

**Brief Report**

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# Quantitative proteomics and protein network analysis of A549 lung cancer cells affected by miR-206

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MiR-206 acts as a potential tumor suppressor during carcinogenesis and a regulatory factor in osteoblasts differentiation, but its modulatory mechanism remains unclear. In this study, we used a quantitative proteomics method, difference gel electrophoresis (DIGE), to profile the protein variation in A549 lung cancer cells with and without miR-206 transfection. We identified a total of 17 differently expressed proteins including 5 up-regulated and 12 down-regulated proteins affected by miR-206 in A549 cells. We further constructed a protein network linked 17 differently expressed proteins with 106 computationally predicted miR-206 targets, and identified 8 "hub" genes (*CALR*, *CTSD*, *ENO1*, *HSPA5*, *CDC42*, *HSPD1*, *POLA1*, and *SMARCA4*) within the network, which may represent important miR-206 functional gene targets. In conclusion, in this study, we identified several candidate functional target genes for miR-206, which is helpful to further explore its mechanisms during carcinogenesis and osteogenesis, and we also proposed a novel proteomic strategy to identify functionally important gene targets for microRNA.

**Keywords:** microRNA, gene target, proteomics, bioinformatics, miR-206

**1. Introduction**

MiR-206 is considered a "myomiR" as it is specifically expressed in skeletal muscle (1). MiR-206 acts as a positive regulator of skeletal muscle differentiation, and a negative regulator of osteoblasts differentiation (2). Accumulating evidence also suggests a tumor suppressor function for miR-206, as it is frequently downregulated in many human malignancies (3). Several oncogenes (such as estrogen receptor 1, cyclinD2) and osteogenesis regulators (such as connexin 43) have been identified and confirmed as targets of miR-206 (3,4). However, its exact regulatory mechanisms during carcinogenesis and osteogenesis remain to be explored further.

Identification of novel functional miRNA targets of miR-206 is central to further understand its modulation on cellular functions. Currently, most of the studies on miR-206 target identification are based on computational prediction algorithms. According to our knowledge, until now, there is still no experimental strategy for miR-206 target identification reported. Therefore, in this study, we adopted a difference gel electrophoresis (DIGE) based technology to compare the protein profiling of lung cancer A549 cells with and without miR-206. Then we bioinformatically constructed a protein network using experimentally identified miR-206-related proteins with computationally predicted targets, in order to screen functional gene targets and provide novel mechanism clues for miR-206.

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**2. Materials and Methods****2.1. Cells line and miR-206 transfection**

A human lung carcinoma epithelial-like cell line A549 was obtained from the Cell Bank of Shanghai

Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) at 37°C in 5% CO<sub>2</sub>. A549 cells were first cultured to reach 50-75% confluence and transfected with miR-206 mimics or negative controls (Shanghai GenePharma, Shanghai, China) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were harvested 48 h later. MiR-206-transfected cells after 48 h were collected for further analyses.

### 2.2. miR-206 expression in A549 cells detected by real-time PCR assay

Total RNA was isolated from A549 cells using Trizol<sup>®</sup> (Invitrogen). The miR-206 expression was quantified by real-time PCR using TaqMan miRNA assays according to the manufacturer's directions. U6 small nuclear RNA (snU6) was used to normalize the expression data of miR-206.

### 2.3. DIGE

A549 cells with miR-206 mimic and with vector transfection for 48 h were collected and solubilised with a lysis buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, pH 8.5) for protein extraction. The protein concentration was determined using a 2D Quant kit (GE Healthcare). Fifty µg of protein extracts from each group were labeled with 400 pmol Cy3 or Cy5. Fifty µg of protein extracts by combining equal amounts (25 µg) from each group was labeled with 400 pmol Cy2 as an internal standard. Then, Cy2-, Cy3-, and Cy5-labeled samples were combined and diluted with a rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer, 40 mM DTT) for isoelectric focusing (IEF). After equilibration, SDS-PAGE isolation was performed. The Cydye-labeled images were scanned on a Typhoon Trio apparatus (GE Healthcare, USA), and were then post-stained by Coomassie Blue G350 (GE Healthcare, USA). Images were analyzed by ImageMaster 6.0 DIGE-enable software package (GE Healthcare, USA), spots with more than 2.0-fold intensity changes between two groups were defined and selected for protein identification.

### 2.4. In-gel tryptic digestion and mass spectrometry

Excised spots of interest were destained with 25 mM NH<sub>4</sub>CO<sub>3</sub>/50% ACN at 37°C for 30 min, and dehydrated in 100% ACN for 10 min. Proteins were digested by sequencing grade-modified trypsin (Promega, Madison, WI, USA) overnight at 37°C. Extracted peptides were mixed with CHCA for mass spectrometry (MS). MS spectra were acquired using an ABI 4700 proteomics analyzer MALDITOF/TOF mass spectrometer (Applied

Biosystems, USA) operating in a result-dependent acquisition mode. MS spectra were searched against a human subset of the Swiss-Prot database for protein identifications using GPS explorer software (Applied Biosystems).

