Knockdown of miR-21 in human breast cancer cell lines inhibits proliferation, in vitro migration and in vivo tumor growth

Li Xu Yan¹,²†, Qi Nian Wu¹,²†, Yan Zhang¹,²†, Yang Yang Li¹,², Ding Zhun Liao¹,², Jing Hui Hou¹,², Jia Fu¹,², Mu Sheng Zeng¹,³, Jing Ping Yun¹,², Qiu Liang Wu¹,², Yi Xin Zeng¹,³, Jian Yong Shao¹,²,³*

Abstract

Introduction: MicroRNAs (miRNAs) are a class of small non-coding RNAs (20 to 24 nucleotides) that post-transcriptionally modulate gene expression. A key oncomir in carcinogenesis is miR-21, which is consistently up-regulated in a wide range of cancers. However, few functional studies are available for miR-21, and few targets have been identified. In this study, we explored the role of miR-21 in human breast cancer cells and tissues, and searched for miR-21 targets.

Methods: We used in vitro and in vivo assays to explore the role of miR-21 in the malignant progression of human breast cancer, using miR-21 knockdown. Using LNA silencing combined to microarray technology and target prediction, we screened for potential targets of miR-21 and validated direct targets by using luciferase reporter assay and Western blot. Two candidate target genes (EIF4A2 and ANKRD46) were selected for analysis of correlation with clinicopathological characteristics and prognosis using immunohistochemistry on cancer tissue microarrays.

Results: Anti-miR-21 inhibited growth and migration of MCF-7 and MDA-MB-231 cells in vitro, and tumor growth in nude mice. Knockdown of miR-21 significantly increased the expression of ANKRD46 at both mRNA and protein levels. Luciferase assays using a reporter carrying a putative target site in the 3’ untranslated region of ANKRD46 revealed that miR-21 directly targeted ANKRD46. miR-21 and EIF4A2 protein were inversely expressed in breast cancers ($r_s = -0.283, P = 0.005$, Spearman’s correlation analysis).

Conclusions: Knockdown of miR-21 in MCF-7 and MDA-MB-231 cells inhibits in vitro and in vivo growth as well as in vitro migration. ANKRD46 is newly identified as a direct target of miR-21 in BC. These results suggest that inhibitory strategies against miR-21 using peptide nucleic acids (PNAs)-antimiR-21 may provide potential therapeutic applications in breast cancer treatment.

Introduction

Breast cancer (BC) is by far the most frequent cancer of women (23% of all cancers), with an estimated 1.15 million new cases worldwide in 2002 [1]. It is still the leading cause of cancer mortality in women [1]. Despite research and resources dedicated to elucidating the molecular mechanisms of BC, the precise mechanisms of its initiation and progression remain unclear.

MicroRNAs (miRNAs) are small non-coding RNAs (20 to 24 nucleotides) that post-transcriptionally modulate gene expression by negatively regulating the stability or translational efficiency of their target mRNAs [2]. After the discovery of miRNAs, and findings indicating that they play a role in cancer, the concept of "oncomirs" was proposed [3]. In particular, miR-21 [miRBase: MIMAT0000076] has emerged as a key oncomir, since it is the most consistently up-regulated miRNA in a wide range of cancers [4-7].

Functional studies showed that knockdown of miR-21 in MCF7 cells led to reduced proliferation and tumor
growth [8,9]. Knockdown of miR-21 in MDA-MB-231 cells significantly reduced invasion and lung metastasis [10]. These data clearly implicate miR-21 as a key molecule in carcinogenesis, but functional studies that demonstrate cause and effect relationships between miR-21 and target genes are lacking. Given that miRNAs usually target multiple genes post-transcriptionally, miR-21 is likely to exert its effects by regulating many genes involved in BC.

The inhibition of miRNAs using antisense oligonucleotides (ASOs) is a unique and effective technique for investigating miRNA functions and targets. Peptide nucleic acids (PNAs) are artificial oligonucleotides constructed on a peptide-like backbone. PNAs have a stronger affinity and greater specificity for DNA or RNA than natural nucleic acids, and are resistant to nucleases [11]. PNA-based ASOs can be used without transfection reagents, and are highly effective and sequence-specific. They provide long-lasting inhibition of miRNAs, and show no cytotoxicity up to 1 μM [11]. Therefore, we used a PNA miR-21 inhibitor for in vivo investigation.

In this study, we explored the role of miR-21 in the malignant progression of human BC by assaying in vitro and in vivo function after miR-21 knockdown. We also searched for miR-21 targets using gene prediction-based and systematic screening approaches. Two potential target genes eukaryotic translation initiation factor 4A2 (EIF4A2) [NCBI: NM001967] and ankyrin repeat domain 46 (ANKRD46) [NCBI: NM198401] were selected for correlation analysis between protein levels and clinicopathological characteristics as well as prognosis using immunohistochemistry (IHC) on cancer tissue microrays (TMAs).

Materials and methods

Tissue specimens and TMAs construction

In situ hybridization analysis was performed on fresh samples from BC or fibroadenoma (FA) tissues with paired normal adjacent tissues (NATs, > 2 cm from tumor tissues) obtained from Sun Yat-sen University Cancer Center (SYSUCC) (Guangzhou, China) between January and March 2009. For IHC staining of miR-21 predicted target genes, formalin-fixed paraffin-embedded tissues were obtained from 99 randomly selected BC patients without neoadjuvant therapy at SYSUCC from January 2000 to November 2004, from whom informed consent and agreement, and clinicopathological information was available. A pathologist reviewed slides from all blocks, selecting representative areas of invasive tumor tissue to be cored. Selected cores were analyzed in duplicate using a MiniCore Tissue Arrayer (Alphelys, Passage Paul Langevin, Plaisir, France) with a 1-mm needle. The diagnosis and histological grade of each case were independently confirmed by two pathologists based on World Health Organization classification [12]. The clinical stage was classified according to the American Joint Committee on Cancer (AJCC) tumor-lymph node-metastasis (TNM) classification system [13]. The study was approved by the Research Ethics Committee of SYSUCC (Reference number: YP-2009168). The clinicopathological characteristics and follow-up data of the patients are summarized in Table 1.

