Proteomic Analysis of Inhibitor of Apoptosis Protein-like Protein-2 on MCF-7 Cell Growth

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Research

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Abstract

Backgrounds

Although inhibitor of apoptosis protein-like protein-2 (ILP-2) is regarded as a novel growth enhancer for breast cancer, its mechanism on breast cancer cell growth remains elusive. This study analysed the expression profiles of proteins with association to ILP-2 protein during Michigan Cancer Foundation-7 (MCF-7) breast cell growth for its mechanism on breast cancer cell growth.

Methods

Isobaric tags for relative and absolute quantification analysis (iTRAQ) was applied on siRNA-5 and negative control groups of MCF-7 breast cancer cells to analyse protein expression profile correlated to ILP-2 during MCF-7 cell growth. Verification of iTRAQ data was by done by Western blot.

Results

4065 proteins were identified in the MCF-7 cells, with 241 of differentially expressed proteins (DEPs) (fold change ≥ 1.20 or ≤ 0.83 and $P < 0.05$). A total of 156 upregulated and 85 downregulated proteins were found in the siRNA-5 group versus negative control group. These DEPs were associated with the extracellular matrix receptor interaction. Proteins from the top 10 biological processes were associated with signal transduction, regulation of cell proliferation, and immune system processes. The expression of AGA (N(4)-(beta-N-acetylglucosaminy)-L- asparaginase), MT1E (metallothionein-1E) and TD02 (tryptophan 2,3-dioxygenase) increased when the protein expression of ILP-2 was knocked-down.

Conclusions

These results suggest that ILP-2 promotes MCF-7 cell growth by regulating cell proliferation, signal transduction, and immune system processes.

Background

Breast cancer is a common malignancy in women [1]. Although the incidence of breast cancer is inferior to lung cancer in developed regions, breast cancer is the most cause of death in underdeveloped regions [2]. About 252,710 breast cancer cases were newly diagnosed in the United States in 2017 [1]. Although therapies for breast cancer have improved, more cases have emerged in recent years when compared to that in 2008 based on key risk factors, such as alcohol consumption, obesity, breast condition and age [3]. Early detection of breast cancer and identification of new treatment methods are therefore needed.

Targeted therapy is gaining interests, with recent discovery of breast cancer drug targets such as human epidermal growth factor receptor 2, epidermal growth factor receptor and mammalian target of rapamycin signalling pathway [4–6]. High ILP-2 expression is found in breast cancer cells and tissues, and is an important growth enhancer for breast cancer [7]. The experiments in our lab found that ILP-2 is

a serological biomarker for breast cancer and a novel growth enhancer for breast cancer [8]. But the mechanistic of how ILP-2 promotes breast cancer cell growth is still unknown.

Isobaric tag for relative and absolute quantitation (iTRAQ) is an accurate and reliable proteomics technique for quantitative analysis. It has been applied in proteome investigation by combining liquid chromatography and tandem mass spectrometry (LC-MS/MS). These tags can be covalently linked to amino acids (including N-terminal amino acids and lysine side chain amino acids) by stable isotopically labelled molecules [9–12].

In this present study, we analysed the expression profiles of proteins with association to ILP-2 protein during MCF-7 cell growth for its mechanism on breast cancer cell growth, and ascertained as to whether ILP-2 could be a new drug target for breast cancer-targeted treatment.