### 2.5. Network construction and "hub" gene identification

MiR-206 targets were first computationally predicted by PicTar, miRanda, and TargetScan. Predicted target genes overlapped among at least two prediction algorithms were combined with differently expressed proteins identified in proteomic study. Then the combined gene sets was used to search for their protein-protein interaction links by accessing the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins, <http://string-db.org>) and KEGG (Kyoto Encyclopedia of Genes and Genomes, <http://www.genome.jp/kegg>) databases. The links among miR-206 target or related genes were constructed into a network and visualized by Cytoscape software.

### 2.6. Statistics

The expression levels of miR-206 in real-time PCR experiment and spots intensity changes in DIGE between groups were compared with *t*-test. *p* value less than 0.05 was considered statistically significant. The connectivity of genes within the network was compared with *Z*-test.

## 3. Results and Discussion

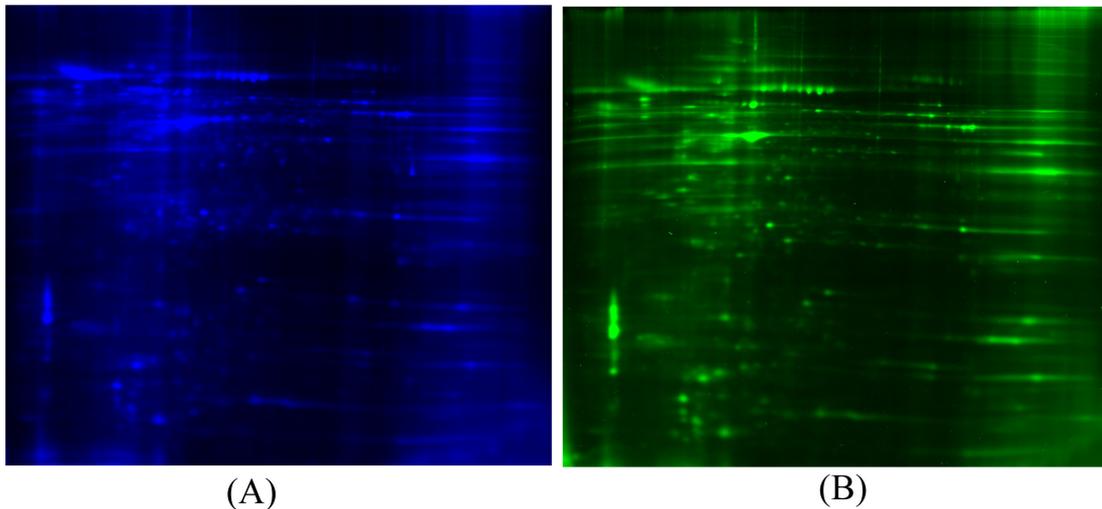
For A549 cells have a relatively low level of miR-206 expression, the protein changes induced by miR-206 down-regulation would be small, therefore, in this study we only investigated the effect of ectopic overexpression of miR-206 on the protein profiling of A549 cells. By real-time PCR, we validated that the cellular level of miR-206 was significantly increased by miR-206 mimic transfection, when compared with vector transfection and the blank control.

Through a DIGE-based quantitative proteomics tool, we compared the profiling of A549 cells induced by miR-206 overexpression (Table 1). This proteomic strategy identified 17 differently expressed proteins including 5 up-regulated and 12 down-regulated proteins (Figure 1). As expected, most of the differently expressed proteins belong to indirect target genes for miR-206, except Annexin IV (ANXA4) belongs to predicted miR-206 target genes.

Next, we computationally predicted miR-206 targets by three different algorithms (PicTar, miRanda, and TargetScan), a total of 106 genes overlapped among at least two prediction sets were defined. We constructed a network linking 106 predicted genes and 17 experimentally identified proteins (Figure 2A).

