Novel Direct Targets of miR-19a Identified in Breast Cancer Cells by a Quantitative Proteomic Approach

Mamoru Ouchida\textsuperscript{1,3,9}, Hirotaka Kanzaki\textsuperscript{1,3,9}, Sachio Ito\textsuperscript{1}, Hiroko Hanafusa\textsuperscript{1}, Yoshimi Jitsumori\textsuperscript{1}, Seiji Tamaru\textsuperscript{2}, Kenji Shimizu\textsuperscript{1}

\textsuperscript{1}Department of Molecular Genetics, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan, \textsuperscript{2}Central Research Laboratory, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan, \textsuperscript{3}Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, California, United States of America

Abstract

The miR-17–92 cluster encodes 7 miRNAs inside a single polycistronic transcript, and is known as a group of oncogenic miRNAs that contribute to tumorigenesis in several cancers. However, their direct targets remain unclear, and it has been suggested that a single miRNA is capable of reducing the production of hundreds of proteins. The majority of reports on the identification of miRNA targets are based on computational approaches or the detection of altered mRNA levels, despite the fact that most miRNAs are thought to regulate their targets primarily by translational inhibition in higher organisms. In this study, we examined the target profiles of miR-19a, miR-20a and miR-92-1 in MCF-7 breast cancer cells by a quantitative proteomic strategy to identify their direct targets. A total of 123 proteins were significantly increased after the endogenous miR-19a, miR-20a and miR-92-1 were knocked down, and were identified as potential targets by two-dimensional electrophoresis and a mass spectrometric analysis. Among the upregulated proteins, four (PPP2R2A, ARHGAP1, IMPDH1 and NPEPL1) were shown to have miR-19a or miR-20a binding sites on their miRNAs. The luciferase activity of the plasmids with each binding site was observed to decrease, and an increased luciferase activity was observed in the presence of the specific anti-miRNA-LNA. A Western blot analysis showed the expression levels of IMPDH1 and NPEPL1 to increase after treatment with anti-miR-19a, while the expression levels of PPP2R2A and ARHGAP1 did not change. The expression levels of IMPDH1 and NPEPL1 did not significantly change by anti-miR-19a-LNA at the mRNA level. These results suggest that the IMPDH1 and NPEPL1 genes are direct targets of miR-19a in breast cancer, while the exogenous expression of these genes is not associated with the growth suppression of MCF-7 cells. Furthermore, our proteomic approaches were shown to be valuable for identifying direct miRNA targets.

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Introduction

MicroRNAs (miRNAs) are endogenous small non-coding single-stranded RNAs, 19 to 23 in length [1,2]. MiRNAs have been suggested to have oncogenic or tumor suppressive functions through their negative post-transcriptional regulation of protein-coding genes [3,4]. Many miRNAs exhibit binding activity to the 3’ untranslated region (3’UTR) of target mRNAs as a result of sequence complementarity. It has been estimated that the miRNAs in a whole cell regulate approximately 30% of all protein-coding genes. A single miRNA is also capable of reducing the production of hundreds of proteins [5]. Therefore, by targeting multiple transcripts and affecting the expression of numerous proteins, miRNAs play key roles in cellular development, differentiation, proliferation and apoptosis [6–9]. Several studies have also demonstrated that more than 50% of miRNAs are located in cancer-associated genomic regions [10], thus suggesting that miRNAs may also play an important role in cancer.