Methods

Cell culture and collection

MCF-7 was obtained from the American Type Culture Collection (ATCC, USA). Cell lines were cultured in RPMI-1640 Medium (Thermo Fisher HyClone, USA) with 10% fetal bovine serum (FBS) (Gibco, MA, USA), and 1% Penicillin Streptomycin Mixture (Beijing solarbio Science & Technology Co., Ltd.) in 5% CO₂ at 37°C. MCF-7 cell lines were divided into siRNA-5 group (knocked-down group, KD group) and negative control (NC) group. Following manufacturer's instructions, small interfering RNA-5 (5'-3'sense: CUAUACGAAUGGGAAUUUGATT; 5'-3'antisense: UCAAAUCCCAUUCGUAUAGTT), NC (5'-3'sense: UUCUCCGAACGUGUCACGUTT; 5'-3'antisense: ACGUGACACGUU CGGAGAATT) (GenePharma, Shanghai, China) and Lipofectamine 2000 (Invitrogen, USA) were individually mixed at 1:1 (volume ratio) and stored for 20 minutes at room temperature. Two groups of mixed small interfering RNA and Lipofectamine 2000 were added to MCF-7 cells in the logarithmic growth phase, and cultivated in 5% CO₂ for 24 hours at 37°C. The small interfering RNA had a final concentration of 100 nM [13, 14]. MCF-7 cells were washed with 0.01M PBS (phosphate buffer saline) to remove the medium. Afterwards, a lysis buffer (7 M urea and 4% SDS (sodium dodecyl sulfate)) containing 1 mM PMSF (Phenyl- methane-sulfonyl fluoride) was added to each group of cells (1×10⁷ cells). The cells were lysed on ice for 30 minutes. The supernatant of the mixed solution was collected for analysis.

ITRAQ assays

SDS-PAGE and Protein Digestion

Total proteins of the above mixed solution from the two cell groups were extracted. Protein concentrations of isolated proteins in the above two group cells were determined by a BCA (bicinchoninic acid) protein assay kit (Appygen Technologies Inc., Beijing, China). Protein concentrations of samples were adjusted to a constant level using the dilution. Total protein (20 µg) per lane was separated on a

10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gels were cut into gel slices, and the gel bands were digested by an in-gel trypsin method.

Reductive Alkylation and Protease Digestion

Dithiothreitol (20 mM final concentration) was added to extracted protein samples (100 µg/µL), and the samples incubated at 37°C for 60 minutes. Iodoacetamide (40 mM final concentration) was added, and the samples incubated in the dark at room temperature for 40 minutes. Pre-cooled acetone (volume ratio of 5:1) was added to the samples, and the mixture placed in - 20°C for 2 hours. The sediment was collected by 10,000g centrifugation at 4°C for 20 minutes and re-dissolved in 100 mM tetraethylammoniumbromide buffer. Trypsin (1 mg trypsin/50 mg protein) was added to the protein samples, and settled at 37°C for overnight.

Isotope labeling

Protease-digested proteins were labeled with 113, 117, 114, and 118. The NC group was labeled with 113 and 117, whereas the siRNA-5 group was labeled with 114 and 118. These were the two biological replications. 100 µg peptides were labeled with an iTRAQ reagent tube [15, 16].

First dimensional separation of peptides

Separation of peptides was performed by ultra-high pressure liquid chromatography (UPLC) (Waters Corp., Milford, MA, USA) with a 2.1×150 mm X Bridge BEH300 column (Waters Corp., Milford, MA, USA). The moving phase was a mixture of water (pH adjusted to 10 with ammonia and formic acid), and acetonitrile was isocratically transmitted using a pump at a flowrate of 0.4 mL/min. The wavelength of the ultraviolet absorbance detector was 214/280 nm. The projects used for gradient elution are listed in Table 1. A total of 10 fractions were collected according to different retention times. Rotation vacuum concentrators (Christ RVC 2-25, Martin Christ, Germany) were used for concentration, and dissolved in buffer solution (pH adjusted to 10 with ammonia and formic acid) for further analysis.

LC-MS/MS

Labeled peptides were analysed using a NanoAcquity UPLC system (Waters Corporation, Milford, MA) combined with a quadrupole- Orbitrap mass spectrometer (Q-Exactive) (Thermo Fisher Scientific, Bremen, Germany), incorporating a C18 column (75 µm×25 cm, Thermo, USA). The mobile phase was a mixture of water, with 2% acetonitrile and 0.1% formic acid isocratically delivered using a pump at a flowrate of 300 nL/min. The schemes used for gradient elution are shown in Table 2. Full-scan mass spectrometry (m/z 350-1300) was acquired with a first mass resolution of 70K, and second resolution of 17.5K. The framsscan was applied with data-dependent acquisition (DDA), Top 20. Fragmentation was used for high-energy collision dissociation (HCD). The microscan was recorded using dynamic exclusion of 18 s.

