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The cell growth suppressor, mir-126, targets IRS-1

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ABSTRACT

miRNAs are a family of approximately 22-nuleotide-long noncoding RNAs involved in the formation and progress of tumors. Since traditional methods for the detection of miRNAs expression have many disadvantages, we developed a simple method called polyA RT PCR. With this method, we detected a series of miRNAs and found that mir-126 is one of the miRNAs underexpressed in breast cancer cells. Flow cytometry analysis showed that mir-126 inhibited cell cycle progression from G1/G0 to S. Further studies revealed that mir-126 targeted IRS-1 at the translation level. Knocking down of *IRS-1* suppresses cell growth in HEK293 and breast cancer cell MCF-7, which recapitulates the effects of mir-126. In conclusion, we developed a simple method for high-throughput screening of miRNAs and found that mir-126, a cell growth suppressor, targets *IRS-1*.

microRNAs (miRNAs) are a class of approximately 22-nuleotide noncoding RNAs which are expressed in many organisms [1,2]. Mature miRNAs arise from one arm of endogenous hairpin transcripts by sequential processing in the nucleus and cytoplasm [3], which inhibit the translation of target mRNAs through imperfect base pairing with the 3′-untranslated regions (3′-UTRs) or degrade their target mRNAs through perfect or near-perfect base pairing [4,5]. miRNAs have important roles in many biological processes, such as cell death [6], proliferation [7], differentiation [8], etc. Increasing evidences show that mutation and aberrant expression of some specific miRNAs are associated with the formation and progress of many types of cancers, including chronic lymphocytic leukemia [9], colorectal neoplasia [10] and cancer breast [11–13].

Considering the numerous disadvantages of traditional miRNAs detection methods, such as stem-loop PCR and Northern blot, we developed a new method suitable for high-throughput miRNA screening. Using this method, we assayed a series of miRNAs in breast cancer cells, and found that mir-126 was one of the miRNAs down-regulated in breast cancer cells. Mir-126 belongs to a highly conserved mircoRNA family and locates at intron 5 of EGFL7. miRNA microarray studies also find that mir-126 correlates with the classification of breast cancer samples [13].

In this study, we further looked into the function of mir-126 and found that mir-126 inhibited cell cycle transition from G1/G0 to S and targeted insulin receptor substrate-1 (IRS-1).

Materials and methods

Cell lines and transfection. All cell lines were obtained from the American Type Culture Collection. Cell transfection was mediated by Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. All plasmid DNA for transfection was extracted by DNA Midiprep or Midiprep kit (Qiagen).

Plasmids expressing the shRNA and mir-126. Using HEK293 genomic DNA as the PCR template, the DNA fragment encoding mir-126 pre-miRNA (flanking upstream and downstream 30–50 nt) was amplified and inserted into the expressing vector pSilencer4.1CMV-puro (Ambion). DNA fragments for anti-IRS-1 shRNAs were generated by annealing two complementary oligonucleotides and cloned into pSilencer4.1CMV-puro as reported previously [14]. Sequences for oligonucleotides used are listed in Table 1.

MTT assay. HEK293 or MCF-7 cell were grown in 96-well plates. pSilencer4.1CMV-based plasmids or mature miRNA oligos (GenePharma, Shanghai, China) were transfected into HEK293 or MCF-7 cells with Lipofectamine2000. Seventy-two hours after transfection, the cells were harvested for a standard tetrazolium bromide (MTT) assay. For MCF-7 cells, 2 μg/ml puromycin was added 36 h before the harvest to enrich plasmid-transfected cells. All assays were performed in Octuplicate and repeated at least three times.