There are a large number of miRNA targets which have been identified by bioinformatics studies [11–13], and many other miRNA targets have been experimentally identified [14]. The target prediction is primarily based on the sequence complementarity between the 5’ end of the mature miRNA and the 3’UTR of the target gene(s). Since there are many cases of both false-positive and false-negative miRNA targets predicted by the current software programs, it is critically important to confirm the miRNA targets by experimental assays [15]. The most extensively used approaches to the target identification of miRNAs include cDNA microarray and real-time PCR-based methods. Considering that the miRNAs are thought to regulate gene expression by translational inhibition, rather than mRNA degradation [1], these methods might thus be problematic when trying to identify direct miRNA targets [16–18]. Consequently, a proteomic approach would provide major advantages for identifying direct targets of miRNAs.
The miR-17–92 cluster is one of the best known oncogenic miRNAs, called oncomir-1 [19], which is a polycistronic miRNA encoding miR-17-5p, -17-3p, -18a, -19a, -20a, -19b and -92-1 [20]. These miRNAs are categorized into four separate families according to their characteristic seed sequence: the miR-17 family (miR-17-5p, miR-17-3p, miR-20a), the miR-18 family (miR-18a), the miR-19 family (miR-19a and miR-19b) and the miR-92 family (miR-92-1) [21]. The overexpression of the miR-17–92 cluster has been observed in multiple tumor types [22,23]. MiR-17–92 is thought to have an oncogenic function in lung cancer and lymphomas [19,24], whereas the correlation between the expression of miR-17–92 and breast cancer remains unexplored.

In this study, we examined the overexpression of miR-17–92 in MCF-7 breast cancer cells. To identify the direct targets of miR-17–92, we performed profiling of the changes in protein expression that occurred after knocking down miR-17–92 in these breast cancer cells using two-dimensional electrophoresis (2-DE). By global proteomic profiling, we identified 123 putative targets of miR-17–92. In subsequent validation studies, we demonstrated that a subset of these targets were direct targets of miR-19a.

Results

The Expression of the miR-17–92 Cluster in MCF-7 Cells, and its Inhibition by an Anti-miRNA-locked Nucleic Acid (LNA)

To identify targets of miR-17–92, a 2-DE-based quantitative proteomic strategy was adopted (Fig. 1A). First, we screened 12 human breast cancer, lymphoma and synovial sarcoma cell lines to find cells that highly express miR-17–92. The expression of miR-17–3p, -18a, -19a, -19b-1, -20a and -92-1, which comprise the miR-17–92 cluster, in the 12 cell lines was analyzed using TaqMan real-time PCR. The relative expression level of each miRNA was calculated using the mean of the expression level in the 12 cell lines. We found that all of these miRNAs, except miR-18a, were expressed at a significantly highly level in MCF-7 breast cancer cells compared to the other cell lines (Fig. 1B). We hypothesized that knockdown of miR-17–92 would increase the protein products of its cognate target genes. In order to inhibit miR-17–92 expression, we transfected the MCF-7 cells with an anti-miRNA-LNAs against miR-19a, miR-20a, miR-92-1 or a negative control. The luciferase activity was significantly decreased in all of the targets (Fig. 4B).

Verification of the Direct Targets of miR-19a and miR-20a

To confirm that miR-19a or miR-20a directly regulates the expression of the target proteins, we performed luciferase assays. First, the nucleotide sequences of the miRNA binding sites on the 3’ UTR of these target mRNAs were obtained from the GenBank database (depicted in Fig. 4A). The 3’ UTR of these targets were individually cloned downstream of the luciferase ORF in the pTK-hRG vector (Fig. S4). Next, each construct was transfected into MCF-7 cells. The luciferase activity significantly decreased in all of the targets (Fig. 4B).

In order to validate the activity by another luciferase assay, each construct was co-transfected into MCF-7 cells with anti-miRNA-LNA or control LNA. The luciferase activity was significantly increased for all candidate targets after miRNA inhibition (Fig. 4C), which was in contrast to the previous luciferase assay.

The protein expression changes of candidate targets after treatment with an anti-miRNA-LNA were examined by a Western blot analysis in MCF-7 cells in order to confirm the data obtained from the luciferase assay. The expression levels of IMPDH1 and NPEPL1 were both increased after treatment with anti-miR-19a, while the expression levels of PPP2R2A and ARHGAP1 did not change (Fig. 5A).

Quantitative real-time PCR was performed as the last validation step to examine whether there were any changes in the expression...
of IMPDH1 and NPEPL1 at the mRNA level. As expected, the expression levels of IMPDH1 and NPEPL1 were not significantly changed by anti-miR-19a-LNA at the mRNA level (Fig. 5B), while the miR-19a expression was decreased following the anti-miR-19a-LNA treatment (Fig. 2A). Taken together, our results indicate that miR-19a directly affects the post-transcriptional regulation of the IMPDH1 and NPEPL1 genes.