ITRAQ data analysis

The MS/MS data of iTRAQ were analysed using Protein Discoverer™ Software 2.1 (Thermo Scientific) and searched in Uniprot-proteomes-homo-sapiens-70611.fasta. The database had 70611 entries, and date of download was July 15th, 2016. Result-filtered parameters were used to control peptide level false discovery rates (FDR) $\leq 1\%$. Protein quantification was performed by only unique peptides, and normalisation on protein medians was applied to rectify experimental deviation, which was accomplished by retrieval software. The ratios of the samples were weighted, and normalised by contrasting the negative control group (sample tagged as 113 and 117) to the denominator for protein quantitation. Regarding the quantitative changes, $a \geq 1.2$ or ≤ 0.83 -fold change takeout and P -value (t-test) < 0.05 were set for DEPs.

Gene Ontology (GO, <http://www.geneontology.org/>) and Kyoto Encyclopaedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/>) pathway were applied to annotate, and classify all authenticated proteins. DAVID 6.8 Functional Annotation Tool (<http://david.abcc.ncifcrf.gov/>) was arranged to process the DEPs for the enrichment analysis. Fisher Exact statistic methodology was used to filter results. The ClueGO of Cytoscape software (<http://www.cytoscape.org/>) was performed to assess GO biological network. String (Search Tool for the Retrieval of Interacting Genes) v10.0 (<http://www.string-db.org/>) was applied to analyse protein-protein interaction, and a high coefficient value of 0.7 was used as a cut-off. The expression patterns of DEPs (fold change ≥ 1.2 or ≤ 0.83 and $P < 0.05$) were identified by Cluster analysis using h-cluster (<https://pypi.python.org/pypi/hcluster/0.2.0>) [9, 17].

Western blot

Cells of siRNA-5 and NC groups (5×10^6 cells per cell group) were separately collected, and western blot analysis conducted [18-20]. Total proteins were extracted from cells with RIPA buffer (150 mM NaCl, 20 mM Tris, 0.1% SDS, pH 7.5, 1% deoxycholate and 1% Triton X-100) containing protease inhibitors. Protein concentration was determined by a BCA Protein Assay kit (Appygen Technologies Inc., Beijing, China). Briefly, total protein (30 μ g) per lane was separated on a 10% SDS-polyacrylamide gel, and transferred onto polyvinylidene fluoride membranes (Merck KGaA, Darmstadt, Germany). The membranes were blocked using Tris-buffered saline and Tween 20 (TBST) and 5% skim milk powder for 2 hours, and incubated using primary antibodies against ILP-2 (rabbit IgG, 1:1,000; cat. no. Ab9664; Abcam, Cambridge, UK), MT1E (mouse IgG, 1:500; cat. no. MAD794Hu21; Cloud-Clone Corp, Wuhan, China), TDO2 (rabbit IgG, 1:500; cat. no. ab84926; Abcam, Cambridge, UK), AGA (rabbit IgG, 1:500; cat. no. ab 231021; Abcam, Cambridge, UK), Tubulin (rabbit IgG, 1:1000; cat. no. 11224-IAP; Proteintech, Shanghai, China) and GAPDH (mouse IgG, 1:2500; cat. no. 60004-1-Ig; Proteintech, Shanghai, China) overnight at 4°C. On the second day, the membranes were washed using TBST, and incubated using a goat anti-mouse immunoglobulin G-horseradish peroxidase (anti-mouse IgG, 1:5000; cat. no. SA001; Auragene Biotech, Hunan, China), and peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L) (anti-rabbit IgG, 1:5000; cat. no. ZB-2301; ZSGB-BIO, Beijing, China) for 1 hour at room temperature. An enhanced chemiluminescence detection system (SuperECL-Plus, Appygen Technologies, Beijing, China) was used to visualise immunoreactive proteins. The protein was visualised by chemical

chemiluminescence imaging system (Beijing Sage Creation Science Inc., Beijing, China). The images were analysed with ImageJ. Tubulin, GAPDH was used as internal control.