**Table 1. Differently expressed proteins in miR-206-transfected A549 cells compared with vector-transfected cells in DIGE analysis**

Protein names	Gene names	Accession Number	Mascot scores	Fold (miR-206/vector)
GLUD1 protein	GLUD1	Q14400	78	3.0
Prelamin-A/C	LMNA	P02545	102	2.5
Cathepsin D	CTSD	P07339	131	3.5
78 kDa glucose-regulated protein	HSPA5	P11021	122	4.0
NADH dehydrogenase [ubiquinone] iron-sulfur protein 3	NDUFS3	O75489	278	2.2
Transitional endoplasmic reticulum ATPase	VCP	P55072	118	-3.1
Peroxisome oxidoreductin-6	PRDX6	P30041	240	-4.2
Eukaryotic translation initiation factor 4H	EIF4H	Q15056	86	-2.8
Galectin-1	LGALS1	P09382	150	-3.7
Sorcin	SRI	P30626	86	-2.0
Calreticulin	CALR	P27797	180	-2.5
14-3-3 protein epsilon	YWHAE	P62258	110	-3.1
Prohibitin	PHB	P35232	128	-2.2
Protein disulfide-isomerase	P4HB	P07237	280	-3.5
Stathmin	STMN1	P16949	80	-2.5
Alpha-enolase	ENO1	P06733	90	-2.3
Annexin A4	ANXA4	P09525	171	-5.0

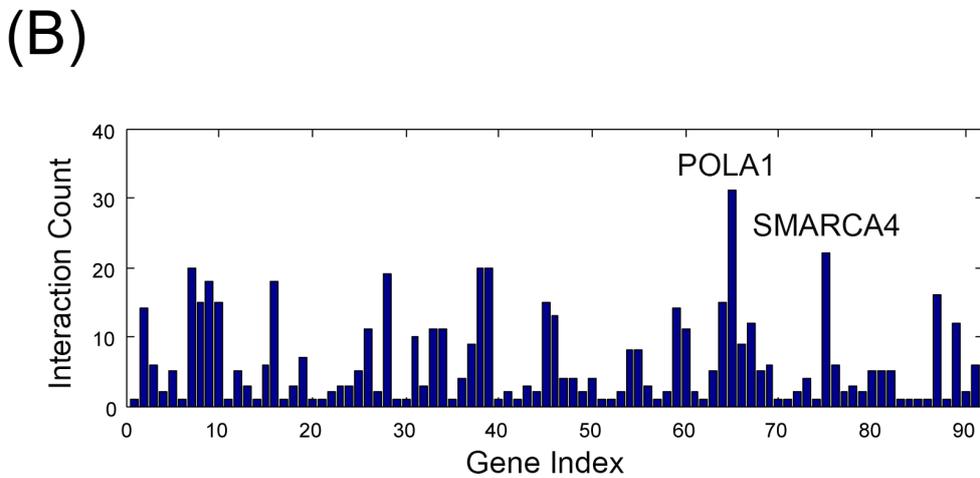
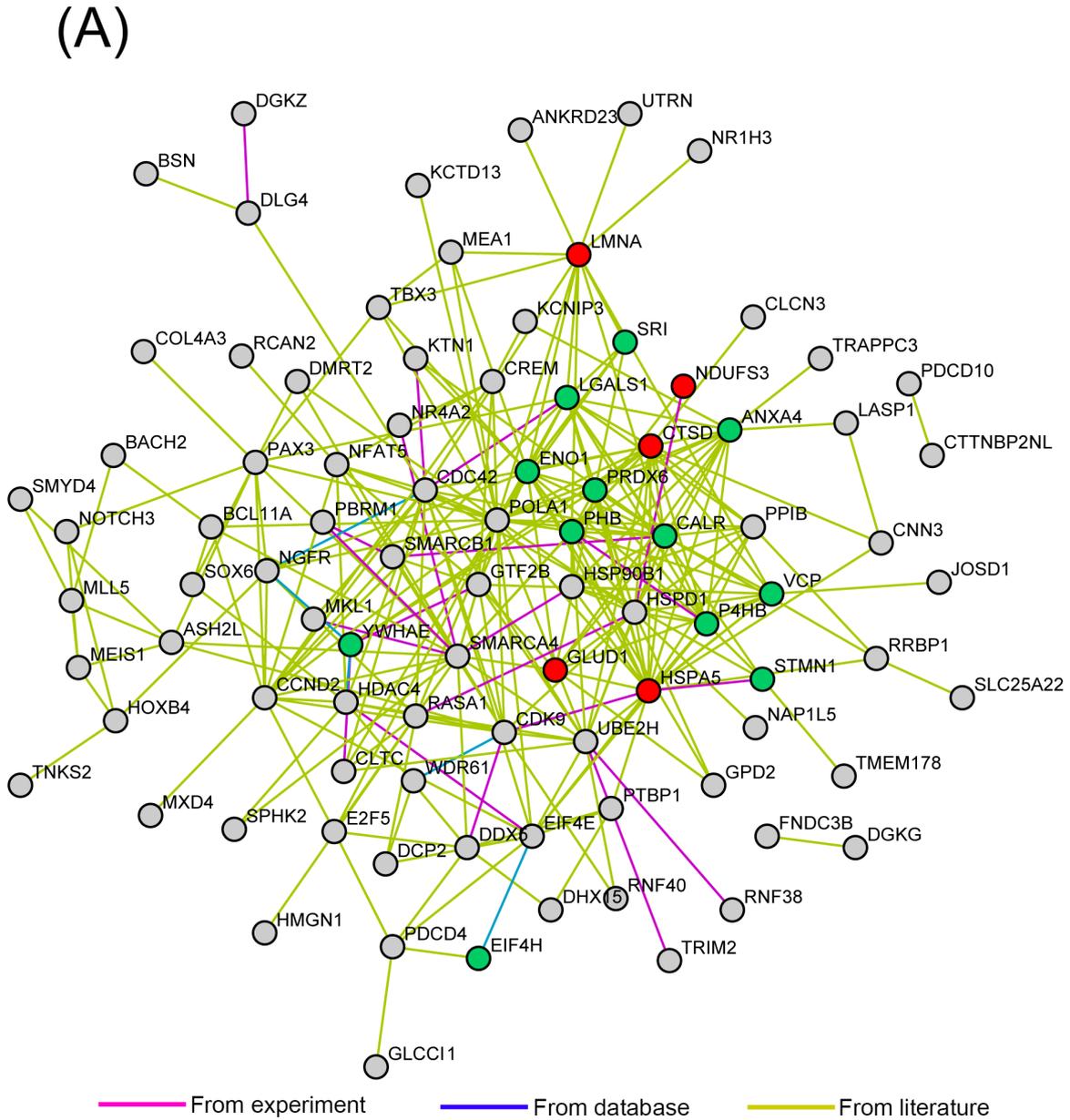
**Figure 1. Representative images of DIGE analysis on A549 cells with and without miR-206. (A) Cy5 labeling A549 cells with miR-206 transfection; (B) Cy3 labeling A549 cells with vector transfection.**