In order to examine the effects of the IMPDH1 and NPEPL1 genes on growth suppression of breast cancer cells, the GFP expression vectors bearing the IMPDH1 or NPEPL1 gene were transfected into MCF-7 cells by electroporation. By observing GFP fluorescence at 24 hours after electroporation, it was confirmed that the transfection efficiency between these transfectants was almost equivalent (approximately 80%). These cells were counted and split onto 96- and 6-well plates, and the cell growth was measured using the cell proliferation reagent WST-1 and Trypan Blue exclusion test at 24, 48 and 72 hours after the split. If these genes are associated with tumorigenesis under the control of miR-19a, then the cells with the exogenous expression of these genes will show a decreased cell growth. However, cells transfected with the IMPDH1 or NPEPL1 gene did not show a decreased growth in comparison to the cells transfected with a control vector (Fig. 6).

Discussion

Recently, the association between the abnormal expression of miRNAs and tumorigenesis was reported. However, the molecular mechanisms by which miRNAs can modulate tumor growth or metastases remain unknown. In particular, the activities and importance of the miR-17-92 cluster are largely unknown in breast cancer. One of the reasons may be our limited knowledge of miRNA targets. The most characteristic feature of miRNAs is the
Novel Targets of miR-19a by Proteomic Approach

The results of our proteomic analysis were compared to the predicted results for potential targets of miR-19a, -20a and -92-1 using the Pictar (http://www.pictar.org/), TargetScan (http://targetscan.org/), and MiRanda (http://www.microrna.org/microrna/home.do) software programs. Although only a few putative targets were identified within these three prediction programs, there is a possibility that false-positives or negatives were generated from the search using the computational prediction systems based on miRNA seed regions. Thus, we performed additional experimental analyses to increase the accuracy of the prediction programs and the likelihood of detecting genuine direct targets of miRNAs. Finally, the candidate proteins which were identified by the 2-DE analysis were validated by luciferase assays and a Western blot analysis to eliminate the possibility of false positive results.

Four novel candidate targets (PPP2R2A, ARHGAP1, IMPDH1, and NPEPL1) were identified by our proteomic approach. Their miRNA binding sites were shown to be important for their translation, as indicated by the luciferase activity assays. The luciferase activity of the plasmids with each target site was decreased (Fig. 4B), and increased luciferase activity was observed in the presence of the specific anti-miRNA-LNA (Fig. 4C). The IMPDH1 and NPEPL1 proteins were confirmed to be potential direct targets of miR-19a by a Western blot analysis (Fig. 5A). As miR-19a was expressed at a significantly highly level in MCF-7 cells, it is considered that the IMPDH1 and NPEPL1 proteins might function as tumor suppressors if they are associated with tumorigenesis under the control of miR-19a. However, we could not find any growth suppression effects of these genes on MCF-7 cells. In the present study, we could not validate the impact of miRNAs in this cluster on any tumor suppressor proteins, although we tried to identify some genes that have tumor suppressor activity that are direct targets of miR-17-92.

IMPDH1 (Inosine-5-prime-monophosphate dehydrogenase) catalyzes the rate-limiting step in the de novo synthesis of guanine nucleotides, i.e., the formation of xanthine monophosphate from inosine monophosphate [25]. IMPDH1 is a ubiquitously expressed enzyme, functioning as a homotetramer, and it may play an important role in cyclic nucleotide metabolism within photoreceptors. Mutations and decreased expression of the IMPDH1 gene are responsible for the disease phenotype of autosomal dominant retinitis pigmentosa [26,27]. The bulk of GTP within photoreceptors is generated by IMPDH1, and dysfunction of this enzyme might give rise to neurodegeneration [28]. Decreased expression of the IMPDH1 enzyme by aberrant overexpression of miR-19a might play a role in some cases of this disease. In general, guanine nucleotides are synthesized through the de novo pathway in T- and B-lymphocytes, while there are two synthetic pathways, the de novo pathway and a salvage pathway, in epithelial cells. Some IMPDH inhibitors, e.g. mycophenolic acid and mycophenolate mofetil, are known to have immunosuppressive activity through the de novo synthesis of guanine nucleotides in lymphocytes. IMPDH inhibitors are also known to have antiviral activity and tumor suppressor activity. Therefore, the inhibition of IMPDH1 by miR-19a may represent a potential strategy for antitumor-, antiviral- and immunosuppressive therapies.