qPCR analysis

To extract the total RNA, wash the cell sample twice with 1mL PBS in a cell culture dish, add 1mL RNA isolater Trizol (Vazyme, Nanjing, China) and pipette evenly, transfer it to a 1.5mL RNase free EP tube to fully lyse the cells. Let stand at room temperature for 10 min; add 200uL chloroform, shake vigorously for 15s, and stand at room temperature for 10 min; centrifuge at 4°C 12000rpm for 15 min, collect the upper colorless liquid phase in a new 1.5mL RNase free EP tube; add 500 uL isopropanol, shake and mix well. After 10 minutes at room temperature, centrifuge at 4°C 12000rpm for 10 minutes, discard the supernatant; add 1mL 75% alcohol, and gently shake to wash the precipitate, centrifuge at 4°C 12000rpm for 5 min, discard the supernatant, and repeat twice; dry at room temperature for 10-15min; add 50uL of DEPC (diethyl pyrocarbonate) water to dissolve the RNA. Measure the OD260/OD280 of RNA on the nucleic acid protein detector to detect the purity of total RNA, and run the RNA sample in 1% agarose gel electrophoresis to detect RNA integrity. Perform RNA reverse transcription according to the instructions of Bester qPCR RT Kit (DBI Bioscience, Germany). Denature the RNA by keeping it at 65°C for 5 minutes and put it on ice immediately for later use. Add the components to a 0.2ml RNase free EP tube in the following order to prepare a reverse transcription reaction solution, 2 µL 5×RT Buffer, 0.5 µL RT Enzyme Mix, 0.5 µL Primer Mix, 1 µg RNA, make the reaction solution up to 10µL by adding RNA-free Water. For reverse transcription reaction, put the above-mentioned mixture on a PCR machine immediately, perform reverse transcription reaction at 37°C for 15 minutes, and at 98°C for 5 minutes, and store the reversed transcribed RNA (the cDNA) at 4°C for immediate use or at -20°C for long-term use. For QPCR experiments, follow the instructions of Bestar SybrGreen qPCR Mastermix kit (DBI Bioscience, Germany). Prepare 20µL reaction solution by the following component accordingly, 10µL Bestar SybrGreen qPCR Mastermix, PCR Forward Primer (10µM) 0.5µL, PCR Reverse Primer (10µM) 0.5µL, DNA template 1µL, ddH₂O 8µL. Run Real Time PCR amplification using the following thermal conditions, pre-denaturation at 95°C for 2min; PCR cycle (40 times) at 95°C for 10s, 60°C for 30s, and 72°C for 30s; melting curve hold at 95°C for 1min and 55°C for 1min.

Statistical analysis

Histograms were constructed using GraphPad Prism 5. Statistical analysis was performed by conducting t-test with SPSS statistics 17.0 (SPSS, Chicago, USA). A level of $P < 0.05$ was considered statistically significant.

Results

Proteomic expression profiling is associated with ILP-2 in MCF-7 cells

ITRAQ-based proteomics analysis was used to evaluate the protein expression profiles related to ILP-2 during MCF-7 cell growth. LC-MS/MS analysis produced 55180 matched spectra, 20857 peptides and

19551 unique peptides (Table S1-2, Figures 1A-C). A total of 4065 proteins were identified by at least one unique peptide with a confidence coefficient above 95% (Table S1). All proteins were grouped by GO analysis according to biological process, cellular component, and molecular function (Table S2). Single-organism, cellular and metabolic processes were the main categories for these proteins in the biological processes. Cell, organelle and cell part accounted for a large portion in the cellular component, and the catalytic activity and binding were the two most dominant categories in the molecular function (Figure 1A). KEGG analysis showed that these proteins were mainly involved in the pathway of global and overview maps, signal transduction and translation, which were related to metabolism, genetic information processing, and environmental information processing (Figure 1B). There were a greater number of proteins on cancer, endocytosis, spliceosome, and RNA transport in the top 20 pathways that were primarily included (Figure 1C).

