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miR-19a-3p Functions as an Oncogene by Regulating FBXO32 Expression in Multiple Myeloma

Ying Li[1], Song Gao[1], Wenjing Xue[1], Yanna Ma[1], Yuesheng Meng[1], Dawei Zhang[2]

[1]Department of Hematology, Jinshan Hospital of Fudan University, Jinshan, Shanghai, PR China
[2]Department of General Surgery, Jinshan Hospital of Fudan University, Jinshan, Shanghai, PR China

Background: Multiple myeloma remains a virtually incurable hematologic malignancy, which is featured with the aberrant growth of malignant plasma cells.

Aims: To elucidate the functions of miR-19a-3p in multiple myeloma.

Study Design: Cell study.

Methods: Cell counting kit-8 assay was performed to detect cell viability, and flow cytometry was conducted to detect cell apoptosis. Bioinformatics analysis predicted miR-19a-3p-associated biological function, pathway, core regulatory network, and target genes. Luciferase reporter assay verified the target sequence of miR-19a-3p regulating FBXO32.

Results: miR-19a-3p is upregulated in multiple myeloma cells ($p<0.01$) and patients with multiple myeloma ($p<0.001$). Overexpressed miR-19a-3p significantly increased cell viability ($p<0.05$) and inhibited cell apoptosis ($p<0.01$). FBXO32 is a target gene of miR-19a-3p ($p<0.01$). Moreover, FBXO32 is downregulated in MM, and it significantly decreased cell viability ($p<0.05$) and promoted cell apoptosis ($p<0.01$). FBXO32 significantly rescued the influence of miR-19a-3p-inhibiting cell apoptosis ($p<0.05$).

Conclusion: miR-19a-3p promoted cell proliferation and inhibited cell apoptosis by degrading the target FBXO32 mRNA in multiple myeloma.

Multiple myeloma (MM), accounting for approximately 13% of hematological malignancies, is featured with the aberrant growth of malignant plasma cells (1). Previous research achievements have greatly increased life expectancy and quality of life. However, MM remains a virtually incurable hematologic malignancy (2). MM pathogenesis is involved in a series of biological processes, such as gene mutations, chromosomal abnormalities, epigenetic modifications, cell proliferation, tumor-microenvironment, and evolution of drug-resistant tumor cells (3, 4). Identifying essential genes in disease progression is of the most significant importance for identifying new therapeutic approaches and prolonging the prognosis in patients (5, 6).

MicroRNAs (miRNAs), 18-24 nucleotides in length, are a kind of noncoding RNA. miRNAs regulate posttranscription gene expression by degrading or repressing target mRNAs (7, 8). miRNAs mediate various biological functions such as differentiation, proliferation, apoptosis, and migration (9, 10). A series of cancer-associated miRNAs have recently been profiled in MM using microarray analysis (10). Previous findings demonstrated that miR-19a-3p acts as a novel poor prognostic indicator in MM (11). However, the mechanism of miR-19a-3p regulating cellular function in myeloma cells has not been elucidated.

FBXO32 (atrogin-1) belongs to the F-box protein family (12). Recent findings have reported that FBXO32 is down-regulated in cancers and may function as a tumor suppressor (13-17). Besides, emerging studies suggest that FBXO32 is a novel apoptosis regulator (18). However, the upstream regulatory factor of FBXO32 in tumorigenesis remains unclear.

This study showed that miR-19a-3p is significantly overexpressed and significantly suppressed cell apoptosis in MM cells. FBXO32 was negatively regulated by miR-19a-3p. Besides, our results demonstrated that *FBXO32* was a miR-19a-3p target gene in MM cells. FBXO32 rescued the function of miR-19a-3p inhibiting cell apoptosis in MM cells. These results suggested that miR-19a-3p promoted MM cells development by regulating FBXO32 expression.