The NPEPL1 (probable aminopeptidase-like 1) gene is located on chromosome 20q13.32, and has been deposited in the NCBI database [NCBI Gene ID, 79716]. However, no information about the function of the protein has been reported so far.

The miR-17-92 cluster was identified as an oncogenic cluster of miRNAs with multifaceted functions in cell survival, proliferation and differentiation [19,24,29]. This cluster is known to be upregulated in lung cancer [24] and B-cell lymphomas [19], and...
downregulated in senescent cells [30]. The suppressor of cytokine signaling-1 (SOCS-1) gene [31] has been reported as a target of miR-19a, and retinoblastoma-like protein 2 (RBL2) [32], hypoxia-inducible factor 1-alpha (HIF1α) [33] and Ras-related protein Rab-14 (RAB14) [34] have been identified as direct targets of miR-17-92. Recently, HMG box-containing protein 1 (HBP1) [35] and zinc-finger and BTB domain containing 4 (ZBTB4) [36] were reported to be targets of miR-17-92 and to be correlated with the prognosis in breast cancer. Recently, miR-19a was identified as a key molecule responsible for the oncogenic activity of the cluster, and was shown to reduce the tumor suppressor PTEN level, and consequently activate the AKT/mTOR (mammalian target of rapamycin) pathway [37,38].

Although miRNAs are largely known to repressively regulate protein expression, it has been reported that some miRNAs can also upregulate translation [39]. In this study, we focused on the repressive gene regulation of the miRNAs as a result of their binding to the 3’UTR of target genes, and identified only proteins that were downregulated by miR-17-92, while many upregulated proteins were also detected by the 2-DE analysis. Further experiments will be needed to determine whether any of these upregulated genes are targets of miR-17-92, and how some miRNAs are able to upregulate translation. Furthermore, a previous study demonstrated that the suppression of miR-17-92 induces complete growth arrest in anaplastic thyroid cancer cells [40], while the overexpression of one of its members, miR-20a, induces senescence in mouse embryonic fibroblasts [41], indicating that special attention needs to be paid to the possible cell type-specific responses to miR-17-92.

In conclusion, we found that miR-17-92 is overexpressed in MCF-7 breast cancer cells, and performed direct target profiling of miR-17-92 in these breast cancer cells. We identified 123 genes that were candidate targets of miR-19a, miR-20a or miR-92-1 using a quantitative proteomic approach, and performed sub-

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Table 1. Selection of candidate genes by computer-aided algorithms to search for miRNA target sites.

| Gene symbol | UniProt accession | MS/MS analysis | Target prediction |
|-------------|-------------------|----------------|------------------|
|             |                   | MS/MS score | Peptides | AA\(^1\) coverage | MW\(^2\) (kDa) | pl | PicTar | Target Scan | MiRanda |
| PPP2R2A     | P63151            | 58.4          | 4         | 4                  | 10             | 52 | 5.82   | 17          | -        | 17, 20a |
| ARHGAP1     | Q07960            | 41.35         | 3         | 3                  | 6               | 50 | 5.65   | 17, 19a, 19b| 19a      | -       |
| IMPDH1      | P20839            | 29.81         | 3         | 3                  | 6               | 55 | 6.43   | 19a, 19b   | -        | 19a     |
| NPEPL1      | Q8NDH3            | 18.26         | 1         | 1                  | 2               | 56 | 6.41   | 19a, 19b   | -        | -       |