Identification of DEPs when ILP-2 was knocked-down in MCF-7 cells

DEPs were identified in cells transfected with siRNA-5 and NC groups to analyse proteins with dynamic changes in relation to ILP-2 during MCF-7 cell growth. A total of 241 DEPs (fold change >1.20 or <0.83 and $P < 0.05$) were found from siRNA-5 versus NC groups (Figures 2A-C). Among them, 156 proteins were upregulated and 85 were downregulated when siRNA-5 transfected group were compared with NC group (Figures 2B, 2C). The DEPs of siRNA-5 versus NC groups are selectively listed in Table 3 according to the FC (fold change, KD/NC) and the P -values (fold change ≥ 1.66 , $P \leq 0.05$).

Functional analysis of proteins in relation to ILP-2 when ILP-2 was knocked-down in MCF-7 cells

In order to indicate any difference between biological pathways, DAVID tool was used to perform GO functional enrichment analysis. 241 proteins enriched into GO term were involved in terpenoid backbone biosynthesis, protein digestion and absorption, ECM-receptor interaction, synthesis and degradation of ketone bodies, and lysosome (Figure 2C). KEGG pathway analysis was used to assess significant enrichment of lysosome, ECM-receptor interaction, butanoate metabolism, synthesis and degradation of ketone bodies, terpenoid backbone biosynthesis, protein digestion and absorption (Figure 2D, 2E). 29 proteins from the top 30 biological processes were further analysed, which were GO annotated proteins in these DEPs, and a functional interaction network was obtained. Results showed that the altered proteins associated with ILP-2 were mainly involved in signal transduction, regulation of cell proliferation, and regulation of immune system process during breast cancer cell MCF-7 growth (Figure 2F).

Heatmap of DEPs when ILP-2 was knocked-down

H-cluster was arranged to perform cluster analysis, and illustrate the expression patterns of these DEPs (fold change ≥ 1.20 or ≤ 0.83 and $P < 0.05$). Heatmap of DEPs revealed similar expression patterns to KD and NC groups (Figure 3A). Based on the expression patterns, DEPs were grouped into 10 clusters (Figure 3B). These clusters had an overall upregulation pattern from NC group to KD group, which were sub-clusters 2, 3, 6, 7, 8, 9 and 10 (including 1, 17, 2, 2, 4, 1 and 1 protein). However, the other clusters were

downregulated from NC group to KD group, which were sub-clusters 1, 4 and 5 (including 10, 7 and 3 proteins).

Western blot analysis of DEPs

Western blot analysis was performed to validate iTRAQ results of AGA, MT1E, and TDO2 proteins. There was a strong protein expression of AGA, MT1E and TDO2 when ILP-2 was knocked-down (Figure 4). These results were consistent with iTRAQ data, and suggest that the protein expressions of AGA, MT1E, and TDO2 are associated with ILP-2 accelerating breast cancer cell MCF-7 growth.

qPCR analysis of DEPs

qPCR analysis was performed to validate iTRAQ results of AGA, MT1E, and TDO2 mRNAs. They were all significantly highly expressed after ILP-2 was knocked-down (Figure 5).

Interactive network of DEPs

The STRING software was used to identify DEPs from the top 10 biological processes, and analyse the change in protein interaction networks related to ILP-2 in MCF-7 cell growth. 42 DEPs were analysed, which were associated with nodes in the protein interaction network. These proteins were proteins with annotation information in the string database, 16 of which constituted an interaction network containing AGA and GAA, HMGCS1 and COL3A1 (Figure 6).

Discussion

Breast cancer is a multifactorial, multistep and heterogeneous disease [21]. Some biological processes actively promote breast carcinogenesis and breast cancer growth, including protein signal transduction and other complex biological processes in breast cancer cell [22–25]. In this study, we used iTRAQ analysis to identify 4065 proteins when ILP-2 was knocked-down in breast cancer cell line, MCF-7. We found that these identified proteins had an important role on endocytosis and cellular signal transduction. Results from our study indicate that breast cancer cell MCF-7 growth is closely associated with ILP-2 protein-involved processes such as genetic information, endocytosis, and RNA transport.