Locked nucleic acid (LNA)-based in situ hybridization for miRNA

To study the spatial and temporal expression of miRNAs with high sensitivity and resolution, the miRNA chromogenic in situ hybridization (CISH) and fluorescein in situ hybridization (FISH) protocol [14] were optimized (Additional file 1).

Transfection of LNA-antimiR-21 into BC cells

MCF-7 and MDA-MB-231 cells were maintained in Dulbecco’s modified Eagle’s medium, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (GIBCO-Invitrogen, Carlsbad, CA, USA). For transfection, the LNA-antimiR-21 or LNA-control (Exiqon A/S, Skelstedet, Vedbaek, Denmark) was added to the culture media at a final concentration of 50 μM 4 hours before transfection. Cells were then transfected using Lipofectamine 3000 (Invitrogen) following the manufacturer’s instructions. Transfection efficiency was evaluated by western blotting.

Table 1 Clinicopathological characteristics and follow-up data for 99 patients with BC

| Characteristics | Number of patients/Number analyzed (%) |
|----------------|----------------------------------------|
| Median age (range) | 48 (30 to 74) (years) |
| Histological type* | Ductal 93/99 (94%) |
| | Lobular 1/99 (1%) |
| | Other 5/99 (5%) |
| Histological grade* | I 22/99 (22%) |
| | II 58/99 (58%) |
| | III 19/99 (20%) |
| Lymph node status at time of primary diagnosis | Metastasis 57/99 (58%) |
| | No metastasis 42/99 (42%) |
| AJCC clinical stage** | I 8/99 (8%) |
| | II 68/99 (69%) |
| | III 23/99 (23%) |
| Overall survival (median, range) | 74 (6 to 112) (months) |
| Alive without evidence of cancer | 45/99 (68%) |
| Alive with cancer | 14/99 (14%) |
| Died of cancer | 40/99 (40%) |
| Died of other disease | 0/99 (0%) |

* According to the WHO classification of BC. ** According to the AJCC staging system; AJCC, American Joint Committee on Cancer; BC, breast cancer; WHO, World Health Organization.
were delivered at a final concentration of 50 nM using Lipofectamine 2000 reagent (Invitrogen).

**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and colony formation assay**
Growing cells (2 x 10^5 cells per well) were seeded into 96-well plates. At 24 h after LNA-transfection, cells were stained with 20 μl sterile MTT dye (5 mg/ml; Sigma-Aldrich Corp., St. Louis, MO, USA), followed by 4 h at 37°C. After supernatant removal, 150 μl of dimethyl sulphoxide (Sigma) was added and thoroughly mixed for 15 minutes. Absorbence was measured with a microplate reader (SpectraMax M5; Molecular Devices Corp., Silicon Valley, CA, USA) at 490 nm. For colony formation assays, cells were seeded in six-well plates (0.5 x 10^6 cells per well) and cultured for two weeks. Colonies were fixed with methanol for 10 minutes and stained with 1% crystal violet (Sigma) for 1 minute. Each cell group was measured in triplicate.

**Wound healing assay**
Cells cultured in the presence of 50 nM LNA-antimiR-21 or LNA-control for 24 h were allowed to reach confluence before dragging a 1-ml sterile pipette tip (Axygen Scientific, Inc., Union City, CA, USA) through the monolayer. Cells were washed to remove cellular debris and allowed to migrate for 24 h or 48 h. Images were taken at time 0 h, 24 h, and 48 h post-wounding using a digital camera system (Leica DFC 480; Leica Microsystems, Bannockburn, IL, USA). The motility of the cells was determined as repaired area percentage [15]. Each cell group was measured in triplicate.

**Validation of tumor growth-promoting activity of miR-21 in an animal model**
Five- to six-week-old female BALB/c-nude mice (Slaccas Shanghai Laboratory Animal Co., Ltd., Shanghai, China) were used for experimental tumorigenicity assays. To facilitate estrogen-dependent xenograft establishment, each mouse received 17-estradiol (20 mg/kg; Sigma) intraperitoneally once a week. One week after treatment, equivalent amounts of MCF-7 cells, treated with PNA-antimiR-21 or PNA-control (100 nM for 48 h; Panagene, Inc., Yuseong-gu, Daejeon, Korea) without transfection reagents according to the manufacturer’s protocol, were injected subcutaneously (10^7 cells/tumor) into the left axilla of nude mice [16]. Mice were weighed, and tumor width (W) and length (L) were measured every day. Tumor volume was estimated according to the standard formula: V = (L/2) x W^2, as described previously [17]. Animals were killed nine days after initial growth of the MCF-7 xenografts was detectable, and tumors were extracted. In all experiments, the ethics guidelines for investigations in conscious animals were followed, with approval from the local Ethics Committee for Animal Research.

**mRNA array and data mining**
MCF-7 and MDA-MB-231 cells were transfected either with LNA-antimiR-21 or with LNA-control at a final concentration of 50 nM. Total RNAs were isolated from MCF-7 cells 48 h post transfection and from MDA-MB-231 cells 36 h post transfection, respectively, using Trizol Reagent (Invitrogen). The mRNA expression profile was performed using human genome oligo array service V1.0 (Catalog Number 400010; CapitalBio, Beijing, China) as described [18]. Each sample was analyzed once, and the CapitalBio data preprocess, normalization and filtering were as previously described [18]. Ratios were defined as marginal signal intensity when there was a substantial amount of variation in the signal intensity within the pixels from 800 to 1,500. All the microarray data have been deposited to the Gene Expression Omnibus (GEO) [19] and are accessible through GEO Series accession number [GEO: GSE20627].

**Relative quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**
For validation of mRNA array and quantitative analysis of miR-21 as well as potential target genes, qRT-PCR was used as previously described [20]. The primers for qRT-PCR are in Additional file 2. The relative expression was calculated using the equation relative quantification (RQ) = 2 \Delta \Delta CT [21].

**Computational prediction of miR-21 target genes**
Predicted miR-21 targets were identified using the algorithms of TargetScan 5.1 [22], miRBase Targets V5 [23], miRNAMap 2.0 [24], PicTar [25] and miRanda 3.0 [26].

