis and Cell Counting—Following trypsinization, cells were permeabilized, stained with propidium iodide, and analyzed on an EPICS XL-MCL (Coulter Corp. flow cytometer). Cell cycle analysis was performed using MultiPlus AV MultiParameter data analysis software (Phoenix Flow Systems). Cells were seeded in 6-cm dishes and assessed 3 days after miR-7 or miR-NC transfection by light microscopy. Five representative fields of view were photographed for each condition. Cells in each field of view were counted manually.

Microarray Expression Profiling and Analysis—Total RNA was isolated from A549 cells transfected for 24 h with miR-7 or miR-NC (30 nM) using TRIzol reagent (Invitrogen) and RNeasy columns (Qiagen) and assessed for quality and integrity using a 2100 Bioanalyzer (Agilent Technologies). Gene expression profiling by microarray hybridization was performed with two experimental replicates by the Lotterywest State Microarray Facility using Human Genome U133 Plus 2.0 array chips (Affymetrix). The raw data were processed using GeneSifter software (VizX Labs). An “all groups must pass” restriction was imposed, with a threshold quality score of “P” (present) required for inclusion in subsequent analysis. Data were normalized to the all means fluorescence and were \log_2 -transformed. Pairwise analysis of the probe intensities of miR-7- and miR-NC-treated sample data sets was performed using Student's *t* tests (two-tailed, unpaired), and used to identify transcripts that were significantly up-regulated or down-regulated with miR-7 transfection ($p < 0.05$) by at least a factor of 2. These represented potential miR-7 target transcripts.

Investigation of the enrichment of gene sets for predicted miRNA targets was conducted using the L2L microarray analysis tool (27). miRanda (28), PicTar (29), and TargetScan (21) were used for miR-7 target predictions. Analysis of the enrichment of gene sets for functional Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was performed with GeneSifter software (VizX Labs). Microarray expression data have been deposited in the Gene Expression Omnibus under Accession Number GSE14507.

RESULTS

EGFR 3'-UTR Contains Multiple, Specific Target Sequences for miR-7—As EGFR expression is regulated in part via *cis*-acting 3'-UTR mRNA stability sequences (30), we sought to identify miRNAs that could regulate EGFR gene expression in human cells. TargetScan (21) identified three putative miR-7 target sites (A, B, and C, Fig. 1A). The 3'-end of each site contains the hexamer motif UCUUCC, which is complementary to the seed region (nt 2–7) of human miR-7 (hsa-miR-7) (Fig. 1B). When contextual features of miRNA target sites associated with functionality were taken into account, such as their proximity to AU-rich sequences and positioning away from the center of 3'-UTRs (20), EGFR sites B and C ranked in the top 6% and 4%, respectively, of all predicted miR-7 target sites in TargetScan. Target site A ranked only in the top 56% of TargetScan predicted sites for miR-7. This suggested that sites B and C were more likely to represent functional targets of miR-7 than site A. The miRanda algorithm (28) also predicted EGFR to be a miR-7 target, whereas PicTar (29) did not. Although miR-7 is normally expressed in the brain, lens, pituitary, and hypothalamus (31–33), its expression is significantly decreased in pituitary adenomas and in a panel of central nervous system cancer cell lines relative to normal tissue (34, 35). This raises the possibility that it may function as a tumor suppressor in these systems by inhibiting oncogene expression. The three putative EGFR 3'-UTR miR-7 target sites are poorly conserved between human, mouse, and rat (Fig. 1B). Binding sites that are not conserved between species are often excluded in an attempt to reduce the number of false-positives in target

prediction sets. However, the evolution of miRNAs and their target mRNAs suggests that this exclusion could also increase the rate of false-negative predictions (19). For instance, in mice, miR-7b regulates *Fos* oncogene translation via a 3'-UTR target site that is not present in human *Fos* mRNA (36).

To investigate the interaction between miR-7 and its predicted EGFR mRNA 3'-UTR target sites, we generated reporter vectors containing sequences with complementarity to miR-7 downstream of a luciferase open reading frame (Fig. 1C). These included a sequence with perfect complementarity to the miR-7 sequence (miR-7 target), the full-length wild-type EGFR 3'-UTR (EGFR 3'-UTR), four wild-type EGFR 3'-UTR sequences (A–D), and the same four EGFR sequences with three point mutations predicted to disrupt miR-7 binding in each of the seed match regions (Fig. 1D). In human EGFR-overexpressing NSCLC cells (A549) transfected with synthetic miR-7 precursor (miR-7), expression of the perfect target reporter was reduced, an effect that was not observed following transfection with a negative control miRNA precursor (miR-NC) (Fig. 1E). Similarly, miR-7, but not miR-NC, reduced expression of a reporter that contained the full-length EGFR 3'-UTR (Fig. 1E).

We next investigated the relative contribution of each putative miR-7 target site in the EGFR 3'-UTR to the regulation of gene expression by miR-7. In A549 cells, miR-7 reduced the expression of reporters bearing putative target sites B and C, but not of the corresponding mutant reporters (Fig. 1F). In contrast, putative target site A did not mediate a change in reporter expression by miR-7 (Fig. 1F). This suggested that site A alone was not a target for miR-7 binding. The presence of sites B and C in the same reporter construct (plasmid construct EGFR D, Fig. 1C), conferred additive, although not synergistic, repression with miR-7, which was not observed with the EGFR D mutant reporter (Fig. 1F). Together, these data indicate that the EGFR 3'-UTR is a specific target of miR-7 and that two of the three predicted miR-7-binding sites in the EGFR mRNA 3'-UTR are likely to be specific targets of miR-7. Furthermore, these data suggest that miR-7 may repress EGFR expression via target sites B and C in an additive fashion.

miR-7 Inhibits EGFR Expression in Human Cancer Cell Lines and Has Reduced Expression in U87 Glioblastoma Cells—We next sought to determine the effect of miR-7 on EGFR mRNA and protein expression in EGFR-positive A549, U87 (glioblastoma), MDA-MB-468 (breast cancer), and DU145 (prostate cancer) cell lines. Compared with transfection with miR-NC precursor, transfection with miR-7 precursor reduced EGFR mRNA expression significantly in A549, U87, MDA-MB-468, and DU145 cells at 24 h post-transfection by qRT-PCR (Fig. 2A), consistent with miR-7 promoting EGFR mRNA decay. On the other hand, Lee and co-workers (36) observed that miR-7 regulated translation of *Fos* mRNA in the mouse hypothalamus. These contrasting results suggest that miR-7 may regulate the stability and/or translation of target mRNAs. Furthermore, compared with transfection with either transfection reagent alone or miR-NC, transfection with miR-7 for 72 h reduced EGFR protein expression in A549, U87, MDA-MB-468, and DU145 cells, shown by immunoblotting (Fig. 2B). This effect was observed with concentrations of miR-7 precursor as low as