MATERIALS AND METHODS

Cell lines and clinical specimens

Cells (MM.1S, U266, RPMI8266, IM9, H929, and W63) were purchased from the American Type Culture Collection (MD, USA). Bone marrow (BM) specimens were provided by 8 healthy donors (5 males, 3 females; age range=25-55 years) and 8 patients with MM (4 males, 4 females; age range=30-56 years) from (for blind review). Clinical samples were purified with CD138 MicroBeads

Address for Correspondence: Dawei Zhang, Department of General Surgery, Jinshan Hospital of Fudan University, Jinshan, Shanghai, PR China
e-mail: zhang_daw@126.com

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ORCID iDs of the authors: Y.L. 0000-0002-4605-6603; S.G. 0000-0003-4875-7902; W.X. 0000-0002-1599-6176; Y.M. 0000-0001-5117-8747; Y.M. 0000-0001-8839-8487; D.Z. 0000-0002-9722-5147.

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according to the Miltenyi Biotec protocol. Ethical approval was obtained from Jinshan Hospital of Fudan University. Cells were grown in the Roswell Park Memorial Institute 1640 medium, containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL).

Quantitative real-time polymerase chain reaction

We extracted total RNAs (cells and BM specimens) using TRizol reagent (Invitrogen, MD, USA) following the manufacturer's instructions. The SYBR Green primers are listed in Table 1. Reverse transcription of complementary DNA and quantitative real-time polymerase chain reaction (RT-qPCR) was performed using Takara reagent (Takara, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were conducted as the standard for normalization. The $2^{-\Delta\Delta CT}$ method was used to determine mRNA expression levels.

TABLE 1. The primer sequences

Gene	Sequence (5'→3')
miR-19a-3p-forward primer	GGGGGGGTGTGCAAATCT
miR-19a-3p-reverse primer	GTGCGTGTCGTGGAGTCG
FBXO32-forward primer	GCCTTTGTGCCTACAACTGAA
FBXO32-reverse primer	CTGCCCTTTGTCTGACAGAAT
GAPDH-forward primer	GGAGCGAGATCCCTCCAAAAT
GAPDH-reverse primer	GGCTGTTGTCATACTTCTCATGG
U6-forward primer	GCTTCGGCAGCACATATACTAAAAT
U6-reverse primer	CGCTTCACGAATTTGCGTGTCAT

Transfection

Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA) was responsible for synthesizing the miR-19a-3p mimics (cat. no. MIMAT0000073) and inhibitors (cat. no. MIMAT0021837). FBXO32 overexpression vector was synthesized by Sangon Biotech (Shanghai, China). Vectors were transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Cell counting Kit-8 assays

Cell Counting Kit-8 (CCK-8) (DOJINDO, Japan) reagent was used to test cell viability. U266 and H929 cells (5×10^3 per well) were seeded on plates and incubated for 24 hours, 48 hours, or 72 hours. The medium was replaced with 10 μL CCK-8 reagent and 100 μL fresh medium. After 1 hour at 37°C, the wavelength of optical density values was 450 nm.

Flow cytometry

The FITC/PI reagent (KeyGen Biotech Co, Nanjing, China) was used to detect cell apoptosis. FlowJo_V10 (Becton-Dickinson,