**Luciferase reporter assay**
The 3’ untranslated region (3’ UTR) of mRNA sequence of ANKRD46 containing predicted miR-21 binding site was amplified by PCR. PCR primers were listed in Additional file 2. After amplification, PCR products were cloned into the pMIR-REPORT (Applied Biosystems, Foster City, CA, USA), resulting in the pMIR-REPORT-3’ANKRD46. Mutation of ANKRD46 was introduced in the predicted miR-21 binding site by a QuickChange site-directed mutagenesis kit (Stratagene, Foster City, CA, USA). Wild-type EIF4A2 and mutant EIF4A2 were cloned into pMD19-T Simple Vector by Takara Bio-technology CO., LTD. (Dalian, Liaoning, China) and then were individually subcloned downstream of the luciferase coding sequence in the pMIR-REPORT (Applied Biosystems). All constructs were verified by DNA sequencing.
For reporter assays, wide-type or mutant reporter constructs (15 ng) were cotransfected into 293T cells in twelve-well plates with miR-21 or miR-control (50 nM; GenePharma, Shanghai, China) and Renilla plasmid (5 ng) using lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase activities were measured by using a Dual Luciferase Assay (Promega, Madison, WI, USA) 24 h after transfection. Firefly luciferase values were normalized to Renilla, and the ratio of firefly/Renilla was presented.

Immunoblot analysis

Cells were harvested and lysed in radioimmune precipitation buffer (Upstate, Lake Placid, NY, USA) at the indicted time post-transfection. Antibodies used for immunoblot analysis were against ANKRD46 (1:500 dilution; sc-87548, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), EIF4A2 (1:1000 dilution; ab31218, Abcam, Cambridge, UK) and GAPDH (1:3,000 dilution; sc-32233, Santa Cruz Biotechnology), as a loading control. All bands were detected using a SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

Immunohistochemical staining

IHC and scoring of the estrogen receptor (ER), progesterone receptor (PR) and CerbB2 were performed as previously described [20]. Slides were incubated with primary antibodies against ANKRD46 (1:150 dilution; sc-87548, Santa Cruz Biotechnology); or EIF4A2 (1:700 dilution; ab31218, Abcam). All slides were processed simultaneously in identical conditions per the manufacturer’s instructions. Three observers independently determined consensus scoring of EIF4A2 and ANKRD46 immunostaining using a semi-quantitative estimation according to the percentage of positive cells and the intensity of staining as described previously [27]. With these data, the composite score was obtained by adding the values of the staining intensity and relative abundance [28]. Samples with scores lower than the median score were grouped as low protein expression [29].

Statistical analysis

Spearman’s rank correlation test was used for correlation analysis between predicted target gene protein levels and endogenous miR-21 levels measured previously by qRT-PCR [20]. Pearson’s Chi-Square tests were used to compare target gene expression levels to clinicopathological characteristics. Survival curves were estimated by the Kaplan-Meier method and log-rank test. All analysis used SPSS 16.0 for Windows (SPSS Inc, Chicago, IL, USA). All tests were two-tailed, and the significance level was set at \( P < 0.05 \).

Results

miR-21 is overexpressed in BC tissues and cell lines

Expression of miR-21 was detected in the cytoplasm in cancerous and luminal epithelial cells, and occasionally in fibroblasts. In BC patients, an increase in miR-21 staining intensity was observed in BC and FA tissues compared with corresponding NATs (Figure 1a). Parallel detection by FISH is shown in Additional file 3. Consistent with the CISH results, quantitative analysis indicated that miR-21 expression was significantly increased by 4.44- to 2.02-fold in BC tissues compared with NATs \( (P = 0.019, n = 4) \), and increased in FA tissues by 3.03- to 1.89-fold \( (P = 0.008, n = 4) \). FISH and qRT-PCR were used to measure miR-21 levels in five BC cell lines (MCF-7, MDA-MB-231, MDA-MB-453, MDA-MB-435, and SK-BR-3) and one non-tumorigenic epithelial cell line (MCF-10A). Consistent with previous findings [8,10,30], miR-21 overexpressed in MCF-7, MDA-MB-231 and MDA-MB-453 cell lines from 6.48- to 4.04-fold compared with MCF-10A cell line \( (P < 0.01) \) (Figure 1d).

LNA-antimiR-21 inhibits BC cell growth, proliferation and migration in vitro

MCF-7 and MDA-MB-231 cell lines were selected to investigate miR-21 functions and targets by using sequence-specific functional inhibition of miR-21, because both cell lines express higher levels of miR-21 compared with MCF-10A cells. Optimal doses and time points for transfection of LNA reagents were determined by evaluating miR-21 levels using qRT-PCR. Knockdown of miR-21 reduced miR-21 levels by 98% in MCF-7 cells, and 77% in MDA-MB-231 cells \( (P < 0.01) \) (Figure 2a). LNA-antimiR-21 led to a decrease in MCF-7 cell growth (Figure 2b) and proliferation (29%, \( P = 0.003 \), Figure 2c). Similar inhibition of cell growth and proliferation effects (51%, \( P = 0.011 \), Figure 2c) was also observed in MDA/LNA-antimiR-21 cells (data not shown). In vitro wound healing assays showed that wound repair in MCF/LNA-antimiR-21 and MDA/LNA-antimiR-21 was delayed compared with MCF/LNA-control and MDA/LNA-control cells (data not shown). Knockdown of miR-21 suppressed MCF-7 cell migration by up to 69% \( (P = 0.013) \), and MDA-MB-231 migration by 51% \( (P = 0.001) \), compared with the LNA-control at 24 h after wound scratch (Figure 2d). These data demonstrate the tumorigenic properties of miR-21 in regulating cell growth, proliferation and migration.