Differential expressions of proteins from different control group cells have a positive role on breast cancer growth [26–28]. 241 DEPs of 4065 proteins were found from ILP-2 knocked-down group versus NC group. Results showed that these DEPs related to ILP-2 are of great importance to MCF-7 cell growth and enriched in pathways related with assess significant enrichment of lysosome, ECM-receptor interaction, butanoate metabolism, and so on.

In our previous study, we demonstrated the rate of cell migration and cell viability to be decreased, and cell apoptosis rate to be increased when ILP-2 expression in MCF-7 cells is inhibited. These results suggest that ILP-2 promotes MCF-7 cell growth via some biological processes [7]. Breast cancer cells growth is associated with the regulation of cell proliferation, signal transduction and immune system

processes[29, 30].The over-expression of MT1E (metallothionein-1E) is a poor prognosis of breast cancer [31, 32]. TDO2 (tryptophan 2, 3- dioxygenase) can promote breast cancer cell migration [33, 34]. AGA (N(4)-(beta- N-acetylglucosaminy)-L-asparaginase) gene mutation may result in a lysosomal storage disease called aspartylglucosaminuria [35, 36]. Our results showed the knock-down of ILP-2 in breast cancer cells to be related to the cell signal transduction, the regulation of cell proliferation and immune system processes. The expressions of AGA, MT1E and TDO2 were upregulated when ILP-2 protein expression was knocked-down. These data show the close relationship between ILP-2 and MCF-7 cell growth.

Proteins usually interact with each other to perform various functions [9]. Protein-protein interaction activity can actively promote breast cancer cell growth [37–39]. Protein-protein interaction analysis is frequently performed using the STRING software [17]. The STRING analysis data indicated that ILP-2 may regulate cell proliferation through complex protein regulatory network relationships.

42 DEPs made up the protein interaction network, among which, 16 proteins, including AGA and GAA, HMGCS1 and COL3A1formed an interactive network.

Thus, ILP-2 has an important role in MCF-7 cell growth via protein interaction.

Conclusion

In summary, our study characterised the protein expression profile of MCF-7 cell growth by iTRAQ. A total of 241 DEPs in 4065 proteins were identified when ILP-2 expression was knocked-down in MCF-7 cells. These DEPs were mainly involved in ECM-receptor interaction. Proteins from the top 10 biological processes were associated with regulation of cell proliferation, signal transduction and immune system processes, and ILP-2 promoted breast cancer cell MCF-7 growth via these processes. Overall, these results provide a comprehensive insight into the biochemical pathways, and regulatory networks related to ILP-2 protein during MCF-7 cell growth. Further studies on how ILP-2 promotes breast cancer cell growth are warranted. Also, the application of co-immune-precipitation and pull-down technology in the exploration of mechanism underlying the acceleration of breast cancer cell growth due to ILP-2 is suggested.

Abbreviations

AGA N(4)-(beta-N-acetylglucosaminy)-L-asparaginase

DEPs differentially expressed proteins

ECM extracellular matrix

FDR false discovery rates

GAPDH Glyceraldehyde-3-phosphate dehydrogenase

GO Gene Ontology

ILP-2 inhibitor of apoptosis protein-like protein-2

iTRAQ isobaric tags for relative and absolute quantification

KD knocked down

MCF-7 Michigan Cancer Foundation-7

KEGG Kyoto Encyclopaedia of Genes and Genomes

LC-MS/MS liquid chromatography coupled with tandem mass spectrometry

MT1E metallothionein-1E

NC negative control

PMSF Phenyl-methane- sulfonyl fluoride

SDS sodium dodecyl sulfate

SDS- PAGE sodium dodecylsulphate polyacrylamide gel electrophoresis

String Search Tool for the Retrieval of Interacting Genes

TBST Tris-buffered saline and Tween 20

TD02 tryptophan 2,3-dioxygenase

Declarations

Availability of data and materials

The datasets used in this study are available from the corresponding author.