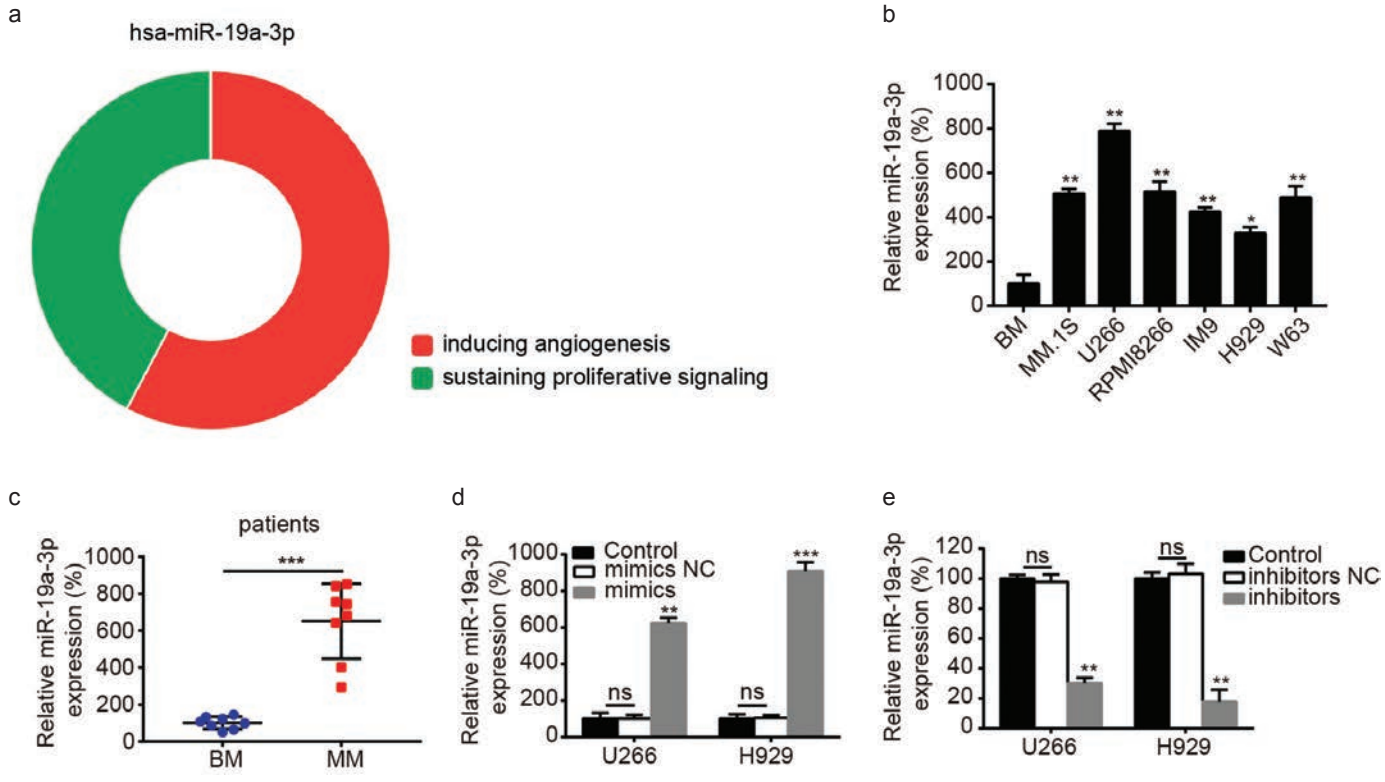

FIG. 1. a-e. Expression of miR-19a-3p in multiple myeloma (MM) cell lines and tissues. Association between miR-19a-3p and hallmarks of cancer from Cancer Hallmarks Analytics Tool (CHAT), which can be accessed at http://chat.lionproject.net/ (a). Relative miR-19a-3p expression levels between normal bone marrow (BM) cells and MM cell lines (b). Relative miR-19a-3p expression levels between normal BM cells and patients with MM (c). miR-19a-3p mimics increased miR-19a-3p expression in U266 and H929 cells (d). miR-19a-3p inhibitors suppressed miR-19a-3p expression in U266 and H929 cells (e). *p<0.05; **p<0.01; ***p<0.001; each bar represented mean±standard deviation from 3 independent experiments