PNA-antimiR-21 inhibits tumor growth in vivo

To address the potential effects of PNA-antimiR-21 in vivo on the growth of BC cells, equal numbers (3 × 10⁵)
Figure 1 Altered expression of miR-21 in different breast tumor types and breast cell lines. (a) CISH detection using miR-21 LNA detection probe (blue) or scramble-miR as negative control were performed on consecutive 8 μM cryo-sections obtained from BC/FA tissues and corresponding NATs. DNA was counterstained with methyl green (green, × 400 magnification). (b) Total RNA was used to quantify miR-21 expression by relative qRT-PCR, normalizing on U6 RNA levels. The graph shows a log2-scale RQ calculated by normalizing the miR-21 expression values in the tumors on those in the NATs. Data indicate the mean (+SD) of four independent samples. * P < 0.05. (c) FISH detection of miR-21 in five BC cell lines (MCF-7, MDA-MB-231, MDA-MB-453, MDA-MB-435, SK-BR-3) and one non-tumorigenic epithelial cell line (MCF-10A). Positive in situ hybridization signals are visualized in green, while blue depicts DAPI nuclear stain (× 1,000 magnification). (d) qRT-PCR analysis show that MCF-7, MDA-MB-231 and MDA-MB-453 cells express higher levels of miR-21 compared with MCF-10A cells. Data are mean (+SD) of three replicates. ** P < 0.01; BC, breast cancer; FA, fibroadenoma; NATs, normal adjacent tissues.
of MCF-7 cells treated with PNA-antimiR-21 or the PNA-control were subcutaneously injected into female nude mice (eight animals per treatment). As seen in Figure 3b and 3c, detectable tumor masses (0.011 ± 0.013 g, mean ± standard deviation, SD) were seen in only 5/8 (62.5%) of mice in the MCF/PNA-antimiR-21 group, while much larger tumors (0.036 ± 0.038 g, mean ± SD) were detected in all mice in the MCF/PNA-control group (P = 0.065, Mann-Whitney test). Both tumor weight and number showed that MCF/PNA-control cells formed larger tumors more rapidly (Figure 3a, c) than MCF/PNA-antimiR-21 cells in nude mice. PNA-antimiR-21 reduced miR-21 expression by 5.72 log₂-scale in MCF-7 cells 48 h post-treatment compared with that in the control (P < 0.01). miR-21 expression in xenograft tumors of PNA-antimiR-21 group was 0.96 log₂-scale higher than that of the control group (P < 0.05; Figure 3e). Notably, MCF/PNA-antimiR-21 tumor cells were decreased in mitotic and pathological mitotic stages compared with MCF/PNA-control cells, indicating a decreased in cell proliferative activity and apoptosis (Figure 3d).

Identification of potential miR-21 targets

It is known that animal miRNAs regulate gene expression by inhibiting translation and/or by inducing degradation of target. In our study, most modulated genes in the mRNA differential expression profiles changed by less than two-fold. Since mRNA levels regulated by less than two-fold may still be miRNA targets, we defined differentially expressed genes as no less than 1.3-fold change [31]. Comparative analysis of LNA-antimiR-21
and LNA-control mRNA profiles showed differential regulation of 394 genes in MCF/LNA-antimiR-21, of which 228 (58%) were up-regulated and 166 (42%) were down-regulated. 321 genes were differentially expressed in MDA/LNA-antimiR-21 cells, of which 190 (59%) were up-regulated, and 131 (41%) down-regulated (Figure 4a). The intersection of MCF/LNA-antimiR-21 and MDA/LNA-antimiR-21 consisted of 27 genes (18 up- and 9 down-regulated; Figure 4b and Table 2).

To further screen potential direct targets, we compared the 27 candidate mRNAs with miR-21 targets predicted by TargetScan 5.1, miRBase Targets V.5,
miRNAMap 2.0, PicTar and miRanda 3.0. Of the 27 mRNAs, 3 were recognized by the algorithms (Table 2). Because miR-21 targets are expected to up-regulated for the LNA-antimiR-21 samples, ANKRD46 and EIF4A2, the two up-regulated genes upon miR-21 knockdown in the two cell lines and predicted by target prediction programs, were selected for further investigation.

**miR-21 directly targets ANKRD46 in BC cells**

The microarrays were validated by qRT-PCR assay. Consistent with the microarray results, qRT-PCR showed increased ANKRD46 and EIF4A2 mRNA levels in MCF-7 and MDA-MB-231 cell lines upon miR-21 inhibition, although the increase of EIF4A2 in MDA/LNA-antimiR-21 cells was relatively modest (Figure 4c). To determine whether miR-21 affects the expression of the potential endogenous target genes, we transfected MCF-7 and MDA-MB-231 cells with LNA-antimiR-21 or LNA-control. Western bolt showed that LNA-antimiR-21 led to up-regulation of endogenous ANKRD46 in both MCF-7 (P = 0.005) and MDA-MB-231 cells (P = 0.004), but no significant up-regulation of EIF4A2 protein (Figure 5c).

We further tested whether miR-21 could directly repress the identified mRNA targets through 3' UTR interactions (Figure 5a). Thus, the full-length 3' UTRs of the human genes ANKRD46 and EIF4A2 were cloned into the downstream of the luciferase gene (pMIR-REPORT), respectively. These vectors were then used to assess whether miR-21 could repress luciferase activity in 293T cells. ANKRD46 3' UTR showed a reduction to 54.8% of total luciferase reporter activity, in presence of miR-21, but EIF4A2 3' UTR did not display significant reduction of luciferase levels, compared with the miR-control (Figure 5b). These results suggest that miR-21 directly targets ANKRD46 in BC cells.

**miR-21 and EIF4A2 proteins are inversely expressed in resected patient tumors in vivo**

We examined ANKRD46 [NCBI: NP940683] and EIF4A2 [NCBI: NP001958] protein levels by IHC on TMAs constructed by the BC cases described in

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**Figure 4** mRNA profiling of miR-21-knockdown BC cell lines. MCF-7 and MDA-MB-231 cells were transfected with 50 nM LNA-antimiR-21 or LNA-control. Total RNAs were isolated from MCF-7 cells 48 h post transfection and from MDA-MB-231 cells 36 h post transfection, respectively, and were hybridized to the human genome oligo array V1.0 (CapitalBio, China). (a) The scatter plot shows the LNA-antimiR-21 expression values normalized to LNA-control values, applying a cut-off of 1.3. Each dot represents one probe set. (b) Unsupervised hierarchical cluster analysis of intersection of mRNAs differentially expressed in MCF-7 and MDA-MB-231 cells upon LNA-antimiR-21 treatment. Rows, mRNAs; columns, biological samples. For each mRNA, red is expression higher than average expression across all samples, green is expression lower than average. (c) Validation of microarray results by qRT-PCR, normalizing on GAPDH RNA levels. MCF/A, MCF/LNA-antimiR-21; MCF/C, MCF/LNA-control; MDA/A, MDA/LNA-antimiR-21, MDA/C, MDA/LNA-control.
Materials and Methods. EIF4A2 was found in the cytoplasm, and ANKRD46 was seen in both the cytoplasm and nucleus (Figure 6a). ANKRD46 low expression was found in 47.5% (staining score < 4; median = 4); EIF4A2 low expression was found in 21.2% (staining score < 7; median = 7) of the 99 BC cases. Next, we investigated the negative regulation of endogenous EIF4A2 and ANKRD46 protein by endogenous miR-21 in vivo. In 99 successfully tested cases out of 113, endogenous miR-21 and EIF4A2 protein levels were inversely expressed in resected patient tumors ($r_s = -0.283, n = 99, P = 0.005$, Spearman’s correlation analysis). However, no significant association between miR-21 and ANKRD46 ($P = 0.181$, Spearman’s correlation analysis) was observed.