\(^1\) AA: amino acid.  
\(^2\) MW: molecular weight.  
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such as proteomic approaches. To identify direct miRNA targets, investigations will be required to clarify the proteins and expression levels. This is accomplished through not only a single miRNA-LNA or control-LNA was co-transfected with the pTK-hRG-target site plasmid. The luciferase activity of anti-miRNA-LNA-treated group was compared to that of the control-LNA treated group. The data are shown as the means + SD. *, p<0.05; **, p<0.01 using a one-way ANOVA followed by Scheffe’s F-test (pTK-hRG-target site vs. pTK-hRG-control). (C) An anti-miRNA-LNA or control-LNA was co-transfected with the pTK-hRG-target site plasmids. The luciferase activity of anti-miRNA-LNA-treated group was compared to that of the control-LNA treated group. The data are shown as the means + SD. *, p<0.05; **, p<0.01 using a one-way ANOVA followed by Scheffe’s F-test (anti-miRNA-LNA vs. control-LNA). doi:10.1371/journal.pone.0044095.g004

Cell Culture

Synovial sarcoma cell lines SYO-1, YaFuSS, and HS-SY-II were provided by Dr. A. Kawai (National Cancer Center, Tokyo, Japan) [42], Dr. J. Toguchida (Institute for Frontier Medical Sciences, Kyoto University, Japan) [43], and Dr. H. Sonobe (National Fukuyma Hospital, Hiroshima, Japan) [44], respectively. Synovial sarcoma cell line HTB-93, breast cancer cell line MCF-7, lymphoma cell line U937, and chronic myelogenous leukemia cell line K562 were purchased from American Type Culture Collection. Hematopoietic cell lines NALM-7, p30/OHKUBO, KOPN-8, NALM-6 and REH were supplied by Hayashibara Inc. (Okayama, Japan). These cell lines were grown in Dulbecco’s modified Eagle’s medium or RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml of penicillin G and 100 μg/ml of streptomycin (Meiji Seika, Tokyo, Japan). All cells were incubated at 37°C in a humidified atmosphere containing 5% CO2.

The knockdown of miR-19a, miR-20a and miR-92 was performed using anti-miRNA LNAs (Gene Design, Inc, Osaka, Japan). The cells were plated in 96 mm culture dishes or 24 well plates and then transfected with anti-miRNA LNA oligonucleotides (30 nM) using lipofectamine 2000 (Invitrogen). A control LNA oligonucleotide against GFP was included in a parallel experiment. The cells were subjected to RNA extraction or luciferase assay 48 hours after transfection, and protein extraction 72 hours after transfection. Cell viability was determined using a WST-1 assay (Roche Applied Science, Mannheim, Germany) at 96 hours after transfection, according to the manufacturer’s instructions.

Materials and Methods

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Quantitative Real-time PCR

Total RNA was extracted using ISOGENE (Nippon Gene, Tokyo, Japan) according to standard guanidium-phenol-chloroform extraction procedures. The quantitative real-time PCR analysis of miRNAs contained within the miR-17-92 cluster was performed with a TaqMan MicroRNA Reverse Transcription Kit, TaqMan Universal PCR Master Mix and TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA, USA). Total RNA (10 ng) was reverse transcribed in a total volume of 15 μL using a TaqMan MicroRNA Reverse Transcription kit. Aliquots of each RT reaction were amplified by PCR in a 20 μL total volume containing 10 μL of the TaqMan 2X Universal PCR Master Mix. The PCR was performed on a 7900 Fast Real-Time PCR System with an initial incubation at 95°C for 15 s and 60°C for 60 s. Each PCR reaction was performed in triplicate a minimum of three times. The expression level, i.e. cycle threshold (CT) value, of each miRNA was normalized to the CT value of a small nuclear RNA, U6B, which was co-amplified as an endogenous control. The ΔCT was calculated as the difference in the CT values between the tested miRNA and the internal control in one sample. The comparisons of miRNA expression levels were conducted using the ΔΔCT method, where the ΔΔCT was the difference in the ΔCT values between two samples and 2−ΔΔCT represents the fold change in miRNA expression. After the ΔCT of the miRNA in human cancer cell lines was averaged, a comparison of the miRNA expression level in the MCF-7 cells to the average value was made using the ΔΔCT method, as shown in Fig. 1B.