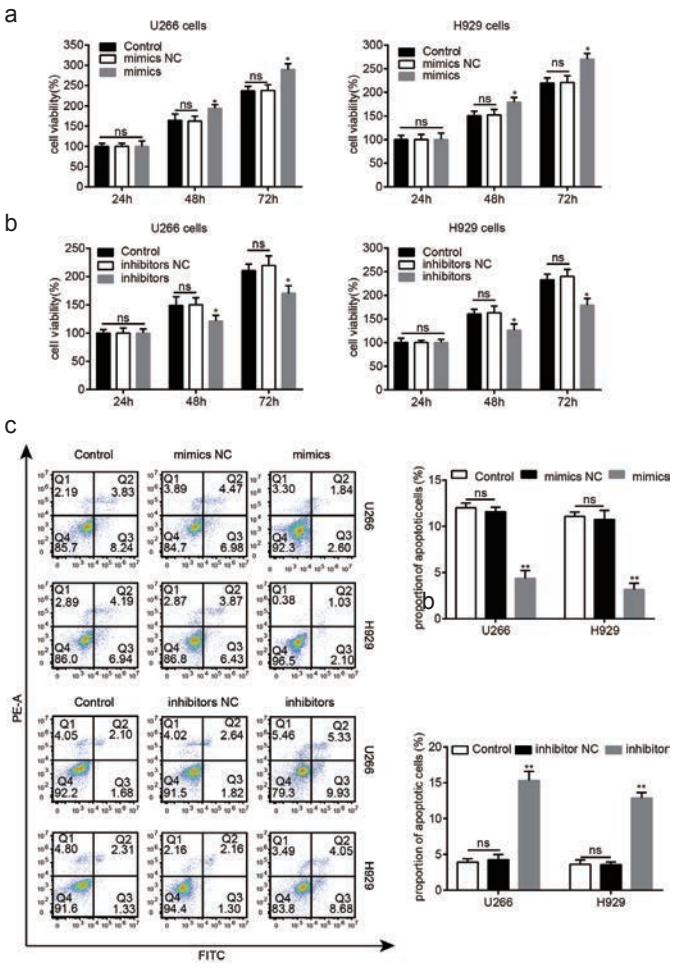

FIG. 2. a-c. miR-19a-3p promoted cell viability and inhibited cell apoptosis in multiple myeloma (MM) cell lines. miR-19a-3p overexpression promoted cell viability in U266 cells and H929 cells (a). miR-19a-3p inhibitors decreased cell viability in U266 cells and H929 cells (b). miR-19a-3p overexpression inhibited cell apoptosis, and miR-19a-3p inhibitors promoted cell apoptosis in U266 cells and H929 cells (c). *p<0.05; **p<0.01; each bar represented mean±standard deviation from 3 independent experiments

New Jersey, USA) software was used to analyze the results. The upper right quadrant represented late apoptotic cells. The right lower quadrant represented viable apoptotic cell.

Bioinformatics analyses

The biological function of miR-19a-3p and FBXO32 in cancer was explored in the Cancer Hallmarks Analytics Tool (CHAT) (19). Targets of miR-19a-3p were analyzed by TargetScan (20), miRDB (21), and DIANA-microT (22) database. Online Venn tools (http://bioinformatics.psb.ugent.be/webtools/Venn/) visualized the overlapping target genes of these databases. Enrichment analysis (gene ontology and pathway) was performed using the Enrichr analysis tool (23). The protein interaction network of miR-19a-3p target genes was analyzed using the STRING analysis tool (https://string-db.org) (24) and visualized by Cytoscape software. The Cytoscape MCODE algorithm further extracted the core subnetwork.

Dual-luciferase reporter assays

The estimated binding site of miR-19a-3p or mutant 3'-UTR sequences were cloned in the XhoI and NotI (Promega Corporation, Madison, WI, USA) restriction sites to the psiCHECK2 vector. The cells were cotransfected with the vectors of wild-type or mutated FBXO32 3'-UTR and the miR-19a-3p NC/mimics/inhibitors. Luciferase assay reagent (Promega Corporation, Madison, WI, USA) was conducted to detect the luciferase activity after 48 hours.

Western blotting

Cleaved caspase 3 and B-cell lymphoma-2 BCL-2 (Abcam, San Francisco, CA, USA) expression levels were detected by western blotting using following the manufacturer's instructions. Cells were lysed with lysis buffer (Cell Signaling Technology, Inc, Danvers, MA, USA). The supernatant protein was collected and normalized, and equivalent protein (30 μg) was electrophoresed and then transferred onto the polyvinylidene difluoride (PVDF) membranes. A blocking buffer was used to block proteins on the PVDF membranes at 4°C overnight. Then, the membranes incubated with the specific antibody and were washed in washing buffer (0.1% Tween 20 in phosphate-buffered saline). Proteins were then visualized by electrochemiluminescence reagents (KeyGen Biotech Co, Nanjing, China). GAPDH (ImmunoWay Biotechnology Company, Plano, TX, USA) expression levels were used as the standard for normalization.