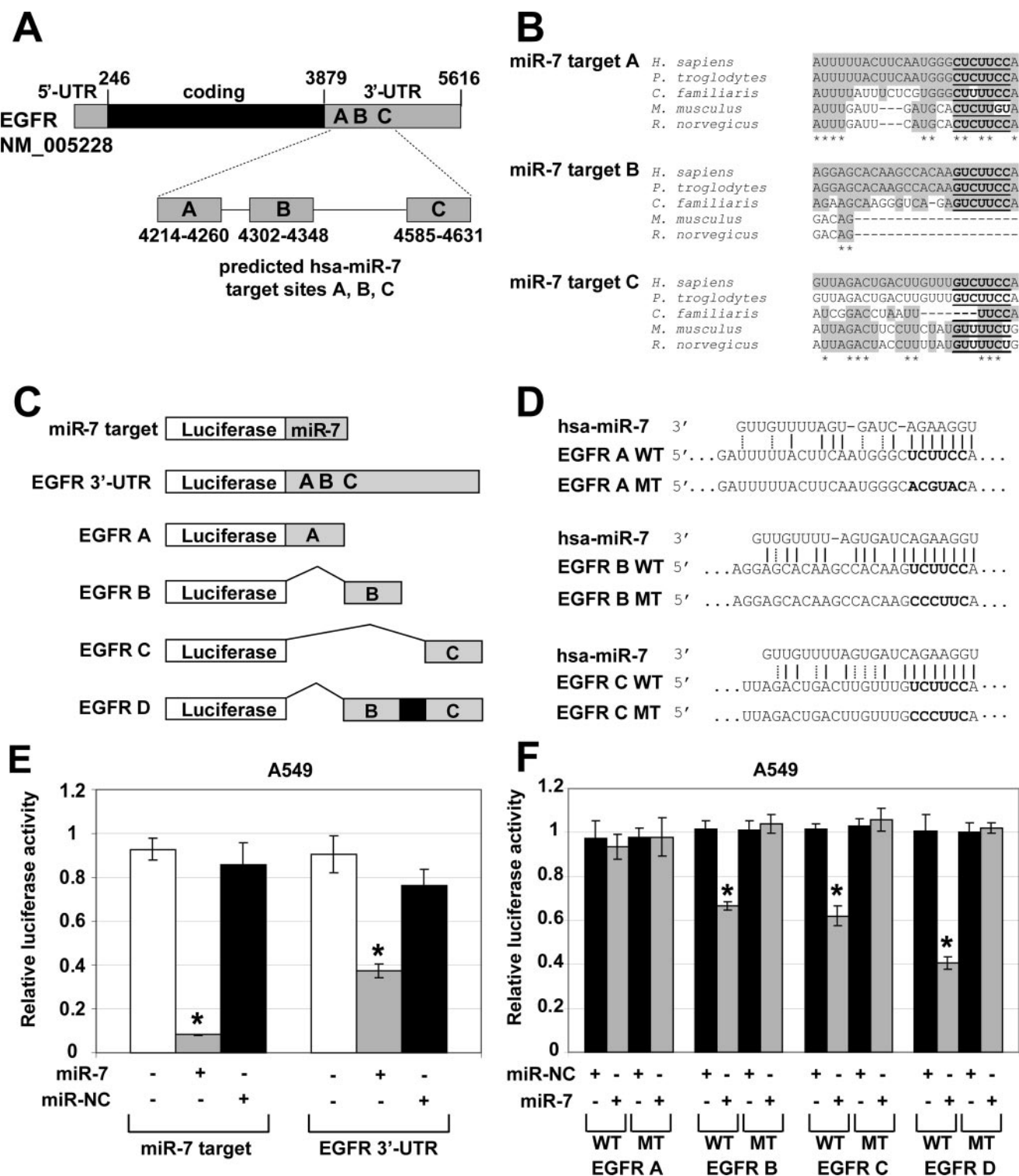


FIGURE 1. Identification of two specific miR-7 target sites within the non-conserved EGFR mRNA 3'-UTR. *A*, schematic representation of the EGFR mRNA with three 3'-UTR miR-7 binding sites (A, B, and C) predicted by TargetScan. *B*, sequence alignment of predicted EGFR 3'-UTR miR-7 target sites showing lack of conservation between human, mouse, and rat. The miR-7 seed target sequence (UCUCCA) is shown in *bold* and *underlined*, and conserved nucleotides are *shaded*. Stars indicate nucleotides conserved across all five species. *C*, schematic representation of firefly luciferase reporter constructs for consensus miR-7 target, full-length wild-type EGFR 3'-UTR, and truncated EGFR 3'-UTR (A–D) miR-7 target sites. *D*, sequence of wild-type (WT) and mutant (MT) EGFR mRNA 3'-UTR miR-7 target sites. Mutations predicted to disrupt miRNA-mRNA binding were made in the seed target region. *E*, luciferase reporter assay to verify activity of miR-7 upon the consensus miR-7 target site and full-length wild-type EGFR 3'-UTR. A549 cells were transfected with consensus miR-7 target or full-length wild-type EGFR 3'-UTR firefly luciferase plasmid and either miR-7 or miR-NC precursor. Relative luciferase expression (firefly normalized to *Renilla*) values are expressed as a ratio of reporter vector alone (\pm S.D.). Data are representative of at least three independent experiments. *, a significant difference from vehicle (Lipofectamine 2000)-treated reporter vector ($p < 0.001$). *F*, luciferase reporter assay with A549 cells that were transfected with WT or MT EGFR target site (A, B, C, or D) 3'-UTR firefly-luciferase reporter, control *Renilla*-luciferase, and either miR-7 or miR-NC precursor. Relative luciferase expression (firefly normalized to *Renilla*) values are the ratio of miR-7-treated reporter vector compared with the same miR-NC-treated reporter vector (\pm S.D.). Data are representative of at least three independent experiments. *, a significant difference from miR-NC-transfected controls ($p < 0.05$).

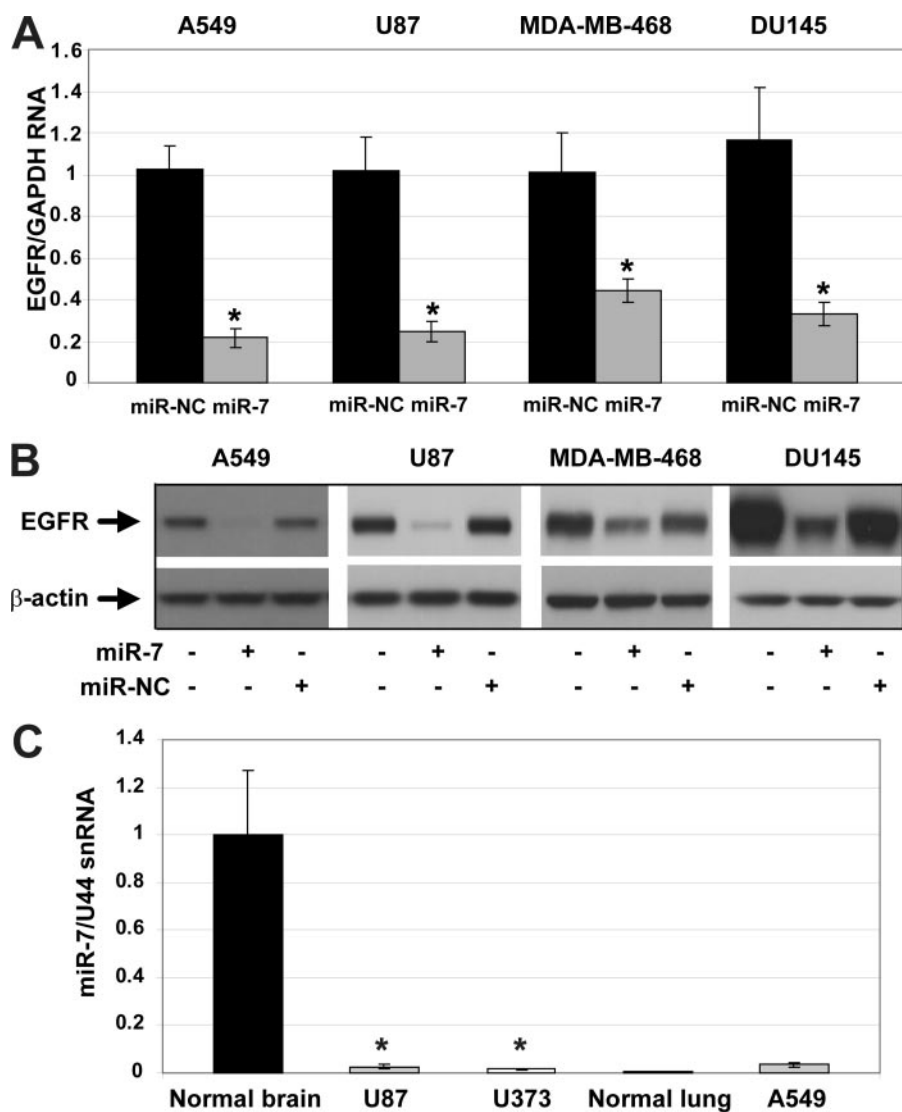


FIGURE 2. miR-7 regulates EGFR expression in cancer cell lines and is down-regulated in glioblastoma cell lines. *A*, quantitative RT-PCR analysis of *EGFR* mRNA expression in A549, U87, MDA-MB-468, and DU145 cells 24 h after transfection with miR-7 or miR-NC precursor. *EGFR* RNA expression was normalized to *GAPDH* RNA expression and is shown as a ratio of miR-NC-transfected cells (\pm S.D.) using the $2^{-\Delta\Delta C_T}$ method. Data are representative of three independent experiments. *, a significant difference from miR-NC-transfected control cells ($p < 0.003$). *B*, immunoblotting detection of EGFR and β -actin expression using 15 μ g of cytoplasmic protein extracts harvested from A549, U87, MDA-MB-468, and DU145 cells 3 days after transfection with miR-7 or miR-NC. *C*, quantitative RT-PCR analysis of miR-7 expression in U87 and U373 glioblastoma cells, and normal lung and A549 cells, compared with normal total brain RNA. 10 ng of total RNA was reverse-transcribed, and miR-7 expression was determined by TaqMan miRNA qRT-PCR assay. miR-7 expression was normalized to U44 snRNA expression, and miR-7 expression was calculated (\pm S.D.) relative to normal total brain RNA using the $2^{-\Delta\Delta C_T}$ method. Data are representative of three independent experiments. *, a significant difference from normal total brain RNA ($p < 0.0001$).