### Table 2 Regulated mRNAs in BC cells after miR-21 knockdown determined by oligo array

| Gene Name | NCBI accession number | Genbank accession number | Description | Fold change | Prediction program |
|-----------|-----------------------|--------------------------|-------------|-------------|-------------------|
| ANKRD46   | NM_198401             | U79297, BC035087         | ankyrin repeat domain 46 | 1.78        | miRBase Targets V5 |
| ACTN4     | NM_004924             | BC005033                 | Alpha-actin 4 (F-actin cross linking protein) | 1.75        |                   |
| NRB1      | NM_000475             | S74720                   | Nuclear receptor 0B1 (Nuclear receptor DAX-1) | 1.60        |                   |
| HIRA      | NM_003325             | BC039835, X89987         | HIRA protein | 1.48        |                   |
| COL18A1   | NM_030582, NM_130444, NM_130445 | AF018081 | Collagen alpha 1(XVIII) chain precursor | 1.43        |                   |
| SAMD11    | NM_152486             | BC024295, AK054643       | sterile alpha motif domain containing 11 | 1.42        |                   |
| EIF4A2    | NM_001967             | AL117412                 | Eukaryotic initiation factor 4A-II | 1.42        | miRNAmap          |
| SFRS16    | NM_007056             | AF042800, AK094681       | Splicing factor, arginine/serine-rich 16 | 1.40        |                   |
| NP_079316 | -                     | BC004930                 | zinc finger protein 614 | 1.37        |                   |
| IL18BP    | NM_173043, NM_005699, NM_173042 | AF110801 | Interleukin-18 binding protein precursor | 1.36        |                   |
| GBF1      | NM_004193             | AK025330, AF068755       | Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1 | 1.35        |                   |
| GNPDA1    | NM_005471             | AJ002231                 | Glucosamine-6-phosphate isomerase | 1.34        |                   |
| SPUD_HUMAN| NM_013349             | -                        | SPUF protein precursor | 1.34        |                   |
| PXN1A1    | NM_032242             | AK127254                 | plexin A1; plexin 1 | 1.32        |                   |
| STMN3     | NM_015894             | AK094112                 | Stathmin 3 (SCG10-like protein) | 1.32        |                   |
| NP_056236 | NM_015421             | AK093383, AL080088       | DKFZP644K062 protein | 1.31        |                   |
| -         | -                     | AK056473                 | adaptor-related protein complex 2, beta 1 subunit | 1.31        |                   |
| AP2B1     | NM_001282             | M34175                   | Histone H2A1 (H2A/I) | -1.47       |                   |
| -         | -                     | CR608156                 | Protein kinase C, iota type | -1.43       |                   |
| HIST1H2AC | -                     | L33881, BC042405, BC022016 | Phosphate carrier protein, mitochondrial precursor (PTP) | -1.39       |                   |
| PRKCI     | NM_002740             | L33881, BC042405, BC022016 | G1/S-specific cyclin E1 | -1.37       |                   |
| SLC25A3   | NM_002635, NM_213611, NM_213612, NM_005888 | BX647062, AK057575 | Guanine nucleotide-binding protein GIl/Gi5/ G(O) gamma-12 subunit | -1.32       | Miranda |
| CCNE1     | NM_057182, NM_001238  | M74093, BC035498         | Serine/threonine-protein kinase DCKML1 | -1.34       |                   |
| DCAMKL1   | NM_004734             | AB002367                 | Guanine nucleotide-binding protein GIl/Gi5/ G(O) gamma-12 subunit | -1.32       | Miranda |
| GNG12     | NM_018841             | AL832431                 | Hypothetical protein FLJ14050 | -1.32       |                   |
| Q8TAY7    | NM_024869             | BC025658                 | Hypothetical protein MGC23280 | -1.31       |                   |
| NP_653284 | NM_144683             | BC015582                 | Growth and transformation-dependent protein | -1.30       |                   |
| NP_055182 | NM_014367             | -                        | Growth and transformation-dependent protein | -1.34       |                   |

Fold-regulation of mRNAs affected by miR-21 knockdown compared with control-transfected cells (MCF-7 and MDA-MB-231) at indicated time points. Fold-changes for all mRNAs are derived from a single microarray experiment, with two relevant genes confirmed by qRT-PCR (Figure 4B). Shown are mRNAs changing by at least 1.3-fold in both cell lines. Genes in bold were selected for candidate gene analyses by qRT-PCR; * marginal signal intensity; NCBI, National Center for Biotechnology Information; GB, GenBank.
Correlation of ANKRD46 and EIF4A2 expression with BC clinicopathological features and prognosis

Using Pearson’s Chi-Square test, we found that, in BC tissues, IF4A2 protein correlated with CerbB2 status ($P = 0.019$), and ANKRD46 protein correlated with ER ($P = 0.021$) and PR ($P = 0.001$, Table 3). No significant correlation was observed between EIF4A2, ANKRD46, or other parameters. The five-year overall survival rate of the 99 BC patients was 59.60% (Figure 6b). The five-year survival rate in patients with high EIF4A2 protein level was 64.10% ($n = 78$), significantly higher than those with a low EIF4A2 protein level (42.86%, $n = 21$; $P = 0.044$, log-rank test; Figure 6b). The five-year survival rate in patients with high ANKRD46 protein was 53.85% ($n = 52$), which was not statistically different from those with low ANKRD46 protein (65.96%, $n = 47$; $P = 0.146$, log-rank test; Figure 6b).