Cell count assay and cell cycle assay by flow cytometry. pSilencer4.1CMV based plasmids were transfected to HEK293 cells grown

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Table 1
Oligonucleotides for cloning mir-126, shRNA and 3′-UTRs of target genes

Primer	Sequence (restriction sites are capitalized)
mir 126 F	aaGGATCCagcgcagcattctggaag
mir 126 F	ggAAGCTTggaggtcaaggctgaggtct
shIRS1-1F	gatccgtcagtctgtcgtccagtattcaagagatactggacgacagactgaccga
shIRS1-1T	agcttcggtcagtctgtcgtccagtatctcttgaatactggacgacagactgacg
IRS1 F	ccTCTAGAtgacctcagcaaatcctcttc
IRS1 T	ccTCTAGActctctccacccaacgtga
TOM1 F	ccTCTAGAtgcttgacccagtgtgactc
TOM1 T	ccTCTAGActgatgagatctcgcccact
RGS3 F	ccTCTAGAgctcagcgttcacaccag
RGS3 T	ccTCTAGAccccatcagccagttcttta
CCNE2 F	ggTCTAGAcctccattgtgagataaggacag
CCNE2 T	ggTCTAGAcacaattagtggtgttttctttttca
muIRS1 F	tcttggatccttcaagtcgcatccatttcagtttgtttac
muIRS1 T	gacttgaaggatccaagagaggaagatatgaggtcctagttg
muCCNE2 F	tcttggatccttcaagtcgaagtcttctgtatagtttg
muCCNE2 T	gacttgaaggatccaagatatagtttcttcaaagggagaagag

in 6 cm dishes for flow cytometry assay, and in 24-well plates for cell counting. Twenty-four hours after transfection, the cells were re-plated into three dishes or three wells. Another 36 h later the cells were harvested for flow cytometry assay or cell counting. Cells were washed with phosphate-buffered saline (PBS) before fixation in citric acid. The fixed cells were treated with 20 μg/ml DNase-free RNaseA in PBS at 37 °C for 30 min, stained with propidium iodide (PI) at room temperature for 5–10 min. The ratio of apoptotic cells and cells in different stages of cell cycle was analyzed by flow cytometry.

Construction of 3′-UTR reporter and luciferase assay. Using genomic DNA from HEK293 cells as the template, DNA fragments of 3′-UTRs from predicted target genes containing potential mir-126 binding sites were amplified and cloned into the XbaI site immediately downstream of the stop codon in the pGL3-promoter vector (Promega). The predicted mir-126 binding sites were replaced with an 18 bp-long fragment by using the overlapping four-primer PCR to produce mutated 3′-UTR pGL3 report plasmids. Oligonucleotides used are listed in Table 1.

HEK293 cells were seeded in 96-well plate. The cells were cotransfected with 5 ng of internal control vector pRL-renilla (Promega), 50 ng of different 3′UTR pGL3-promoter reporters and 150 ng of pSilencer-mir-126 (or pSilencer4.1CMV-negative). Forty-eight hours after transfection, the firefly and Renilla luciferase activities were assayed using Dual-Glo Luciferase assay system (Promega). All experiments were performed in triplicate and repeated at least three times.

Detection of mRNAs, miRNAs and proteins. Whole-cell extracts were prepared using RIPA lysis buffer. SDS-PAGE and Western blotting were performed as described previously [15]. Signals were visualized with SuperSignal West Femto chemiluminescent substrate (Pierce). Antibodies used included anti-IRS-1 (#2382, Cell Signaling), anti-β-actin (A7441, Sigma) and peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology).

Total cellular RNA was extracted using Trizol (Invitrogen). For mRNA detection, *IRS-1* and *beta-actin* mRNA expression were analyzed by the Sybr Green qRT-PCR according to the manufacturer's instructions (Applied Biosystems) or semi-quantitative RT-PCR.

For miRNA detection, polyA tail was added to RNase-free DNase digested total RNA using the *E.coli* polyA polymerase (NEB). Two micrograms of the tailed total RNA was reverse transcribed with ImProm-II (Promega). Conventional PCR or Sybr Green qRT-PCR was used to assay miRNA expression with the specific forward primers and the universal reverse primer complementary to the anchor primer. Anchor RT primer was used as the template for negative control and U6 as internal control. The amplification products were confirmed by DNA sequencing. The primers used were listed in Table 2.