1 nM (data not shown). In addition to investigations of the regulation of EGFR expression by miR-7 in cancer cells, we examined the expression of miR-7 in two EGFR-positive glioblastoma cell lines (U87 and U373) using TaqMan miRNA qRT-PCR assays. We observed that the expression of miR-7 was significantly down-regulated in U87 and U373 cells compared with RNA extracted from total normal human brain tissue (Fig. 2C). This result is consistent with the possibility that reduced miR-7 levels could be associated with elevated expression of EGFR in primary cerebral tumors. We also compared expression of miR-7 in normal brain, normal lung, and normal breast, to its expression in A549, MDA-MB-468, and DU145 cell lines

(Fig. 2C and supplemental Fig. 1), and found very low expression of miR-7 in the normal lung and breast compared with normal brain, suggesting that miR-7 expression is restricted to specific tissues that include those belonging to the central nervous system. Supporting this finding, we observed that miR-7 was expressed at low levels in A549, MDA-MB-468, and DU145 cells compared with normal brain.

Functional Effects of miR-7 in EGFR-positive Human Cancer Cell Lines—Based on our data showing that miR-7 can regulate EGFR expression in cancer cells, we hypothesized that miR-7 might compromise the growth and viability of the same cells. To test this hypothesis, we transfected A549 cells with miR-7 or miR-NC precursors and measured cell cycle progression using propidium iodide staining and flow cytometry, and cell numbers using cell counts at 3 days post-transfection. Transfection with miR-7 induced cell cycle arrest at G₁ (Fig. 3A) and caused a significant decrease in A549 cell numbers and cell viability (Fig. 3B). The reduction in viable cell number with miR-7 transfection did not appear to involve apoptosis, as evidenced by, firstly, the absence of a sub-G₁ cell population by propidium iodide staining and flow cytometry (Fig. 3A) and, secondly, the lack of activation of the executioner caspases 3 and 7 (data not shown). Thus, our results suggest that miR-7 not only inhibits *EGFR* gene expression by targeting its mRNA for degradation but induces a program of gene expression to reduce cell viability and

trigger non-apoptotic cell death.

In view of the important role of EGFR in the protein kinase B (Akt) and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathways, two pathways linked to cancer, we examined the ability of miR-7 transfection to repress Akt and ERK1/2 activity in different cancer cell lines. Following transfection of A549, U87, MDA-MB-468, and DU145 cell lines with miR-7, we observed reductions in basal and EGF-induced phosphorylation of Akt (Fig. 3, C and D, and supplemental Fig. 2A) in all four cell lines. The capacity of miR-7 to regulate ERK1/2 activity appeared to be cell-specific, because miR-7 decreased EGF-stimulated ERK1/2 phosphorylation in U87 cells but did

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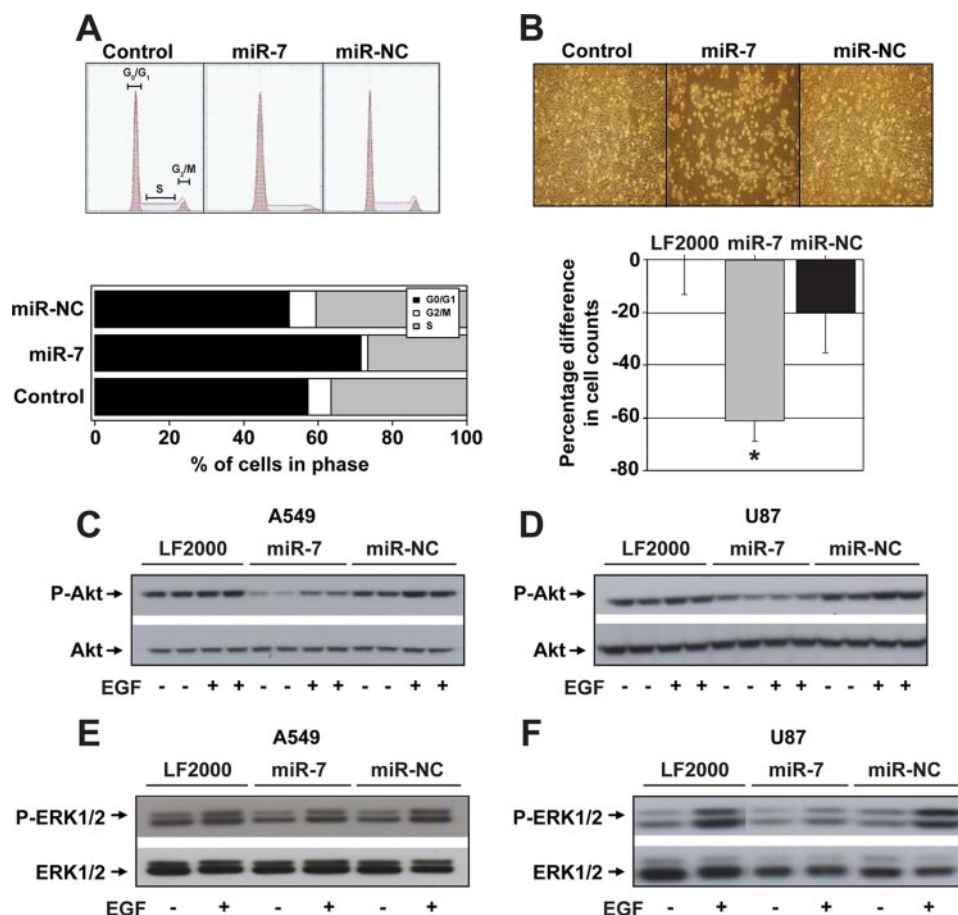


FIGURE 3. miR-7 alters cancer cell cycle progression and cell viability via regulation of Akt and ERK1/2 signaling pathways. A, cell cycle analysis using propidium iodide staining and flow cytometry of A549 cells that had been transfected with miR-7 or miR-NC for 3 days. Cell cycle profile data for the three A549 cell populations (control, miR-7 precursor, miR-NC precursor) are shown from a representative experiment ($n = 3$). B, microscopic assessment of viability of A549 cells 3 days after transfection with miR-7 or miR-NC by light microscopy (40 \times magnification). Bars represent mean percentage difference in cell counts (\pm S.D.) compared with vehicle (Lipofectamine 2000) only. *, a significant difference from LF2000 control ($p < 0.001$). C, immunoblotting detection of P-Akt and Akt expression in A549 cells that had been transfected with miR-7 or miR-NC for 3 days and treated with PBS or EGF (10 ng/ml for 15 min). D, immunoblotting detection of P-Akt and Akt expression in U87 cells that had been transfected with miR-7 or miR-NC for 3 days and treated with PBS or EGF (10 ng/ml for 15 min). E, immunoblotting detection of P-ERK1/2 and ERK1/2 expression in A549 cells that had been transfected with miR-7 or miR-NC for 3 days and treated with PBS or EGF (10 ng/ml for 15 min). F, immunoblotting detection of P-ERK1/2 and ERK1/2 expression in U87 cells that had been transfected with miR-7 or miR-NC for 3 days and treated with PBS or EGF (10 ng/ml for 15 min).

not alter ERK1/2 phosphorylation in A549, MDA-MB-468, or DU145 cells (Fig. 3, E and F, and supplemental Fig. 2B). Together, these observations are consistent with miR-7's ability to inhibit cancer cell cycle progression and reduce cell viability, and suggest that, in addition to the regulation of EGFR expression, miR-7 can regulate the activity of important downstream signaling effectors of EGFR, including Akt and ERK1/2, in multiple EGFR-positive cancer cell lines.

miR-7 Regulates the Expression of Raf1 and Other Members of EGFR Signaling Pathways—miRNAs can have multiple and sometimes functionally related targets (23). We performed microarray analysis using RNA from A549 cells transfected with miR-7 or miR-NC to identify additional genes and biological pathways that could be regulated by miR-7 (Fig. 4A; A549/miR-7 versus A549/miR-NC). Because EGFR mRNA expression was reduced by miR-7 via two specific 3'-UTR target sites (Fig. 2A), it was likely that additional mRNA targets of miR-7 could be identified through a microarray approach.

Microarrays with RNA extracted from miR-7- and miR-NC-transfected A549 cells were performed, with two experimental replicates for each condition. Analysis of the resulting data indicated that 248 transcripts were significantly down-regulated by >2 -fold in the miR-7-transfected cells relative to the miR-NC-transfected cells, whereas 199 transcripts were significantly up-regulated by >2 -fold ($p < 0.05$) (Fig. 4A and supplemental Table S1). The set of down-regulated transcripts was of particular interest, given its potential to contain miR-7 targets. We used the L2L analysis tool (27) (see "Experimental Procedures") to investigate whether there was enrichment of the set of down-regulated transcripts for predicted miR-7 targets. This analysis showed that the set of down-regulated transcripts was significantly enriched by 2.18-fold ($p = 0.025$) with predicted targets of miR-7, but not of any other known miRNA. This enrichment for predicted miR-7 target genes validates our microarray approach using a test employed by other studies that has also been using microarray analysis to identify direct and indirect targets of miRNA action (37).