Discussion

miR-21 is a key molecule in a wide range of cancers, and identifying its functional role in BC has direct clinical implications. We show here that knockdown of miR-21 suppresses cell growth and proliferation of MCF-7 cells in vitro, and suppresses MCF-7 xenograft growth. This result is consistent with the findings of Si et al. [9]. Interestingly, our study suggests that LNA-antimiR-21 also suppresses the growth and proliferation of MDA-MB-231 in vitro, in contrast to a recent report that found no effect of LNA-antimiR-21 on the growth of MDA-MB-231 in vitro or in vivo, although anti-miR-21-treated tumors were slightly smaller than control tumors [10]. One possibility could be differences in transfection efficiency, or miRNA ASO potency. Our results suggest that, as an oncomir, miR-21 also affects cell migration. MCF-7 cells are hormone-sensitive and difficult to culture in vivo. Therefore, we used 17-estradiol to facilitate MCF-7 cells growth in nude mice, which is a common technique. Recently, estradiol was shown to down-regulate miR-21 expression in MCF-7 cells [32], although another study found estradiol-mediated up-regulation of miR-21 in MCF-7 cells [33]. In our study, the miR-21 knockdown effect was reduced from 5.72 log2-scale reduction before cell injection to 0.96 log2-scale reduction after mice sacrifice. Based on our results, we propose that estradiol reduced differences in miR-21 level between MCF/PNA-antimiR-21 and MCF/PNA-control cells, which would explain, in part, why differences in tumor weight between the two groups were not significance ($P = 0.065$). Nonetheless, treatment with anti-miR-21 reduced MCF-7 xenograft growth by approximately 68% for up to nine days. In vivo results suggested that the PNA-based miR-21 inhibitor had a subtle yet reproducible inhibitory effect on tumor growth. MCF-7 xenograft tumor sections demonstrated that miR-21 inhibition induced apoptosis of MCF-7 cells, confirming a previous study [9]. We also showed that miRNA inhibition can be achieved without transfection or electroporation of human BC cell lines.
highlighting the potential of PNA for future therapeutic applications.

ANKRD46, also known as ankyrin repeat small protein (ANK-S), is a 228-amino acid single-pass membrane protein, of unclear function. For the first time, we identify miR-21 as an important regulator of ANKRD46 mRNA and protein levels in BC cells. Our data showed that miR-21 directly interacted with the ANKRD46 3′ UTR and inhibited ANKRD46 expression, though there was no significant association between miR-21 and ANKRD46 in resected patient tumors. This discrepancy may be due to three reasons. First, the artificial luciferase reporter assays do not fully recapitulate miRNA regulation in vivo [34]; second, the expression of ANKRD46 protein in patient tumors reflected specific time-point feature, which maybe different to the in vitro subsequent increase of ANKRD46 protein at the time point of observation (the indicated hours after transfection); third, immunohistochemistry (IHC) is conventional a semi-quantitative method with relatively limited sensitivity. IHC may not be sensitive enough to observe the down-regulation of ANKRD46 by miR-21. Functional

Figure 6 EIF4A2 or ANKRD46 expression and survival in BC patients. (a) Representative TMAs sections stained for ANKRD46 and EIF4A2 from different BC. Examples for staining score 0 (negative), 3, 5 and 7 of marker expression are shown. All images were taken at × 200. (b) Kaplan-Meier survival curve and log-rank test for 99 BC patients showing five-year overall survival. ANKRD46 expression had no significant relationship to patient survival ($P = 0.146$). High EIF4A2 expression was associated with significantly improved OS ($P = 0.044$) in women with BC. TMAs; tissue microarrays; OS, overall survival.
study of ANKRD46 is required in the future to determine weather ANKRD46 is a functional target of miR-21 in BC progression as demonstrated in this study.

EIF4A2, an ATP-dependent RNA helicase, is expressed widely in human tissues [35]. In this study, we found that miR-21 and EIF4A2 protein were inversely expressed in resected BC patient tumors. But we did not find miR-21 binding sites in the EIF4A2 3' UTR and found no significant increase of EIF4A2 protein upon miR-21 knock-down in MCF-7 and MDA-MB-231 cells, although EIF4A2 mRNA increased after anti-miR-21 transfection. Taken together, the data reported here suggest that there maybe unknown indirect interactions between miR-21 and EIF4A2 in BC progression. In adult mice, the expression of the two EIF4A isoforms is dependent on cell growth status, with EIF4A1 expressed in all tissues, while EIF4A2 is expressed only in tissues with a low rate of cell proliferation [36], indicating an anti-proliferative effect for EIF4A2. We for the first time revealed that low EIF4A2 expression correlated with low ERBB2 expression and poor survival of BC patients, suggesting its possible functional role in BC and urging further investigation.

Conclusions
We demonstrate that MCF-7 and MDA-MB-231 cells transfected with anti-miR-21 show growth inhibition in vitro and in vivo, as well as cell migration in vitro. In addition, ANKRD46 is newly identified as a direct target of miR-21 in BC. These results suggest that miR-21 inhibitory strategies using PNA-antimiR-21 may have potential for therapeutic applications in BC treatment.

Table 3 Correlation between target gene protein levels and clinicopathological parameters of 99 BC cases

| Variable                     | EIF4A2        | ANKRD46       |
|------------------------------|---------------|---------------|
|                              | Low (n = 21)  | High (n = 78) |
|                              | p*            |               |
|                              | Low (n = 47)  | High (n = 52) |
|                              | p*            |               |
| Age (years)                  |               |               |
| < 48                         | 8             | 39            | 0.332 | 23 |
| ≥ 48                         | 13            | 39            | 0.0782|
| Pathologic grade             |               |               |
| I                            | 2             | 20            | 0.115 | 14 |
| II, III                      | 19            | 58            | 0.221 |
| Clinical stage**             |               |               |
| I, II                        | 15            | 61            | 0.514 | 41 |
| III                         | 6             | 17            | 0.051 |
| Lymph node status            |               |               |
| Metastasis                   | 14            | 43            | 0.342 | 23 |
| No Metastasis                | 7             | 35            | 0.098 |
| ER status                    |               |               |
| Negative                     | 12            | 36            | 0.371 | 18 |
| Positive                     | 9             | 42            | 0.021 |
| PR status                    |               |               |
| Negative                     | 11            | 28            | 0.170 | 11 |
| Positive                     | 10            | 50            | 0.001 |
| CerbB2 status               |               |               |
| 0,1+                         | 17            | 41            | 0.019 | 29 |
| 2+3+                         | 4             | 37            | 0.832 |

*Two-sided Pearson Chi-Square Test; ** there were no stage IV patients because all the cases selected were surgical patients without neoadjuvant therapy to avoid the effect of the preoperative radiotherapy and/or chemotherapy on miRNAs; EIF4A2, eukaryotic translation initiation factor 4A2 protein; ANKRD46, ankyrin repeat domain 46 protein; ER, estrogen receptor; PR, progesterone receptor; CerbB2, v-erb-b2 erythroblastic leukaemia viral oncogene homolog 2 receptors.