Databases and statistics. We computationally screened target genes of mir-126 with the "Target Scan" program (http://www.targetscan.org/index.html), PicTar (http://pictar.bio.nyu.edu/), miRanda (http://www.microrna.org/microrna/home.do), miRBase (http://microrna.sanger.ac.uk) and miRNAMap (http://mirnamap.mbc.nctu.edu.tw). Results were expressed as means ± SD and differences were tested for significance using 2-sided Student's *t*-test ($n = 8$ for MTT assay and $n = 3$ for flow cytometry, cell counting and luciferase assay).

Results

mir-126 is down-regulated in breast cancer cell lines

We developed a method, named poly(A) RT PCR, suitable for high-throughput screening of miRNAs. The procedure of this method is showed in Fig. 1A. Using this method we detected a series of miRNAs in breast cancer cells, all of them were specific and confirmed by DNA sequencing (data no shown). We found that mir-126 is down-regulated in HEK293 cells and breast cancer cell lines MCF-7 and MDA-MB-231 (Fig. 1B). Since the pre-miR-126 can produce two mature miRNAs—miR-126 and miR-126*, we also detected the expression of mir-126* and found that it was also down-regulated (Fig. 1B). We constructed pSilencer-mir-126 plasmid and found that it could produce mature mir-126 and mir-126* (Fig. 1C).

mir-126 but not mir-126 inhibits cell growth by arresting cells in the G0/G1-to-S-Phase transition*

Overexpression of mir-126 drastically suppressed cell growth in breast cancer cell line MCF-7 as well as HEK293 (Fig. 2A and B), which suggested that the effects of mir-126 on cell growth were not specific to breast cancer cells. In order to identify which mature miRNA functions to suppress cell growth, we chemically synthesized mature miR-126 and miR-126* and analyzed their growth inhibition activities. We found that mature mir-126 but not mir-126* inhibited cell growth (Fig. 2C). Flow cytometry assay showed that overexpression of mir-126 inhibited cell cycle transition from G1/G0 to S phase yet had no effects on cell apoptosis (Fig. 2D).

mir-126 targets IRS-1 at the translation level

To identify possible mir-126 target genes, we performed a computational screen for genes with complementary sites of mir-126 in their 3′-UTR using open access software. Four genes having high prediction scores and are associated with cell growth were chosen for further analysis (Fig. 3A). Plasmid DNA of each pGL3-promoter-based 3′-UTR reporter was co-transfected with pSilencer-mir-126 (or pSilencer4.1CMV-negative control) to examine whether these

Table 2
Sequences of RT-PCR primers

primers	Sequence
mir-126RF	gtccgctcgtaccgtgagtaata
mir-126 starRF	cgcgctcattattacttttggta
Anchor RT primer	cgactcgatccagtctcagggtccgaggtattcgatcgagtcgcactttttttttttttv
Universal rev primer	ccagtctcagggtccgaggtattc
U6F	ctcgcttcggcagcaca
U6T	aacgcttcacgaatttgcgt
IRS-1RT F	gtttccagaagcagccagag
IRS-1RT T	ccTCTAGActctctccacccaacgtga
β-Actin F	ccttcctgggcatggagtcct
β-Actin T	aatctcatcttgttttctgcg