The proportion of miR-7 target predictions present in the set of down-regulated transcripts was different for the three algorithms used (TargetScan, PicTar, and miRanda, Fig. 4B). 32% (96/303) of TargetScan-predicted miR-7 targets, 7% (17/259) of PicTar-predicted miR-7 targets, and 2% (19/1070) of miRanda-predicted miR-7 targets were down-regulated in our miR-7 microarray at the mRNA level. Similarly, 39% (96/248) of miR-7-down-regulated transcripts were predicted by TargetScan, and 8% were predicted by PicTar and miRanda (17/248 and 19/248, respectively). These differences likely reflect the different approaches of the three programs to target site prediction, whereby miRanda predicted a total of 1070 miR-7 target mRNAs, TargetScan predicted 303 target mRNAs, and PicTar predicted 259 target mRNAs. Of the 248 transcripts significantly down-regulated by miR-7, 9 were predicted by the combination of both miRanda and PicTar, 16 were predicted by both miRanda and TargetScan, and 17 were predicted by both PicTar and TargetScan. Furthermore, nine of the transcripts down-regulated in response to miR-7 were predicted by all three programs to be miR-7 targets: *Raf1*, *PSME3*, *PSME3*, *PLEC1*, *CKAP4*, *CNOT8*, *CNN3*, *CAPZA1*, *PFN2*, and *ARF4*. These nine genes

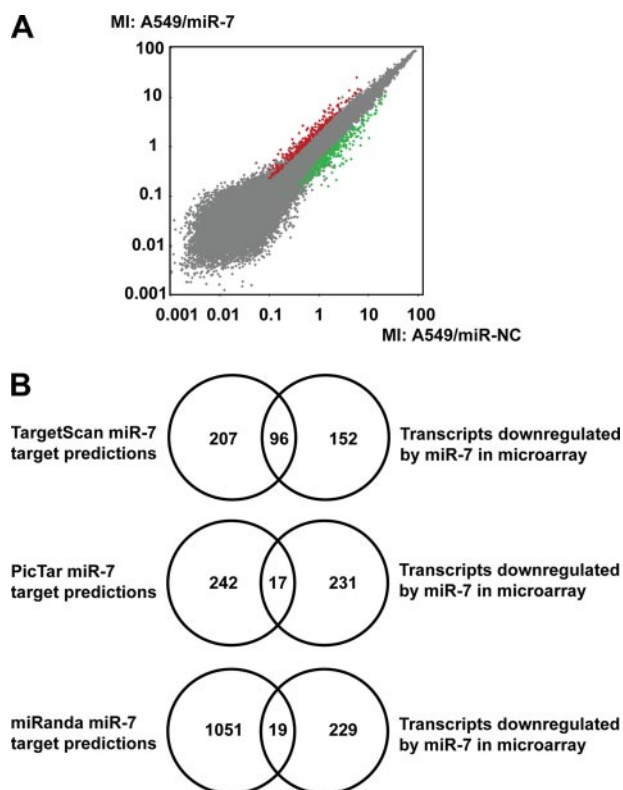


FIGURE 4. Identification of candidate miR-7 target mRNAs by microarray analysis of miR-7 transfected A549 cells. *A*, scatterplot showing log intensities of miR-7-transfected A549 cells (y axis) plotted against the log intensities of miR-NC-transfected cells (x axis). Differentially expressed genes are colored in red (more highly expressed in miR-7-transfected cells) or green (more highly expressed in miR-NC-transfected cells). *B*, intersection of predicted miR-7 target lists (from TargetScan, PicTar, and miRanda) with the 248 transcripts significantly down-regulated by miR-7 in A549 cells.

are of particular interest in view of a recent recommendation that investigation of candidate miRNA targets should be prioritized based on their prediction by two or three of these programs (38).

Of these genes, *Raf1*, whose mRNA expression was significantly down-regulated by 3.47-fold by miR-7 in the microarray experiment ($p = 0.02$), was of interest to us because of the known involvement of Raf1 protein in the EGFR signaling pathway. Raf1 is a downstream effector of EGFR signaling in the Raf-MEK-ERK cascade that is commonly activated by mutations and/or overexpressed in human cancers (39). We hypothesized that miR-7 may coordinately regulate multiple members of the EGFR signal transduction pathway. To confirm the *Raf1* microarray result, we performed qRT-PCR on RNA from A549 cells transfected with miR-7 or miR-NC. This showed down-regulation of the expression of *Raf1* mRNA relative to *GAPDH* mRNA with miR-7 (Fig. 5A), suggesting that miR-7 promotes decay of *Raf1* mRNA. TargetScan predicted that the human *Raf1* 3'-UTR contains two miR-7 target sites (Fig. 5B). To determine whether miR-7 can directly regulate Raf1 expression in cancer cells, reporter assay experiments were conducted with A549 cells. In these experiments, miR-7 reduced the expression of a reporter construct that carried both of the predicted miR-7 target sites from the *Raf1* 3'-UTR but not of a reporter containing an analogous insert with three point mutations in each seed match region (Fig. 5C). This indicated that

the *Raf1* mRNA 3'-UTR is a specific target of miR-7 in cancer cells. Raf1 protein expression was also reduced in A549 and MDA-MB-468 cells transfected with miR-7 compared with untransfected cells and to cells transfected with miR-NC (Fig. 5D). Together, these data provide evidence that, via direct binding to its 3'-UTR, miR-7 regulates the expression of Raf1, a downstream effector of EGFR signaling in the Raf-MEK-ERK cascade.

To further investigate the possible functional roles of miR-7, we performed an analysis on the microarray data to identify KEGG pathways that were significantly enriched with genes that were down-regulated in response to miR-7 (Fig. 6A). The "ErbB signaling pathway," "GnRH signaling pathway," "glioma," "long term potentiation," and "gap junction" pathways were all found to be significantly enriched (supplemental Figs. S3–S7). These results support a role for miR-7 in the regulation of erbB signaling. They are also in line with the expression profile of miR-7, which is brain-specific with the highest expression in the pituitary and hypothalamus, and with the down-regulation of miR-7 in central nervous system tumors, including pituitary tumors (34, 35). In addition to *EGFR* and *Raf1*, several other down-regulated genes from these pathways contain predicted binding sites for miR-7. These include genes involved in calcium signaling (*CALM3* and *CAMK2D*, down-regulated 7.10- and 2.08-fold, $p = 0.006$ and $p = 0.024$, respectively), cytoskeleton reorganization and nuclear signaling (*PAK1*, down-regulated 2.20-fold, $p = 0.006$), and cAMP synthesis and intracellular signaling (*ADCY9*, down-regulated 3.38-fold, $p = 0.005$) (supplemental Table S1). This suggests that miR-7 has the ability to target multiple cell signaling pathways by coordinately regulating the expression of key signaling molecules.

DISCUSSION

We have demonstrated that miR-7 can regulate the expression of multiple effectors of EGFR signaling. As well as directly targeting EGFR and *Raf1*, miR-7 down-regulates the expression of a number of other genes associated with cellular pathways downstream of EGFR. Furthermore, miR-7 has functional effects in cancer cell lines that include inducing cell cycle arrest and reducing cell growth and viability.

EGFR as a Target of miR-7 in Cancer Cells—A major obstacle to the understanding of miRNA signaling and function has been the shortage of validated miRNA targets. This is due in part to the likely ability of a given miRNA to regulate the expression of hundreds or even thousands of target mRNAs, but also to the imperfect complementarity of miRNA and target sequences. Recent work has revealed some of the criteria that support an authentic miRNA-mRNA interaction. We chose to investigate the predicted interaction between miR-7 and the *EGFR* mRNA 3'-UTR, which does not satisfy one of the commonly used criteria for miRNA-target prediction, that is, conservation of the miRNA binding site(s) across species. Our reasoning was based on the evolution of regulatory sequences with increasing species complexity.