Quantitative real-time PCR for the candidate target genes, NPEPL1 and IMPDH1, was performed with ReverTra Ace First Standard cDNA Synthesis Kit (TOYOBO, Osaka, Japan) and POWER SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences were as follows; NPEPL1 forward, 5’-CTC...
TTG ATC GCC TCA CAC ATC-3’ and reverse, 5’-TCA CAC AAG GCT GCG GCT TCT-3’; IMPDH 1 forward, 5’-GCA TGA TG T ACT CAG GAG AGC-3’ and reverse, 5’-ACC CGT AGT GCA AAT CTG TGG-3’. The CT values were normalized to the CT value of the GAPDH gene in the same sample. The expression levels of the target mRNA were also measured using the ΔΔCT method. The expression changes of miR-17-92 or the candidate target genes after treatment with the anti-miRNA LNA were also measured.

Protein Extraction and Two-dimensional Electrophoresis

The cells in exponential growth phase were washed with PBS and harvested by mechanical scraping. Cells were centrifuged, and the cell pellets were solubilized in lysis buffer consisting of 5 M urea, 2 M thiourea, 2% CHAPS, 1% DTT and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). After 3 freeze-thaw cycles, the cells were sonically disrupted for 30 s, and ultracentrifuged at 75,000 g for 30 min at 10°C using an Optima™ TLF Ultracentrifuge (Beckman Coulter, Brea, CA, USA). The supernatant was transferred to a new tube and treated with a ReadyPrep 2D Cleanup Kit (Bio-Rad, Hercules, CA, USA) to remove ions, DNA, RNA, etc. The protein concentration was determined using the RC-DC Protein Assay (Bio-Rad) according to the two-wash standard protocol. Pharmalyte 3–10 for isoelectric focusing was formulated to increase the resolution at the basic end of the isoelectric focusing gel.

Protein spots of interest were manually excised from silver stained gels, and then were destained and dried. In-gel trypsin digestion using a Protein In-Gel Tryptic Digestion Kit (Agilent Technologies, Santa Clara, CA, USA) was done at 30°C overnight. The peptide digests obtained were analyzed with a nano-flow liquid chromatography-ion trap-tandem mass spectrometer (nLC-IT-MS/MS, Agilent 1100 LC/MSD Trap XCT Ultra, Agilent Technologies) in a fully automated manner. The identification of proteins was performed using the Spectrum Mill MS Proteomics Workbench platform (version A.03.02, Agilent Technologies) according to the workflow of Spectrum Mill. The identification parameters were set as follows: database, NCBInr; enzyme, trypsin; monoisotopic masses were used; precursor mass tolerance (peptide tolerance), ±/−2.5 Da; product mass tolerance (MS/MS tolerance), ±/−0.8; the fixed modification was selected as carbamidomethylation (cysteine); the variable modification was selected as oxidation (methionine), two missed cleavages with trypsin were allowed, and the instrument setting was specified as “ESI ion trap”. The probability scores calculated by the software were used as a criterion for correct identification.
were co-transfected with the firefly luciferase reporter plasmid, reverse orientation were used as controls for those with the antimRNA-LNA. The luciferase activity was measured 48 hours after transfection using a dual luciferase reporter assay system (Promega) on a Labosystems Luminoskan RT instrument (Thermo Scientific). The relative luciferase activity was calculated by normalizing the firefly luminescence to the renilla luminescence.

**Western Blotting Analysis**

The cells were lysed in the same buffer used for the 2-DE analysis. A total of 20 μg of whole protein lysates were combined with gel loading buffer, heated to 95°C for 10 min, and then separated on 12% SDS-polyacrylamide gels and electrotransferred to polyvinylidene difluoride membranes (Invitrogen). Membranes were blocked overnight at 4°C in 3% BSA/PBS, and then incubated for 4 hours at room temperature with the following antibodies: 1:1000 rabbit polyclonal anti-PPP2R2A (ab18136, Abcam, Cambridge, UK), 1:250 rabbit polyclonal anti-ARHGAP1 (ab72127, Abcam, Cambridge, UK), 1:200 mouse monoclonal anti-IMPDH1 (H00003614-M01, Abnova, Taipei City, Taiwan) and 1:200 mouse monoclonal anti-NPEPL1 (sc-100556, Santa Cruz Biotechnology). β-actin was used as a loading control and was detected by a 1:1000 mouse monoclonal anti-β-actin antibody (A5316, Sigma, Saint Louis, USA). After washing with PBS/0.05% Tween-20, the membranes were incubated with alkaline phosphatase-conjugated secondary antibodies in PBS. Signals were measured by an enhanced chemiluminescence detection system using a VECTASTAIN ABC-AmP Chemiluminescent Detection Kit (VECTOR LABORATORIES, Inc) and visualized using FLA-3000 (Fujifilm).