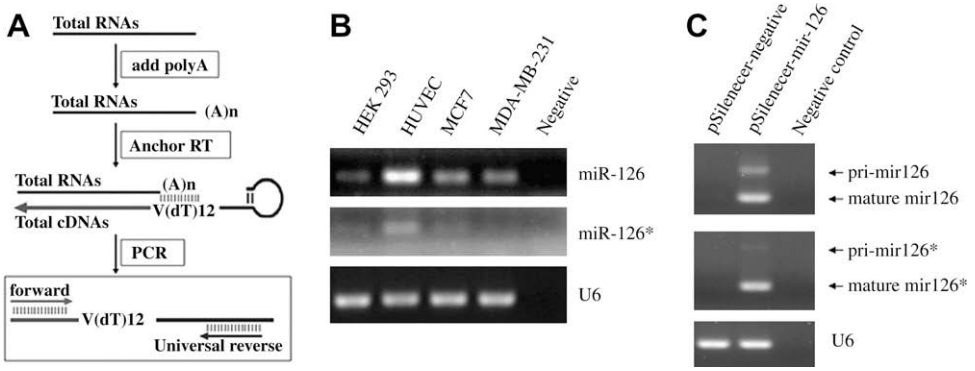

Fig. 1. mir-126 is under-expressed in breast cancer cells. (A) Flow chart of polyA RT-PCR. This method has three major steps: adding polyA tails to total RNAs; reverse-transcription of tailed total RNAs with anchor primer (composed of three parts: 5′ stem-loop, oligo-dT and 3′ anchor base); detection of specific miRNA by PCR with miRNA-specific forward primer and universal reverse primer. (B) Endogenous mature mir-126 and mir-126* were under-expressed in HEK293 and breast cancer cells—MCF-7 and MDA-MB-231. (C) pSilencer-mir-126 plasmid could express mature mir-126 and mir-126*.

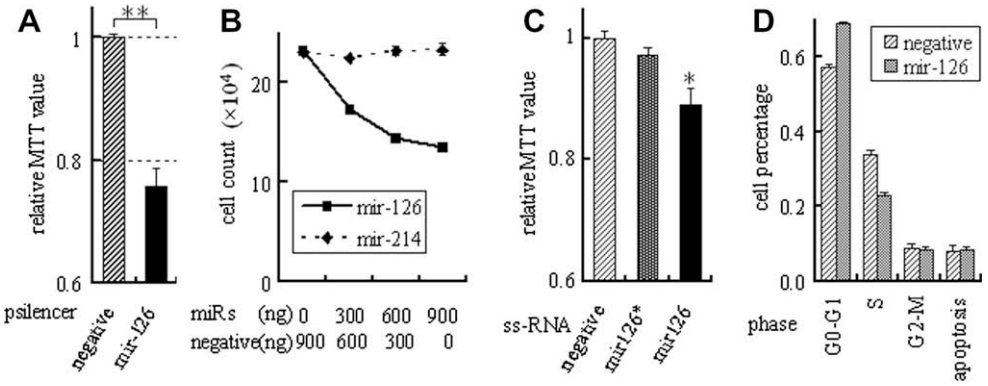

Fig. 2. miR-126 but not miR-126* inhibited cell growth by suppressing cycle phase transition from G0/G1 to S. (A) pSilencer-mir-126 plasmid inhibited cell growth in breast cancer cells MCF-7. MCF-7 cells were transfected with pSilencer-mir-126 and the transfected cells were enriched by puromycin. pSilencer4.1CMV-negative was used as the negative control and cell growth was measured by MTT assay. The value of negative control was set as 100%. (B) Cell growth inhibition of psilencer-mir-126 showed a dose-dependent manner. HEK293 cells were transfected with different ratio of psilencer-mir-126 to pSilencer4.1CMV-negative and viable cell were counted without enrichment by puromycin. (C) The growth of HEK293 cells transfected with synthesized ss-miRNAs was suppressed by mir-126 but not mir-126*. (D) Flow cytometry revealed that mir-126 suppressed cell cycle transition from G1/G0 to S. Compared with the negative control, percentage of cells increased in G1/G0 phase and decreased in S phase. There was no difference in apoptosis.

genes were regulated by mir-126. Among the four genes assayed, luciferase activity with *IRS-1* 3′-UTRs was significantly inhibited by mir-126 (Fig. 3A), while luciferase activity with mutated *IRS-1* 3′-UTR reporter was not inhibited (Fig. 3B and C). This data suggested that mir-126 targets *IRS-1*. We did not find any effects of psilencer-mir-126 on *IRS-1* mRNA level (Fig. 3D), while IRS1 protein level decreased dose-dependently by psilencer-mir-126 transfection (Fig. 3E and F).