Although EGFR overexpression in many cancers often occurs via gene amplification, a subset of tumors with EGFR overexpression does not exhibit *EGFR* gene amplification. In

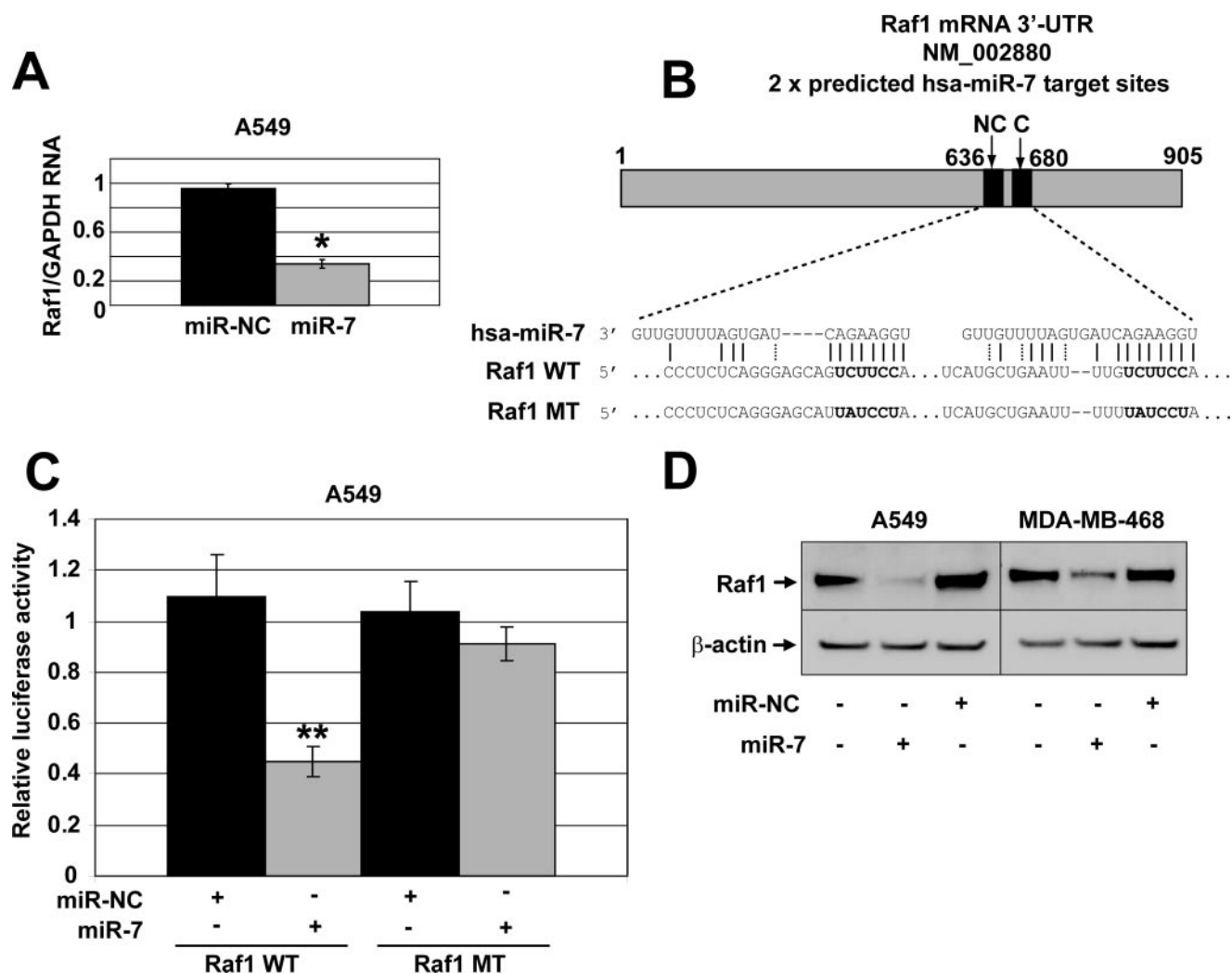


FIGURE 5. miR-7 regulates Raf1 expression via specific binding to the Raf1 mRNA 3'-UTR. *A*, qRT-PCR analysis of Raf1 expression in A549 cells transfected with miR-7 or miR-NC for 24 h. Raf1 expression was normalized to GAPDH expression and data shown relative to miR-NC-transfected cells using the $2^{-\Delta\Delta Ct}$ method (mean \pm S.D., $n = 3$). *, a significant difference from miR-NC control ($p < 0.0005$). *B*, schematic representation of Raf1 mRNA 3'-UTR showing conserved (C) and non-conserved (NC) predicted seed target sites for miR-7 binding. *C*, luciferase reporter gene assay using A549 cells that were transfected with Raf1-WT or Raf1-MT firefly luciferase reporter plasmid DNA, Renilla luciferase plasmid DNA, and either miR-7 or miR-NC, for 24 h. Firefly expression was normalized to Renilla expression. Data are expressed as a ratio of miR-NC-transfected A549 cells (\pm S.D.). Results are representative of those obtained from at least three independent experiments. **, a significant difference from Raf1 WT/miR-NC ($p < 0.05$). *D*, immunoblotting detection of Raf1 and β -actin expression using 15 μ g of cytoplasmic protein extracts harvested from A549 and MDA-MB-468 cells 3 days after transfection with miR-7 or miR-NC.

one study, it was found that $\sim 20\%$ of the EGFR-overexpressing glioblastomas tested lacked EGFR gene amplification (40), suggesting that other, possibly post-transcriptional, mechanisms exist to promote aberrant EGFR expression in cancer cells. We have identified miR-7 as a regulator of EGFR gene expression and signaling in human cancer cells. Furthermore, the normal expression of miR-7 in the brain and endocrine systems, together with the previously reported down-regulation of miR-7 in central nervous system tumors, suggests that miR-7 has the ability to act as a regulator of normal cellular pathways in some systems and as an endogenous tumor suppressor in others.

miR-7 and the Regulation of EGFR Signaling Pathways in Normal and Cancer Cells—Our results show that miR-7 has the ability to coordinately down-regulate the expression of multiple members of EGFR signaling cascades, both directly, as is the case for EGFR and Raf1, and indirectly, and also to induce can-

cer cell cycle arrest and cell death. Our data further suggest that miR-7 may have several tissue-specific functions, with possible roles in the development and progression of gliomas, reproduction, via the production of pituitary gonadotropins, and learning and memory, through regulation of many other signaling and structural proteins in addition to EGFR and Raf1 (Fig. 6B).

A number of recent reports support a role for miR-7 in the brain and in tumor cells. miR-7 has been shown in one study to belong to a subset of miRNAs that are down-regulated in schizophrenia (43). Interestingly, the predicted targets of the down-regulated miRNAs in the study, like the mRNAs down-regulated by miR-7 in the present study, are over-represented in the KEGG functional pathways of "focal adhesion," "regulation of actin cytoskeleton," and "gap junction" (Fig. 6A), and may ultimately influence synaptic plasticity in schizophrenia.

With respect to systems involving EGFR signaling, the MAPK ERK5 is regulated by miR-143 in differentiated adipo-

A

KEGG Pathway	Number of genes from pathway		z-score
	Downregulated	In array	
Glioma	7	64	6.40
Endometrial cancer	6	52	6.12
Non-small cell lung cancer	6	53	6.05
ErbB signaling pathway	7	86	5.25
Prostate cancer	7	90	5.08
Pancreatic cancer	6	73	4.88
Glycerolipid metabolism	5	57	4.66
Pyrimidine metabolism	6	84	4.41
Renal cell carcinoma	5	68	4.11
VEGF signaling pathway	5	68	4.11
GnRH signaling pathway	6	95	4.01
Melanoma	5	71	3.98
Focal adhesion	9	192	3.85
Chronic myeloid leukaemia	5	76	3.78
Apoptosis	5	84	3.49
Colorectal cancer	5	84	3.49
Small cell lung cancer	5	86	3.42
Epithelial cell signaling in <i>H. pylori</i> infection	4	67	3.12
Long-term potentiation	4	68	3.08
Regulation of actin cytoskeleton	8	202	3.06
Melanogenesis	5	99	3.03
Fc epsilon RI signaling pathway	4	74	2.87
Purine metabolism	6	141	2.84
beta-Alanine metabolism	2	24	2.83
DNA polymerase	2	25	2.75
Dorso-ventral axis formation	2	27	2.60
Ether lipid metabolism	2	29	2.46
Gap junction	4	90	2.40
Urea cycle and metabolism of amino groups	2	30	2.40
Olfactory transduction	2	31	2.34
Insulin signaling pathway	5	134	2.25
Glycerophospholipid metabolism	3	64	2.18

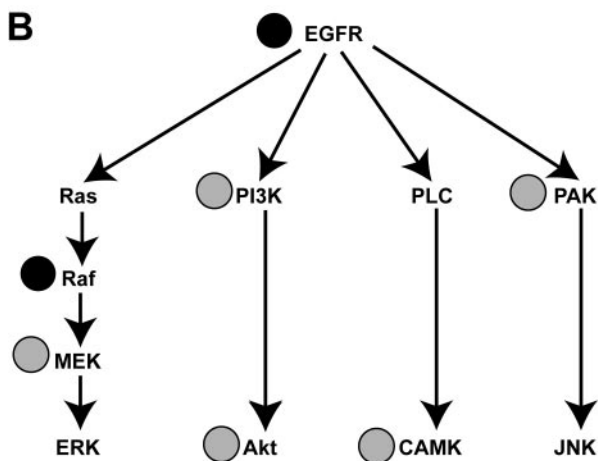


FIGURE 6. Identification of functional pathways enriched with miR-7 target genes. A, table of KEGG pathways significantly enriched (z-score > 2) with genes down-regulated by miR-7 compared with miR-NC in A549 cells. B, schematic model showing coordinate regulation of EGFR signaling by miR-7 via multiple targets. Direct, validated target mRNAs of miR-7 include *EGFR* and *Raf1* (dark circles). Gray circles represent EGFR signaling genes with expression modulated by treatment with miR-7 by unknown mechanism.

cytes and cancer cell lines (41, 42) in much the same way as miR-7 regulates the ERK1/2 pathway, suggesting that miRNAs such as miR-7 and miR-143 have the ability to regulate signaling effectors critical to cell growth and differentiation. Two other reports implicate miR-7 in the regulation of EGFR signaling. In one, miR-7 was shown to control EGFR signaling in *Drosophila* photoreceptor cells (44). This study presented a model in which, upon cell differentiation, EGFR signaling triggers ERK-

mediated degradation of the transcription repressor Yan, thereby relieving Yan-mediated repression of miR-7 expression. Conversely, miR-7 represses Yan expression in photoreceptors via binding to Yan 3'-UTR sequences. This feedback loop thus promotes mutually exclusive expression of Yan and miR-7. However, EGFR is unlikely to represent a direct target of miR-7 in *Drosophila* due to the lack of conservation of the *Drosophila* miR-7 target sites to humans.