Statistical analysis

We analyzed the results using the Statistical Package for the Social Sciences version 21.0 software (IBM SPSS Corp, Armonk, NY, USA). Normality distribution of variables was tested using the Shapiro Wilk test. Mean±standard deviation was used as descriptive statistics for normal distributed data. Median (min-max) was used as descriptive statistics for non-normal distributed data. The *t* test and 1-way analysis of variance tested normally distributed results. Multiple comparisons were made using least significant difference and Student-Newman-Keuls tests. Either χ^2 tests or Fisher exact tests were used to analyze non-normal distributed data. All experiments presented here were conducted at least 3 independent times. $p<0.05$ was considered statistically significant.

RESULTS

Basic expression levels of miR-19a-3p in multiple myeloma cells and patients

Using CHAT, we revealed that miR-19a-3p is associated with inducing angiogenesis and sustaining proliferative signaling (Figure 1a). RT-qPCR assays revealed that miR-19a-3p was increased in MM cells and patients ($p<0.01$) (Figure 1. b, c).

miR-19a-3p regulated cell viability and apoptosis in multiple myeloma cells

To further investigate the biological function of miR-19a-3p in MM, we overexpressed or knocked down miR-19a-3p in U266 and H929 cells ($p<0.01$) (Figure 1. d, e). CCK-8 assays revealed that overexpressed miR-19a-3p significantly promotes cell viability ($p<0.05$). Besides, miR-19a-3p knockdown suppressed cell viability ($p<0.05$) (Figure 2. a, b). Flow cytometry was conducted to detect cell apoptosis in U266 and H929 cells, which showed that miR-19a-3p overexpression significantly inhibits cell apopto-