Knocking down of IRS-1 by shRNA recapitulated the effects of mir-126 on cell growth

We found that all the four cell lines used in our experiments expressed IRS-1 mRNA and protein (Fig. 4A and B). Real-time PCR showed that the expression level of *IRS-1* mRNA in HUVEC was similar to that in MDA-MB-231 but higher than in HEK293 (Fig. 4C). At the protein level, HUVEC cells had the lowest IRS-1 expression (Fig. 4B). And knockdown of IRS-1 caused decrease in cell growth in HEK293 and MCF-7 cells (Fig. 4D–F), which recapitulated the effect of miR-126.

Discussion

miRNAs are difficult to detect due to their small sizes (about 20 nt). Besides Northern blot, most of traditional methods for miRNA detection are based on step-loop PCR. These methods are tedious and costly for high-throughput screening because each miRNA needs one specific revere transcription (RT) reaction. For example, if we used stem-loop RT-PCR to detect 30 miRNAs in four cell lines, 120 reverse transcription reactions would be needed in addition to four reactions for reference gene, which is very sample-demanding. Because there are only 6 bp-complement between miRNA template and its specific RT primer, step-loop PCR often has low specificity and amplification efficiency. So Taqman probes are often used but it is costly. We developed a simple method called poly(A) RT PCR, which has many advantages: (1) it is simple. One sample only needs one RT reaction to detect different miRNAs; (2) the efficiency of RT reaction is high because of the 13 bp-complement between anchor RT primer and tailed RNA templates; (3) it has high specificity because of the additional 12-(dT) distance between the binding sites of miRNA-specific forward primer and the universal reverse primer.

Using this method, we detected a series of miRNAs in breast cancer cell lines and found that mir-126 and mir-126* is down-regulated in breast cancer cell lines MCF-7 and MDA-MB-231 (Fig. 1B). Overexpression of mir-126 can drastically suppress cell growth in HEK293 and breast cancer cell MCF-7 (Figs. 1D and, 2A and B). We found that mature mir-126 but not mir-126* inhibited cell growth (Fig. 2C). Recently Tavazoie et al. reported that mir-126 can reduce metastasis of malignant cells (CN34) by significantly suppressing