While this report was in preparation, a recent report by Kefas and coworkers demonstrated that miR-7 inhibits EGFR expression and the Akt pathway in glioblastomas, tumors shown to have reduced miR-7 expression relative to normal brain tissue (45). The regulation of insulin receptor substrate 2 (IRS2) expression was proposed as a mechanism to explain how miR-7 controls Akt activity. We have confirmed the regulation of IRS2 expression at the protein level by miR-7 in the other cell lines used in our experiments (supplemental Fig. S8). We did not note a decrease in *IRS2* mRNA expression in miR-7-transfected A549 cells in our microarray experiments. Similarly, for each of the cell lines used in this study, we did not observe a significant difference in *IRS2* mRNA expression between cells transfected with miR-7 and miR-NC by qRT-PCR (data not shown), suggesting that miR-7 regulates IRS2 expression at the translational level.

There are a number of key differences between the study by Kefas and coworkers and the present study. Firstly, we evaluated the relative contribution of each of the three predicted miR-7 target sites in the *EGFR* 3'-UTR to the regulation of gene expression by miR-7, show that two of the three predicted miR-7 target sites are directly targeted by miR-7, and demonstrate the specificity of these interactions through mutational analysis of the target site seed regions. Secondly, we observed a reduction in *EGFR* mRNA with miR-7 transfection in multiple EGFR-positive cell lines, using qRT-PCR and microarray analysis, whereas Kefas and coworkers reported no change in *EGFR* mRNA expression with miR-7 transfection. Our finding enabled the use of microarray analysis of miR-7-transfected cells. Thirdly, we did not observe the classic markers of apoptotic cell death with miR-7 transfection, *i.e.* we did not detect a substantial sub-G₁ apoptotic peak or activation of caspases 3 or 7 in miR-7-transfected cells. Fourthly, in addition to a role in the regulation of Akt signaling, our findings implicate miR-7 in the regulation of ERK1/2 signaling in cancer cells, at least in part via its ability to regulate the expression of Raf1 kinase. Furthermore, the regulation of these signaling effectors by miR-7 is shown to occur in a range of EGFR-positive cancer cell line models.

Finally, we have extended our investigation beyond the identification of EGFR as a target of miR-7 to explore additional cellular pathways in which miR-7 may be involved, using microarray analysis of miR-7-transfected cancer cells. This analysis has linked miR-7 to a number of pathways relevant to normal and tumor cells. Furthermore, this analysis suggested several other miR-7 target candidates that are linked to EGFR signaling, including MEK, PI3K, Akt, CAMK, *PAK1*, and *ARF4* (Fig. 6B). The reduced expression of both PI3K and Akt mRNA by miR-7 in our array is interesting. However, in our studies, miR-7 did not significantly alter total Akt protein expression, whereas it did alter Akt phosphorylation. Similarly, the down-

Regulation of EGFR Signaling in Cancer by miR-7

regulation of *MAP2K2* (MEK) expression in the microarray suggests that miR-7 could potentially regulate ERK1/2 activity at a third point in the cascade, in addition to its regulation of EGFR and Raf1.

ADP-ribosylation factor 4 (*ARF4*) is another interesting miR-7 target candidate gene that was down-regulated in the microarray results. The ARF4 protein interacts with EGFR and mediates EGF-dependent activation of the transcription factor AP-1 (46). Therefore, by regulating ARF4 expression, miR-7 could potentially alter a broad program of gene expression through control of EGF-dependent AP-1 activity. Another miR-7-regulated gene, calmodulin kinase II (*CAMK2*), has important roles in the central nervous system relating to long term potentiation and neurotransmitter release (47). This may be significant given that miR-7 is known to be abundantly expressed in the hypothalamus and pituitary, major sites for neurotransmitter secretion. p21/Cdc42/Rac1-activated kinase 1 (*PAK1*) is a serine/threonine kinase that mediates several oncogenic signaling pathways, including EGFR/Akt signaling, to regulate cytoskeletal remodeling, cell motility, cell proliferation, and apoptosis (reviewed in Ref. 48). *PAK1* was down-regulated by miR-7 in our microarray studies and was a predicted target of miR-7. *PAK1* expression is increased in some cancers; in glioblastoma patients its elevated expression is associated with shorter survival time (49). Recently, *PAK1* was shown to be a target of miR-7 in cancer cells (50), suggesting that the reduced expression of miR-7 in glioblastomas might promote the aberrant *PAK1* expression and activity seen in these tumors.

The benefit of abrogating EGFR expression in a variety of human cancer types as a therapeutic approach has been emphasized by recent data showing that the kinase-independent function of EGFR is to prevent autophagic cell death by maintaining the basal intracellular glucose level through an interaction with the sodium/glucose co-transporter 1 (51). To date, clinical trials of EGFR tyrosine kinase inhibitors in a variety of cancers have yielded disappointing results. Co-inhibition of kinase-independent and kinase-dependent EGFR functions could achieve durable clinical responses. Agents such as miR-7 that down-regulate expression of the EGFR as well as some of its signaling effectors may have significant therapeutic potential in a range of human cancer types.

In summary, our data indicate that miR-7 coordinately regulates EGFR signaling at multiple levels and suggest that miR-7 additionally regulates a number of other cellular pathways relevant to normal and tumor cells. The effect of miR-7 action is to inhibit cell cycle progression and reduce cell growth and viability. The reported down-regulation of miR-7 in tumors of the central nervous system, together with its ability to regulate oncogenic EGFR signaling in multiple cancer cell line models, suggests that the therapeutic up-regulation of miR-7 expression in these tumors may inhibit growth and metastasis.

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Supplementary Fig. 1, Quantitative RT-PCR analysis of miR-7 expression in normal breast, MDAMB-468 and DU145 cells compared to normal total brain RNA. 10 ng of total RNA was reverse-transcribed and miR-7 expression determined by TaqMan miRNA qRT-PCR assay. miR-7 expression was normalized to U44 snRNA expression and miR-7 expression was calculated (\pm SD) relative to normal total brain RNA using the $2^{-\Delta\Delta CT}$ method. Data are representative of three independent experiments.

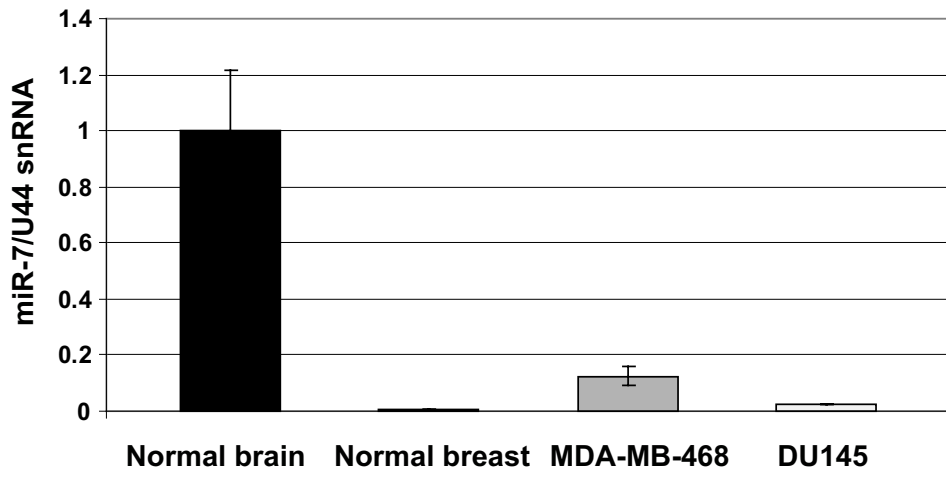
Supplementary Fig. 2. A. Immunoblotting detection of P-Akt and Akt expression in MDA-MB-468 and DU145 cells that had been transfected with miR-7 or miR-NC for 3 d and treated with PBS or EGF (10 ng/ml for 15 min). B. Immunoblotting detection of P-ERK1/2 and ERK1/2 expression in MDA-MB-468 and DU145 cells that had been transfected with miR-7 or miR-NC for 3 d and treated with PBS or EGF (10 ng/ml for 15 min).