Ethical Approval and Consent to participate

Not applicable

Consent for publication

All authors are consent for publication on the manuscript.

Availability of supporting data

<http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD012848>

Competing interests

The authors declare the absence of competing interests.

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Author contributions

SX cultured cell lines, performed proteomic sample preparation, interpreted data and drafted the manuscript. LZ contributed to drafting the revised manuscript. ZZ performed bioinformatics analysis. SW performed Western blotting. SH and RC participated in experimental design, and critically revised the manuscript. MX designed the study, and drafted and critically revised the manuscript. All authors have read and approved the final manuscript.

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Tables

Table 1 LC gradient of first dimensional separation of peptides.

Time (min)	B (%)
0	2
5	5
40	25
45	80
50	80
51	2
60	stop

Table 2 Gradient elution of LC - MS / MS.

Time (min)	B (%)
0	2
70	40
70.1	90
75	90
75.1	2
90	2

Table 3 The part differentially expressed proteins in KD vs. NC group (fold change ≥ 1.66 , $P \leq 0.05$).

Accession Number	Gene Symbol	NC	KD
Q9BYX7	POTEKP	1.0035	2.53
P04732	MT1E	1.069	2.33
P20933	AGA	1.127	2.421
P48775	TDO2	1.01	1.8525
A0A0A0MTQ8	CCDC175	1.078	1.7985

Figures

Figure 1

Functional classification of annotated proteins correlated to ILP-2 in MCF-7 cells. (A)Proteins classified into three main categories by GO analysis: biological process, cellular component and molecular function. The left y-axis indicates the percentage of a specific category of proteins in that category. The right y-axis indicates the number of proteins in thecategory. (B)Proteins classified into five main categories by KEGG analysis: metabolism, genetic information processing, environmental information

processing, cellular processes and organismal systems. The x-axis indicates the percentage proteins within that specific category. A, B, C, D, and E in the y-axis indicate Metabolism, Genetic Information Processing, Environmental Information Processing, Cellular Processes, and Organismal Systems respectively. (C) The number of proteins in the top 20 pathways by KEGG analysis. The right y-axis indicates the number of proteins in the category.

Figure 2

DEPs in the cells of siRNA-5 and NC groups. (A) Volcano plots of DEPs in two comparisons: KD group vs. NC group. (B) Number of DEPs in two comparisons: siRNA-5 group vs. NC group. (C) Histogram of upregulated and downregulated protein GO annotation in siRNA-5 group vs. NC group. The x-axis below indicates the number of proteins annotated to a certain GO term. The x-axis above indicates the proportion of proteins annotated to a certain GO term in the total number of proteins with GO term. (D) The top 10 GO enrichment terms for DEPs. (E) Remarkable enrichments for DEPs by KEGG analysis. (F) The 35 proteins in the top 10 biological processes were analyzed using ClueGO of Cytoscape software, which were GO annotated proteins in these DEPs.

Figure 3

The heatmap and clustering analysis of expression patterns of DEPs. (A)The heatmap of the DEPs. (B)Sub-clusters 2, 3, 6, 7, 8, 9 and 10 (including 1, 17, 2, 2, 4, 1 and 1 proteins): up-regulation from NC group to KD group; sub-clusters 1, 4 and 5 (including 10, 7 and 3 proteins): down-regulation from NC group to siRNA-5 group.

Figure 4

Western blot of the DEPs. Western blot analysis showed the downregulated relative protein expression of ILP-2, and upregulation of AGA, MT1E and TDO2 when ILP-2 was knocked-down. The protein Tubulin and GAPDH was used as reference protein.

Figure 5

The protein-protein interaction network of DEPs in two comparisons: NC group to KD group. In this network, nodes represent proteins, and lines with different color represent the predicted different associations.

Figure 6

qPCR analysis of the DEPs. qPCR analysis showed the downregulated relative mRNA expression of ILP-2, and upregulation of AGA, MT1E and TDO2 when ILP-2 was knocked-down.

Supplementary Files

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- [S1identifiedproteins.xlsx](#)
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