From this network, we identified 8 highly connected "hub" genes with statistical significance ( $p < 0.05$ ) (Figure 2B). DNA polymerase  $\alpha 1$  catalytic subunit (POLA1) and SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 (SMARCA4) had the highest significant correlation of gene connectivity ( $p = 5.0 \times 10^{-5}$  and  $p = 0.0066$ , respectively) within this network.

How to identify genuinely functional target genes remains a fundamental challenge in miRNA mechanism studies (5). Most of the previous studies used computational programs to predict miRNA targets. There are always hundreds of target predicted by these bioinformatics tools, how to select targets for further confirmations still lacks of generally accepted criteria. In addition, computational miRNA target predictions

still have a higher percentage of false-positives and false-negatives.

Recently, accumulating experimental strategies based on gene expression microarray or proteomic tools have been developed to provide more information and clues to identify genuinely functional targets for miRNA (6). For most targets may be repressed by a miRNA at the protein level without being affected at the mRNA level, proteomic tools represent powerful approaches in revealing the full spectrum of miRNA targets (7,8). However, most of the differently proteins found in proteomic studies belong to high or middle abundant proteins. For most of the functional miRNA target effectors are always low abundant proteins such as transcriptors, which is undetectable by even the most sensitive mass spectrometers. Therefore, most varied proteins identified by proteomic tools are always



**Figure 2. Gene network and hub gene analysis.** (A) Protein network of 17 experimentally identified differently expressed proteins in proteomic studies combined with 106 computationally predicted miR-206 targets; (B) "Hub" genes in miR-206-related network.

indirect targets. Among the differently expressed proteins in this study, only ANXA4 is a direct predict target.

Different from previous studies, this study proposed a novel strategy to combine both of predicted and experimental data to identify functional miRNA targets. Using the networks composed, we rank the direct and indirect candidate targets by the connectivity in a miR-206 regulatory network. Absence of "hub" genes would be expected to affect many more other miRNA targeted proteins in gene network (9); therefore, these "hub" genes might represent important regulators for miRNA in mechanism studies.

Among the "hub" genes, *CALR* (*calreticulin*), *CTSD* (*cathepsin D*), *ENO1* (*enolase 1*), *HSPA5* (*heat shock 70kDa protein 5*) were from experimental data, while *CDC42* (*cell division cycle 42*), *HSPD1* (*heat shock 60kDa protein 1*), *POLA1*, *SMARCA4* from the predicted genes. Most of these targets have been proved to be associated with cancer progressions. *CDC42* is a critical  $\beta$ -catenin signaling driver in osteoblasts (10). *HSPD1* has been identified to play an important role in gene networks underlying bone development (11). Therefore, we proposed that these "hub" target genes might help to account for the role of miR-206 as a potential tumor suppressor and osteogenesis inhibitor.

In conclusion, in this study, using miR-206 as an example, we constructed a network using the predicted genes and experimentally identified proteins by a DIGE-based proteomic method. Using this network, we developed a bioinformatic strategy to rank the importance of targets according to their potential role in the miRNA-mediated gene profiling. And the "hub" genes we found for miR-206 in this study deserve further investigation in future.

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