**Exogenous Expression of Target Genes in MCF-7 Cells**

The IMPDH1 and NPEPL1 cDNAs were amplified using PCR with human normal cDNA and primers (IMPDH1 forward, 5′-GGA TCC TCA CAC AAG CCT GCG TCT CTT 9GAG TCA GTA CGG GGG CTG CAG TTC and NPEPL1 forward, 5′-GGA TCC TCA CAC AAG CCT GCG TCT CTT GGA) and cloned into pBluescript. The nucleotide sequences were confirmed with DNA sequencing and the cDNA fragments were digested with restriction enzymes (XbaI for the IMPDH1 and EcoRI and BamHI for the NPEPL1) and cloned into pIRE2-EGFP to generate pIMPDH1-ires-EGFP and pNPEPL1-ires-EGFP plasmids.

The pIMPDH1-IRES-EGFP, pNPEPL1-IRES-EGFP and control plasmids were transfected to MCF-7 cells using the Neon Transfection system (Invitrogen, 1100 V, 30 ms, 2 pulses). After 24 hours, it was confirmed by observing the GFP fluorescence that the transfection efficiency between these cells was almost equal (approximately 80%), and the cells were counted and split into 96-well plates (1×10^4 cells/well) and 6-well plates (5×10^5 cells/well). At 24, 48 and 72 hours after the split, cell viability was measured using 6-well plates and the Trypan Blue excision test and using 96-well plates and a WST-1 assay (Roche Applied Science), according to the manufacturer’s instructions. For the WST-1 assay, the cell growth ratio (%) was calculated by comparing the viability of cells treated with the IMPDH1 or NPEPL1 genes compared to that of cells treated with a control vector.
Statistical Analysis
All experiments were repeated independently a minimum of three times, and the results are expressed as the mean values ± SD. The relative expression of miRNAs, mRNAs or proteins was analyzed by paired t-tests. The other results were assessed by one-way ANOVA followed by Scheffe’s F-test. A value of p<0.05 was considered to indicate statistical significance.

Supporting Information
Figure S1 A representative gel image of MCF-7 cells treated with the anti-miRNA-LNA after fluorescent staining. Protein spots indicated with red circles were positive candidates for regulation by miR-17–92. The molecular weight marker is indicated at left. (TIF)

Figure S2 A representative enlarged gel image and spots of candidate proteins. The protein spots indicated by arrowheads are target spots on the gels. (TIF)

Figure S3 The MS/MS spectra used to identify candidate proteins. The panels show the MS/MS spectra of representative peptides; (A) Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform (PPP2R2A), (B) Rho GTPase-activating protein 1 (ARHGAP1), (C) Inosine-5’-monophosphate dehydrogenase 1 (IMPDH1), (D) Probable aminopeptidase NPEPL1 (NPEPL1). (TIF)

Figure S4 Construction of the luciferase vectors. The miR-17–92 target sites of the candidate genes were cloned downstream of the luciferase ORF at the XhoI restriction site of the p1TK-hRG vector. The miRNA target sites are shown as nucleotide sequences (left) and as boxes (right). Sense (upper) and antisense (lower) strands of complementary sequences indicate the miRNA target site of mRNA 3’ UTR and the corresponding miRNA sequences, respectively. Seven nucleotides (red) on miRNAs show the seed sequences for binding with mRNA. (TIF)

Table S1 Candidate targets of the miR-17–92 cluster identified by LC-MS/MS. (TIF)

Author Contributions
Conceived and designed the experiments: MO KS. Performed the experiments: MO HK SI YJ HH ST. Analyzed the data: HK MO. Contributed reagents/materials/analysis tools: HK MO. Wrote the paper: HK KS MO.

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