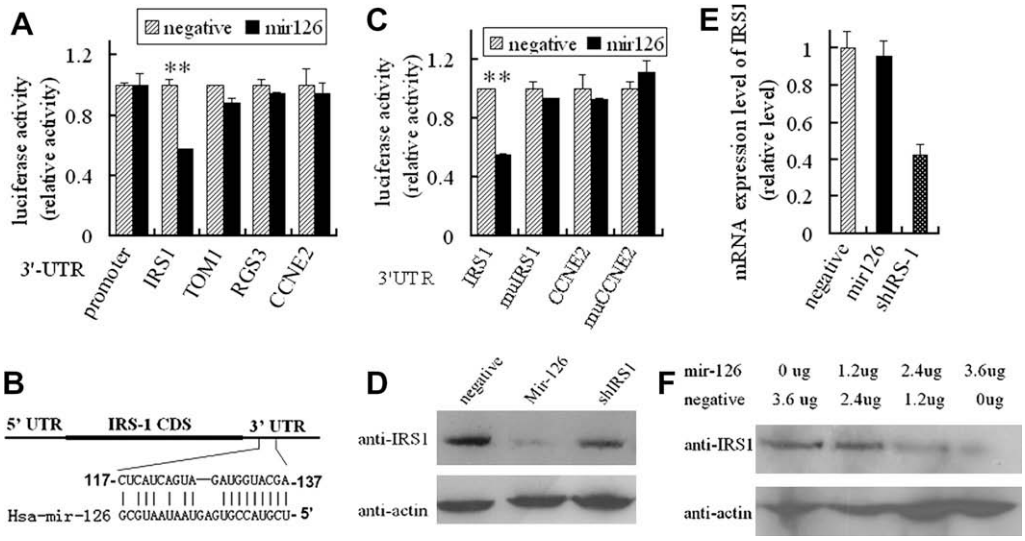

Fig. 3. mir-126 targeted *IRS-1*. (A) mir-126 significantly decreased luciferase report gene activities of *IRS-1* 3′-UTR pGL3-promoter. 3′-UTR report plasmids of 4 computationally predicted target genes were co-transfected with pSilencer-mir-126 (or pSilencer4.1CMV-negative). Firefly luciferase activity of cells was assayed 48 h after transfection and renilla luciferase was used as transfection efficiency correction. Negative control was set as 100%. (B) Schematic structure of mir-126 potential binding sites in 3′-UTRs of *IRS-1* mRNA. (C) Wild-type and mutated 3′-UTR report plasmids of *IRS-1* were cotransfected with pSilencer-mir-126 (or pSilencer4.1CMV-negative). Down-regulation by mir-126 was not observed in the mutated 3′-UTR report plasmids of *IRS-1*. Wild-type and mutated 3′-UTR report plasmids of CCNE2 was used as negative controls. (D) *IRS-1* mRNA was not affected by mir-126, compared to anti-IRS-1 shRNA. HEK293 cells were transfected with psilencer-mir-126, shIRS-1 and negative control respectively, and *IRS1* mRNA expression was detected by Sybr Green qRT-PCR 96 h after transfection. Negative control was taken as 100%. (E) Western blot of IRS-1 protein in HEK293 cells transfected with negative control, mir-126 and anti-IRS-1 shIRS-1, respectively. Anti-actin antibody was used as a loading control (bottom panel). As the anti-IRS-1 shRNAs, mir-126 resulted in decrease of IRS-1 protein. (F) mir-126 dose-dependently decreased IRS-protein expression. HEK293 cells were transfected with different ratio of psilencer-mir-126 to pSilencer4.1CMV-negative and western blot was used to detect IRS-1.