Supplementary Fig. 3, 4, 5, 6 & 7. Functional KEGG pathways significantly enriched (z-score > 2) for genes downregulated by miR-7 in A549 cells compared to miR-NC. These include “glioma” (Supplementary Fig. 3), “ErbB signaling pathway” (Supplementary Fig. 4), “GnRH signaling pathway” (Supplementary Fig. 5), “long-term potentiation” (Supplementary Fig. 6), “gap junction” (Supplementary Fig. 7). Genes marked with asterisks are those with established roles in these pathways that were also significantly downregulated by miR-7 in microarray analysis.

Supplementary Fig. 8. B. Immunoblotting detection of IRS2 and β -actin expression using 15 μ g of cytoplasmic protein extracts harvested from A549, MDA-MB-468 and DU145 cells 3 d after transfection with miR-7 or miR-NC.

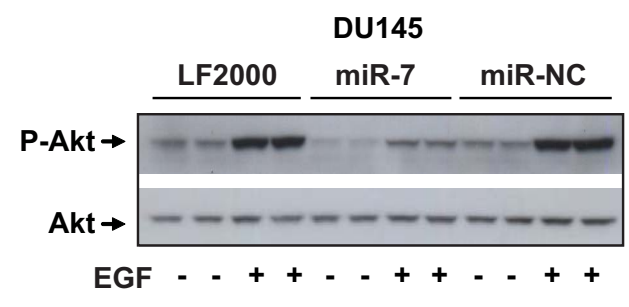
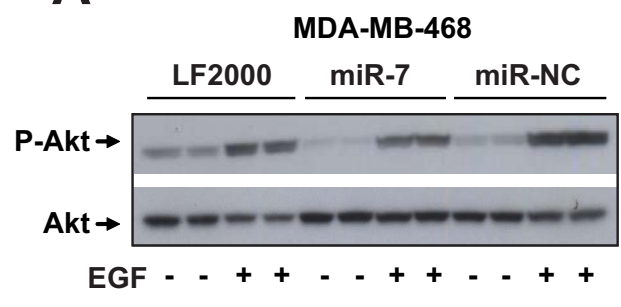
Supplementary Table 1. All genes whose expression is downregulated by more than 2-fold at 24 h in A549 cells transfected with miR-7 compared to miR-NC. The number of predicted miR-7 target sites for each gene according to TargetScan, PicTar and miRanda is shown.

Supplementary Figure 1

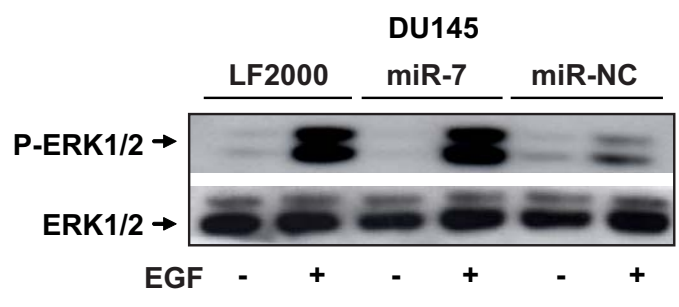
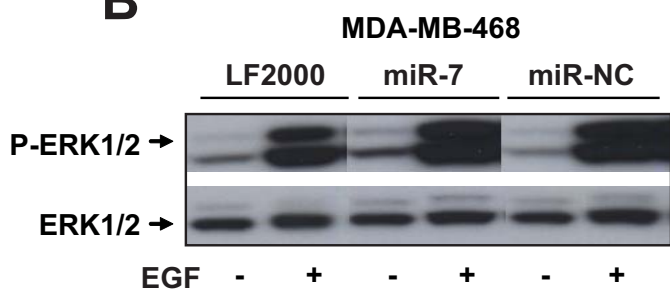