Additional material

Additional file 1: Word document containing the protocol of LNA-based CISH and FISH for miRNA in the present study.

Additional file 2: Excel file containing a table listing RT and PCR primers for miR-21 and putative target genes.

Additional file 3: PDF file comprising a figure showing the expression of miR-21 in human BC tissues and FA tissues by FISH.

Additional file 4: PDF file comprising a figure showing relative miR-21 induction by LNA-antimiR-21 in BC cells.

Table 3 Correlation between target gene protein levels and clinicopathological parameters of 99 BC cases
Abbreviations
3′ UTR 3′ untranslated region; AICC: American Joint Committee on Cancer; ANK-S: ankyrin repeat small protein; ASOs: antisense oligonucleotides; BC: breast cancer; CerbB2: v-erb-b2 erythroblastic leukaemia viral oncogene homology 2 receptors; CSH: chromogenic in situ hybridization; DAPI: 4′,6-diamidino-2-phenylindole; EIF4A2: eukaryotic initiation factor 4A isoform 2; ER: estrogen receptor; F: forward primer; FA: fibroadenoma; FISH: fluorescent in situ hybridization; FITC: fluorescein isothiocyanate; GEO: Gene Expression Omnibus; HIC: immunohistochemistry; L: length; LNA: Locked nucleic acid; miRNAs: microRNAs; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Mut: mutation; NATs: normal adjacent tissues; NBT/BCIP: nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; NCBI: National Center for Biotechnology Information; OS: overall survival; PNA: peptide nucleic acid; PR: progesterone receptor; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; R: reverse primer; RP: reverse transcription primer; SABC-AP: alkaline phosphatase-conjugated streptavidin biotin complex detection system; standard deviation; SYSC: Sun Yat-sen University Cancer Center; TMA: tissue microarrays; TNM: tumor-lymph-node-metastasis; W: width; WT: wild-type.

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Author details
State Key Laboratory of Oncology in Southern China, Sun Yat-sen University Cancer Center, 651 Dong Fong Road East, Guangzhou, 510060, PR China. *Department of Pathology, Sun Yat-sen University Cancer Center, 651 Dong Fong Road East, Guangzhou, 510060, PR China. Department of Experiment Research, Sun Yat-sen University Cancer Center, 651 Dong Fong Road East, Guangzhou, 510060, PR China.

Authors’ contributions
LXY, QNW and YZ carried out the substantial experiment work and drafted the manuscript. JYS designed and financially supported the study. QNW and DZL were responsible for patient samples and tissue array construction. JHH and JF supported the immunohistochemistry. YFL carried out the luciferase reporter assay, JPM, QWW and YXZ helped carry out the research design and critically reviewed the final version of the manuscript for all authors. All authors read and approved the final manuscript.

Competing interests
Miss Li Xu Yan and Mrs Yan Zhang are doctoral degree candidates; Miss Qi Nian Wu and Mrs Yang Yang Li are master’s degree candidates atSYSUCC. Mr Ding Zhen Liao, Mr Jing Hui Hou and Mrs Jia Fu were technicians at SYSC. Dr Mu-Sheng Zeng, Jing Ping Yun, Qiu Liang Wu and Yi Xin Zeng are Professors at SYSUCC. Dr Shao is a Professor and Vice Director at Department of Pathology of SYSUCC. The authors declare that they have not received any reimbursements, fees, funding, or salary, nor hold any stocks or shares in an organization that may in any way gain or lose financially from the publication of this manuscript, either now or in the future. The authors do not hold or are not currently applying for any patents relating to the content of the manuscript. The authors declare that they do not have any other financial or non-financial competing interests.