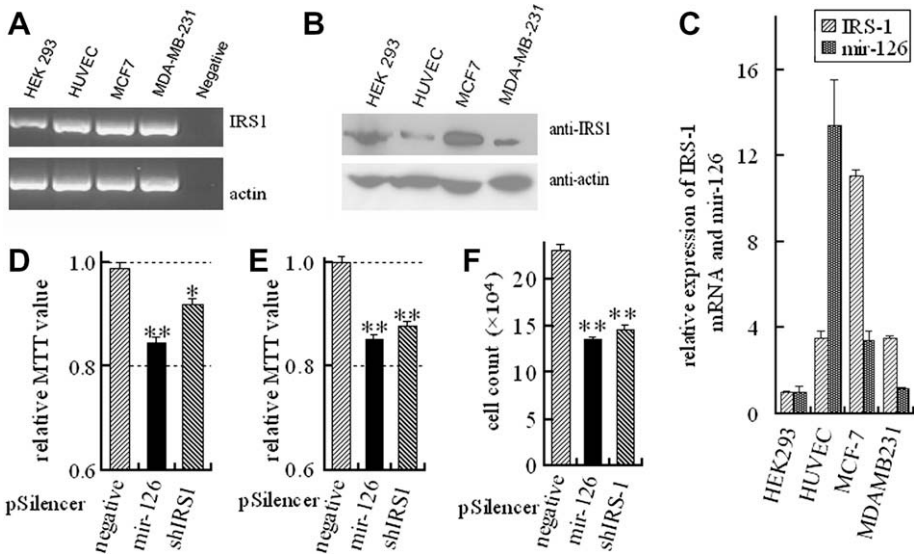

Fig. 4. Knockdown of IRS-1 recapitulates effect of mir-126 on cell growth. (A) The endogenous expression of *IRS1* was detected using semi-qualitative RT-PCR in indicated cell lines. (B) Western blotting assay of endogenous IRS-1 protein in different cell lines. Among the four cell lines, expression in HUVEC was the lowest. (C) Real time PCR of endogenous *IRS-1* mRNA and mir-126 in the four cell lines used in our experiment. Expression of *IRS-1* mRNA was the lowest in HEK293 and the mir-126 was the highest in HUVEC. Expression of IRS-1 and mir-126 in HEK293 is set as 100%. (D) Cell growth was suppressed when IRS1 were down-regulated in breast cancer cell MCF-7. psilencer4.1CMV-based plasmids expressing anti-IRS1 shRNAs were transfected into MCF-7 cells. After enrichment by puromycin, MTT assay was used to analyze the cell growth as described previously. Negative control was set as 100%. (E) Cell growth was suppressed when IRS1 was down-regulated in HEK293. (F) Knockdown of ISR-1 suppressed cell growth. HEK293 cells were transfected with pSilencerCMV4.1-based plasmid DNA and viable cells were counted 60 h after transfection.

growth of tumor volume in mice [16], which also suggests that mir-126 has an important role in cell growth. We demonstrated here that mir-126 suppressed cell growth by inhibiting cell cycle transition from G1/G0 to S phase and had no effects on apoptosis (Fig. 2D).

miRNAs are post-transcriptional negative regulatory elements of genes expression. Harris et al. recently identified vascular cell adhesion molecule 1 (VCAM-1) as a target gene of mir-126 [17]. In order to fully understand the molecular mechanism of growth suppression of mir-126, it is necessary to explore its other target genes associated with cell growth. Among four predicted target genes, *In vitro* luciferase assay suggested that *IRS-1* is the target gene of mir-126 (Fig. 3A–C). Over-expression of mir-126 led to significant decrease of IRS-1 protein but not *IRS-1* mRNA, which confirmed that mir-126 negatively regulated *IRS-1* at the translation level (Fig. 3D–F).

We found that mir-126 and *IRS-1* mRNA were co-expressed in the four cell lines tested (Figs. 2B and 4A). In addition, the expression of IRS-1 protein in HUVEC, HEK293, and MDA-MB-231 cells was negatively correlated with the expression level of mir-126 (Fig. 4B and C). It is noteworthy that MCF-7 highly expresses IRS-1 protein because of its high expression of *IRS-1* mRNA (Fig. 4D), which indicated that IRS-1 is also regulated at the transcription level [18].

As an adaptor of IGF1R, IRS-1 plays an important role in cell growth and proliferation mainly through the PI3K/Akt pathway [19,20]. Constitutive IRS-1 activation is a common phenomenon in tumors, including breast cancer [21]. Up-regulation of IRS-1 in breast cancer cells can cause gain of cell transformation and viability [22]. Moreover reduction of IRS-1 by mir-145 can also inhibit the growth of colon cancer cells [23]. Our results also showed that knockdown of IRS-1 inhibited cell growth in HEK293 and MCF-7, which recapitulates the effect of miR-126 (Fig. 4D–F).

During the time this work was carried on, two other mir-126 target genes associated with cell growth were reported: HOXA9 which promotes growth of leukemic cells [24], and p85beta which is a regulatory subunit involved in 3-kinase (PI3K) signaling [25]. Interestingly, a recent studies showed that HoxA9 can induces insulin-like growth factor-1 receptor (IGF1R) expression [26]. Thus our data and those of the other researchers all suggest that mir-126 may suppress cell growth through intervening the IGF1R-PI3K/Akt signaling pathway by acting on HOXA9, IRS-1 and p85beta.

In summary, we found that as a cell growth suppressor, mir-126 targeted IRS-1 and inhibited the cell cycle phase transition from G1/G0 to S. The method we developed for miRNA detection will find wide application in miRNA research because of the above mentioned advantages.

Acknowledgments

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