Supplementary Figure 2

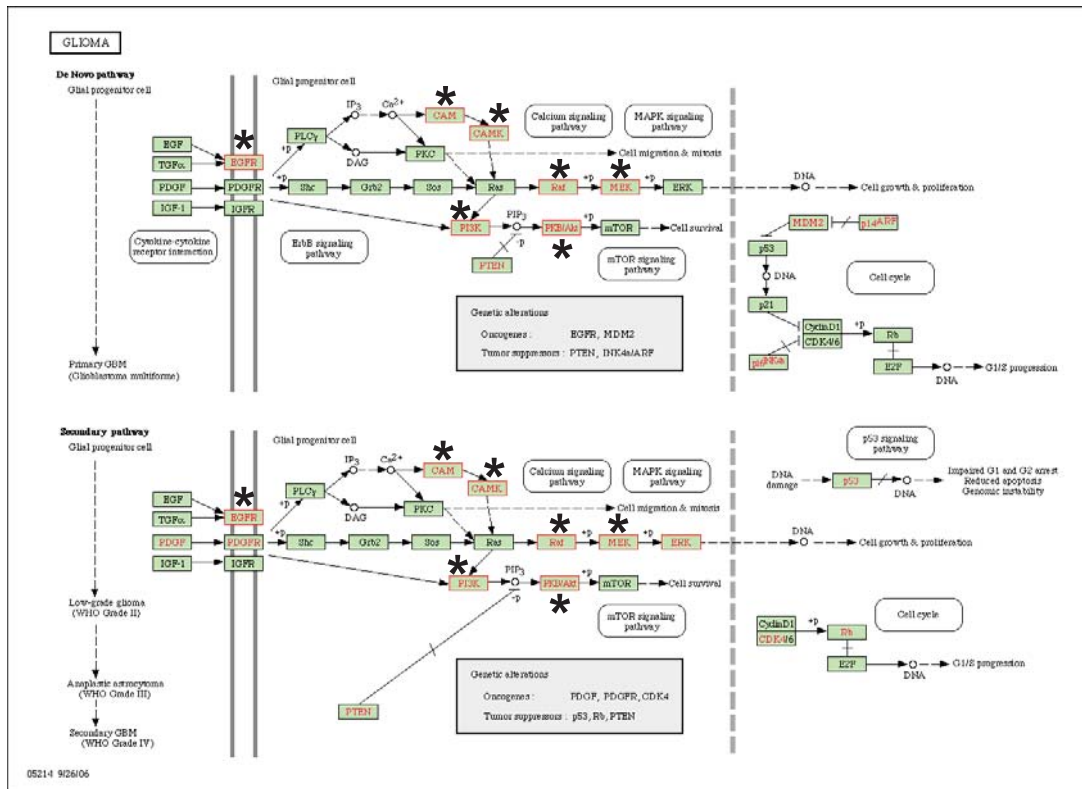
A



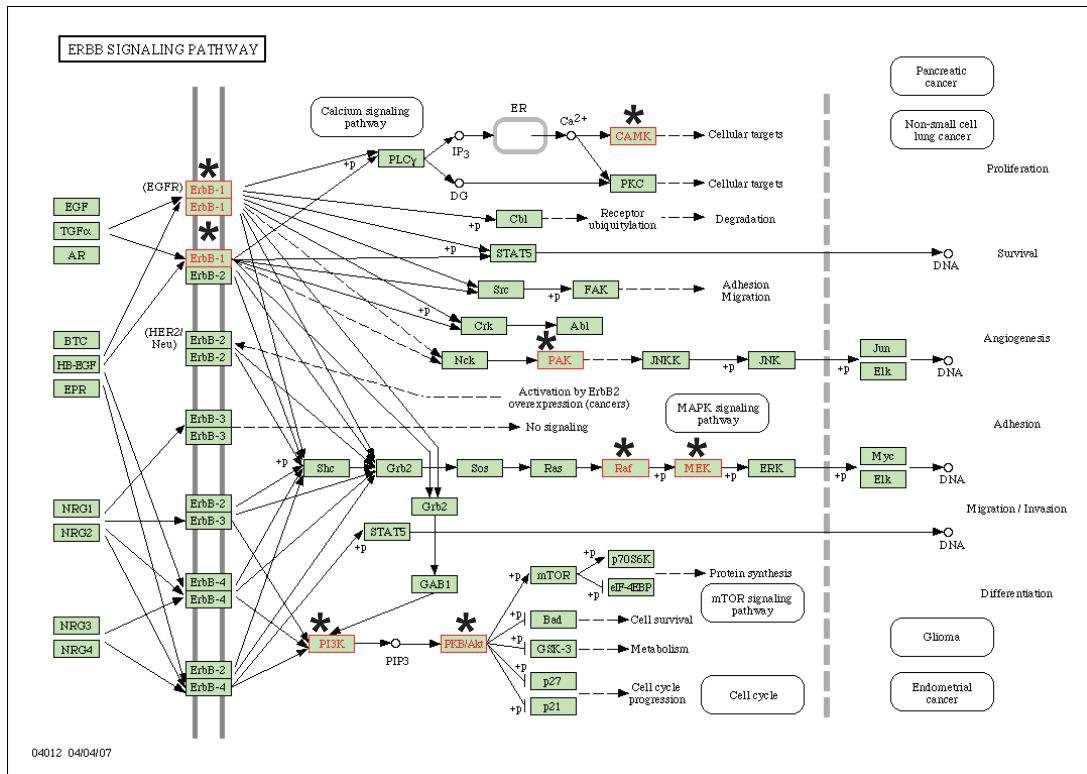
B



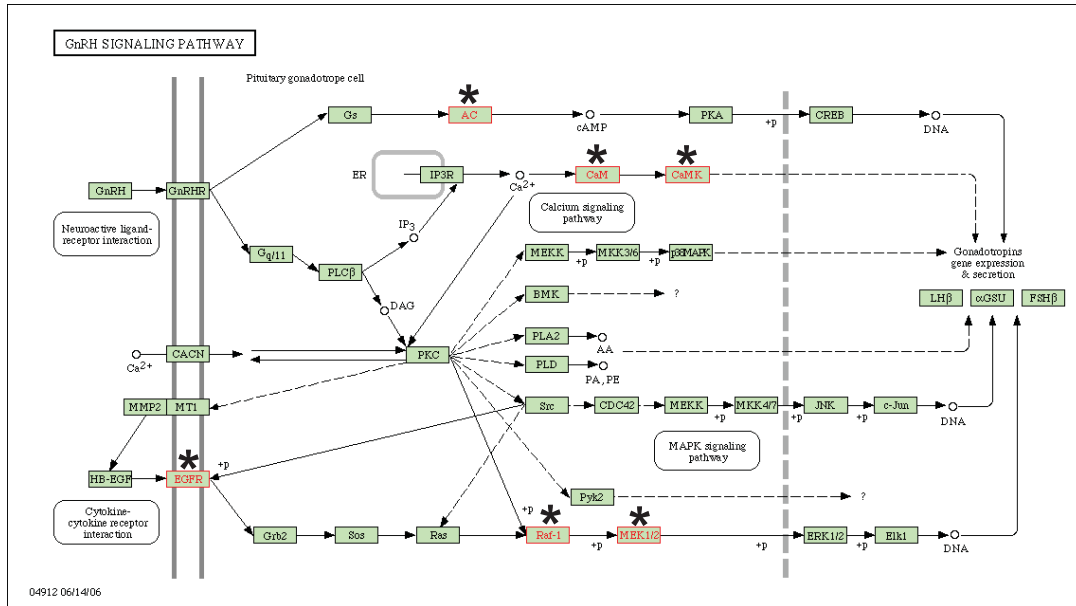
Supplementary Figure 3



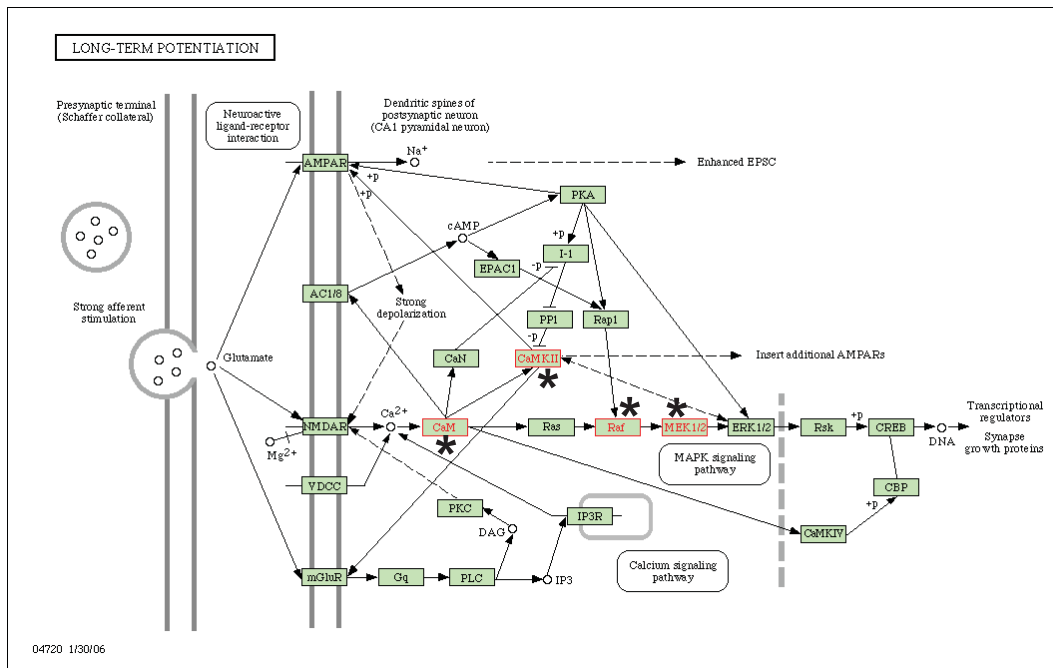
Supplementary Figure 4



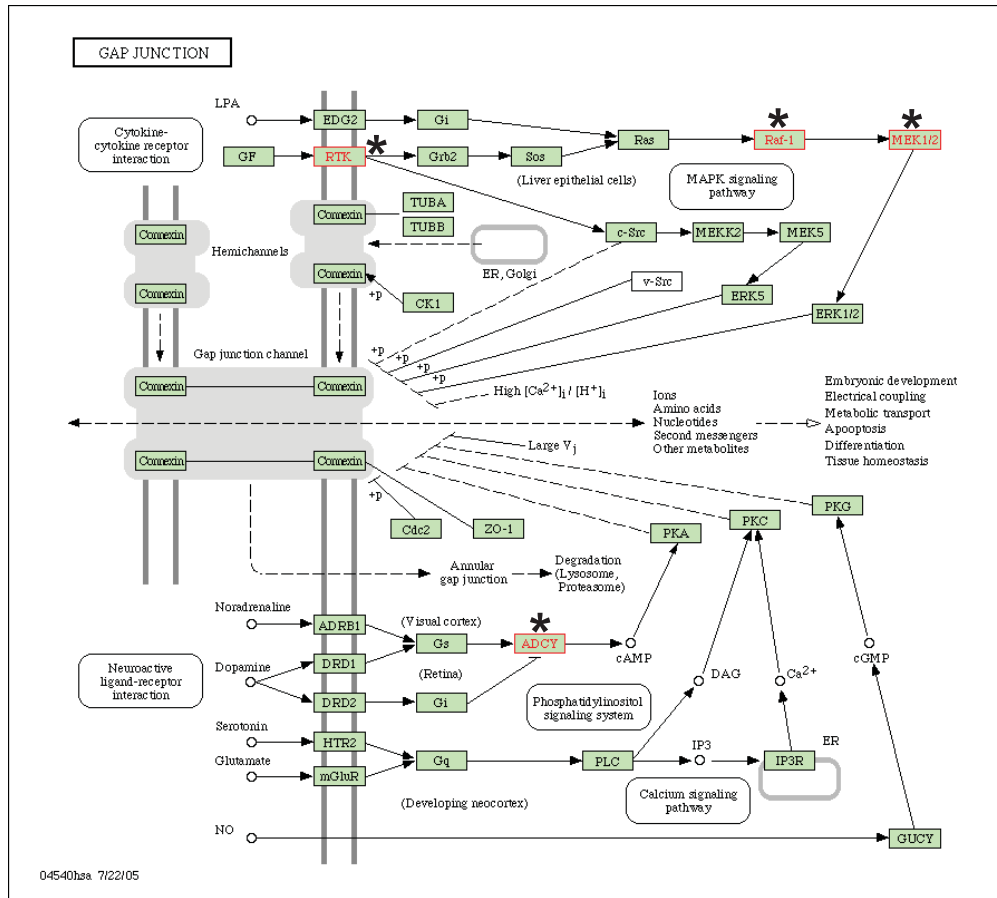
Supplementary Figure 5



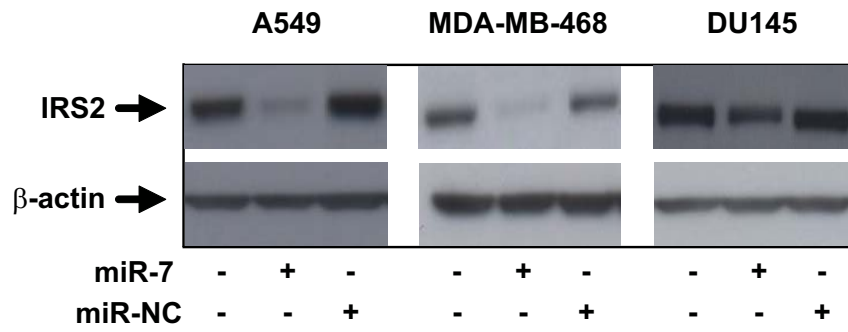
Supplementary Figure 6



Supplementary Figure 7



Supplementary Figure 8



Regulation of Epidermal Growth Factor Receptor Signaling in Human Cancer Cells by MicroRNA-7

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