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References
1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005, 55:74-108.
2. Ambros V. MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. Cell 2003, 113:673-676.
3. Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 2006, 6:259-269.
4. Chan JA, Kichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res 2005, 65:6029-6033.
5. Fulci V, Chiaretta S, Goldoni M, Azzalin G, Canucci N, Tavolaro S, Castellano L, Magrelli A, Cistola F, Messina M, Maggio R, Peragine N, Santangelo S, Mauro FR, Landgraf P, Tuschl T, Weir DB, Chen M, Russo JJ, Ju J, Sheridan R, Sander C, Zavalon M, Guarni A, Foo R, Macino G. Quantitative technologies establish a novel miRNA profile of chronic lymphocytic leukemia. Blood 2007, 109:4494-4501.
6. Yanaihara N, Caplen N, Bowman E, Seike M, Kurimoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calin GA, Liu CG, Croce CM, Harris CC. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 2006, 9:198-199.
7. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magni E, Pedriali M, Fabbri M, Campiglio M, Menard S, Palazzo JP, Rosenberg A, Mussi P, Volinia S, Nencis I, Calin GA, Querzoli P, Negrini M, Croce CM. MicroRNA gene expression deregulation in human breast cancer. Cancer Res 2005, 65:7065-7070.
8. Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A, Lund AH: Programmed cell death 4 (PDCD4) is an important functional target of the miRNA mir-21 in breast cancer cells. J Biol Chem 2008, 283:1026-1033.
9. Si ML, Zhu S, Wu H, Lu Z, Wu F, Mo YY. mir-21-mediated tumor growth. Oncogene 2007, 26:2799-2803.
10. Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res 2008, 18:350-359.
11. Oh SY, Ju Y, Park H: A highly effective and long-lasting inhibition of miRNAs with PNA-based antisense oligonucleotides. Mol Cells 2009, 28:341-345.
12. In Pathology and Genetics of Tumours of the Breast and Female Genital Organs. Edited by: Tavassoli F, Devilee P. Lyon: IARC Press, 2003.
13. Singletary SE, Allred C, Ashley P, Bassett LW, Berry D, Bland KI, Borgen PI, Clark GM, Edge SB, Hayes DF, Hughes LL, Hutter RV, Morrow M, Page DL, Recht A, Thrivurt RL, Thor A, Weaver DL, Weiss HS, Greene FL. Staging system for breast cancer: revisions for the 6th edition of the AJCC Cancer Staging Manual. The Surgical Clinics of North America 2003, 83:803-819.
14. Obermarterer G, Martinez J, Aienius M. Locked nucleic acid-based in situ detection of miRNAs in mouse tissue sections. Nature Protocol 2007, 2:1506-1514.
15. Lee SH, Kurz J, Lin SH, Yu-Lee LY. 16dKDA prolactin inhibits endothelial cell migration by down-regulating the Ras-Ras1-Rac1-Pak signaling pathway. Cancer Res 2007, 67:11094-11053.
16. Wu L, Li Z, Zhang Y, Zhang P, Zhu X, Huang J, Ma T, Li T, Song Q, Li Q, Guo Y, Tang J, Ma D, Chen KH, Qiu X. Adenovirus-expressed human hyperplasia suppressor gene induces apoptosis in cancer cells. Mol Cancer Ther 2008, 7:222-232.
17. Mela SA, Ropero S, Moutinho C, Altona LN, Yamamoto H, Calin GA, Rossi S, Fernandez AF, Carneiro F, Oliveira C, Ferreira B, Liu CG, Villanueva A, Capella G, Schwarz SJ, Shiekhattar R, Esteller M. A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. Nat Genet 2009, 41:365-370.
18. Patterson TA, Lobenhoffer EK, Fulmer-Smentek SB, Collins PJ, Chu TM, Bao W, Fang H, Kawasaki ES, Hager J, Tikhonova IR, Walker SJ, Zhang L, Hurban P, de Longeville F, Fuscoe JC, Tong W, Shi L, Wolffinger RD. Performance comparison of one-color and two-color platforms within the MicroArray Quality Control (MAQC) project. Nat Biotechnol 2006, 24:1140-1150.
19. Gene Expression Omnibus. GEO. [http://www.ncbi.nlm.nih.gov/geo/]
20. Yan LX, Huang XF, Shao Q, Huang MY, Deng L, Wu QL, Zheng YX, Shao JF. MicroRNA mir-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA 2008, 14:2548-2560.
21. Grampantieri L, Ferracin M, Foroni F, Veronesi A, Sabbioni S, Liu CG, Calin GA, Giovannini C, Ferarezi E, Grmi GL, Croce CM, Bolognì L, Negrini M. Cyclin G1 is a target of mir-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. Cancer Res 2007, 67:6092-6099.
22. Grimm A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 2007, 27:91-105.
23. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ: miRBase: tools for microRNA genomics. Nucleic Acids Res 2008, 36:D154-158.
24. Hsu SD, Chu CH, Tsou AP, Chen SJ, Chen HC, Hsu PW, Wong YH, Chen YH, Chen GT, Huang HD. miRNomeMap 2.0: genomic maps of miRNAs in metazoan genomes. Nucleic Acids Res 2008, 36:D165-169.

Yan et al. Breast Cancer Research 2011, 13:R2
http://breast-cancer-research.com/content/13/1/R2
Page 13 of 14
25. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N: Combinatorial microRNA target predictions. Nat Genet 2005, 37:495-500.

26. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS: Human MicroRNA targets. PloS Biol 2004, 2:e63.

27. Song CH, Park SY, Eom KY, Kim JH, Kim SW, Kim JS, Kim IA: Potential prognostic value of heat-shock protein 90 in the presence of phosphatidylinositol-3-kinase overexpression or loss of PTEN, in invasive breast cancers. Breast Cancer Res 2010, 12(R2).

28. Gamallo C, Palacios J, Suarez A, Pizarro A, Navaro P, Quintanilla M, Cano A: Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma. The American Journal of Pathology 1993, 142:987-993.

29. Tuominen VJ, Ruotoistenmaki S, Vitianen A, Jumppanen M, Isola J: Immunofluo: a publicly available web application for quantitative image analysis of estrogen receptor (ER), progesterone receptor (PR), and Ki-67. Breast Cancer Res 2010, 12:R56.

30. Sempere LF, Christensen M, Silahtaroglu A, Bak M, Heath CV, Schwartz G, Wells W, Kauppinen S, Cole CN: Altered MicroRNA expression confined to specific epithelial cell subpopulations in breast cancer. Cancer Res 2007, 67:11612-11620.

31. Matkovich SJ, Van Booven DJ, Youker KA, Torre-Amione G, Diwan A, Eschenbacher WH, Dorn LE, Watson NA, Margulies KS, Dorn GW: Reciprocal regulation of myocardial microRNAs and messenger RNA in human cardiomyopathy and reversal of the microRNA signature by biomechanical support. Circulation 2009, 119:1263-1271.

32. Wickramasinghe NS, Manavalan TT, Dougherty SM, Riggs KA, Li Y, Klinger CM: Estradiol downregulates miR-21 expression and increases miR-21 target gene expression in MCF-7 breast cancer cells. Nucleic Acids Res 2009, 37:4850-4861.

33. Bhat-Nakshatri P, Wang G, Collins NR, Thomson MJ, Geistlinger TR, Carroll JS, Brown M, Hammond S, Siour EF, Liu Y, Nakshatri H: Estradiol-regulated microRNAs control estradiol response in breast cancer cells. Nucleic Acids Res 2009, 37:4850-4861.

34. Eulalio A, Huntzinger E, Izaurralde E: Getting to the root of miRNA-mediated gene silencing. Cell 2008, 132:9-14.

35. Sudo K, Takahashi E, Nakamura Y: Isolation and mapping of the human EIF4A2 gene homologous to the murine protein synthesis initiation factor 4A-II gene Eif4a2. Cytogenet Cell Genet 1995, 71:385-388.

36. Williams-Hill DM, Dunican RF, Nielsen PJ, Tahara SM: Differential expression of the murine eukaryotic translation initiation factor iogenes elf4AII and elf4AIiI is dependent upon cellular growth status. Arch Biochem Biophys 1997, 338:111-120.

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