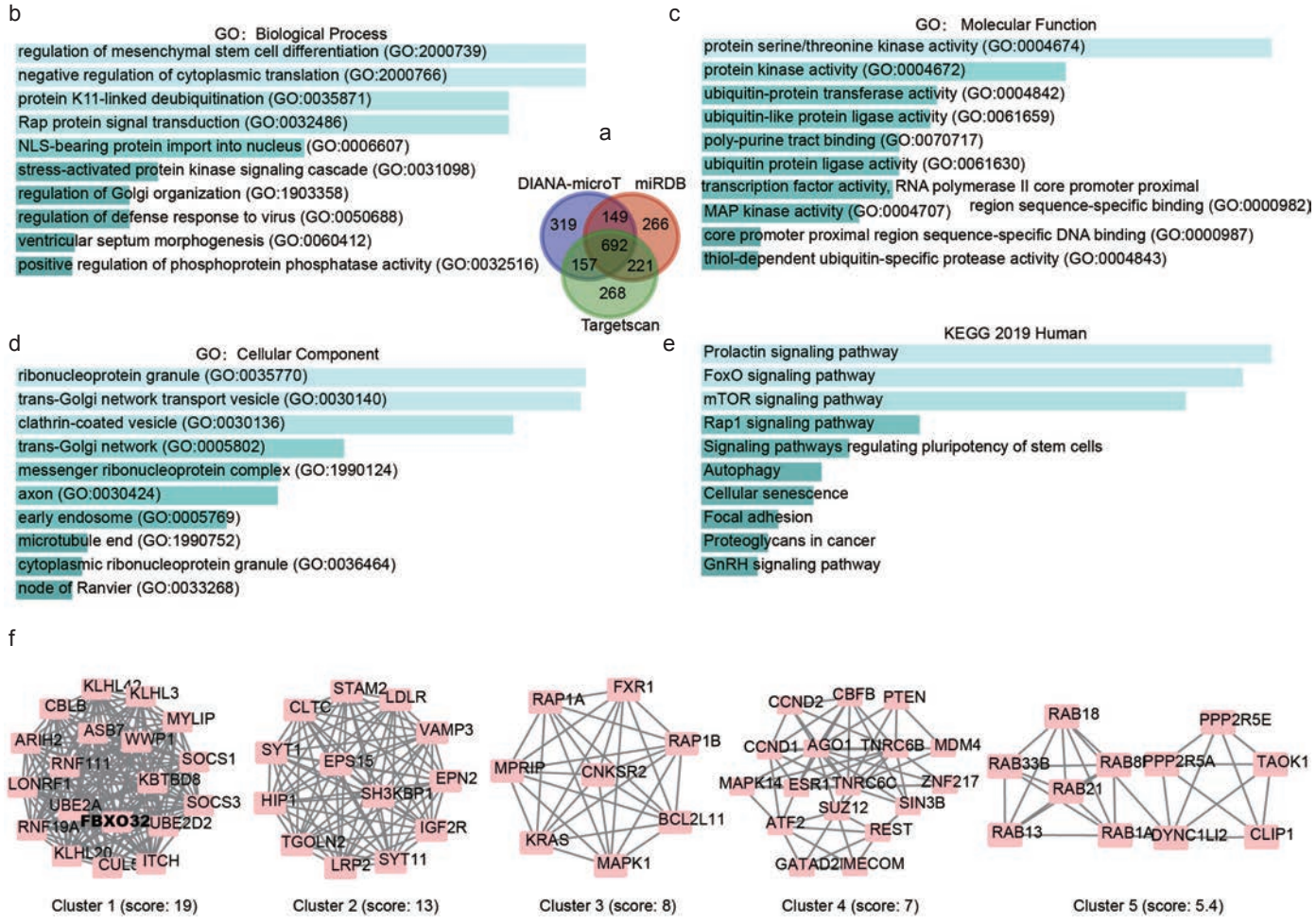

FIG. 3. a-f. Predicting the target genes of miR-19a-3p by TargetScan, miRDB, and DIANA-microT and enrichment analysis. Venn of 692 overlapping 1317 target genes (DIANA), 1328 target genes (miRDB) and 1338 target genes (TargetScan) (a). GO enrichment analysis, and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis revealed miR-19a-3p target genes correlated with multiple biological functions and pathways at Enrichr analysis tool (http://amp.pharm.mssm.edu/Enrichr/) (b-e). Cytoscape software was used to analyze core subnetworks in miR-19a-3p target genes (f)

sis and miR-19a-3p knockdown increased cell apoptosis ($p<0.01$) (Figure 2c).

Predicting miR-19a-3p target genes

miR-19a-3p target genes were estimated through 3 bioinformatics databases, including TargetScan, miRDB, and DIANA-microT. A total of 692 genes were overlapped in 3 bioinformatics databases (Figure 3a). The Enrichr analysis tool was used to analyze the correlated biological function and pathway of these overlapping target genes, the results of which revealed that these target genes are correlated with multiple biological function and pathway, such as negative regulation of cytoplasmic translation, protein kinase activity, and FoxO signaling pathway ($p<0.05$) (Figure 3. b-e). Besides, we analyzed the protein interaction network of these overlapping genes through the STRING analysis tool (Figure S1) and extract core subnetworks using the Cytoscape MCODE analysis tool. Finally, we obtained 5 significant core subnetworks and found that FBXO32 is a core gene in the core subnetworks ($p<0.05$) (Figure 3f).

FBXO32 was a target gene of miR-19a-3p in multiple myeloma cells

In CHAT, FBXO32 is also associated with sustaining proliferative signaling (Figure 4a). Then, we found that FBXO32 3'-UTR sequences matched the "seed sequence" of miR-19a-3p, which indicated FBXO32 is related to the regulation of MM (Figure 4b). Luciferase report system revealed that miR-19a-3p mimics significantly inhibits the activity of FBXO32-wt 3'UTR among H929 and U266 cells ($p<0.05$). miR-19a-3p inhibitors significantly promoted the activity of FBXO32-wt 3'UTR among H929 and U266 cells ($p<0.01$) (Figure 4. c, d). miR-19a-3p does not regulate the activity of FBXO32-mt 3'UTR among H929 and U266 cells (Figure 4. c, d). Besides, RT-qPCR assays revealed that FBXO32 is significantly down-regulated in patients with MM ($p<0.001$) (Figure 4e) and cells ($p<0.05$) (Figure 4f). miR-19a-3p mimics significantly inhibited FBXO32 mRNA expression ($p<0.05$) (Figure 4g). miR-19a-3p inhibitors significantly promoted FBXO32 mRNA expression in U266 and H929 cells ($p<0.001$) (Figure 4h).

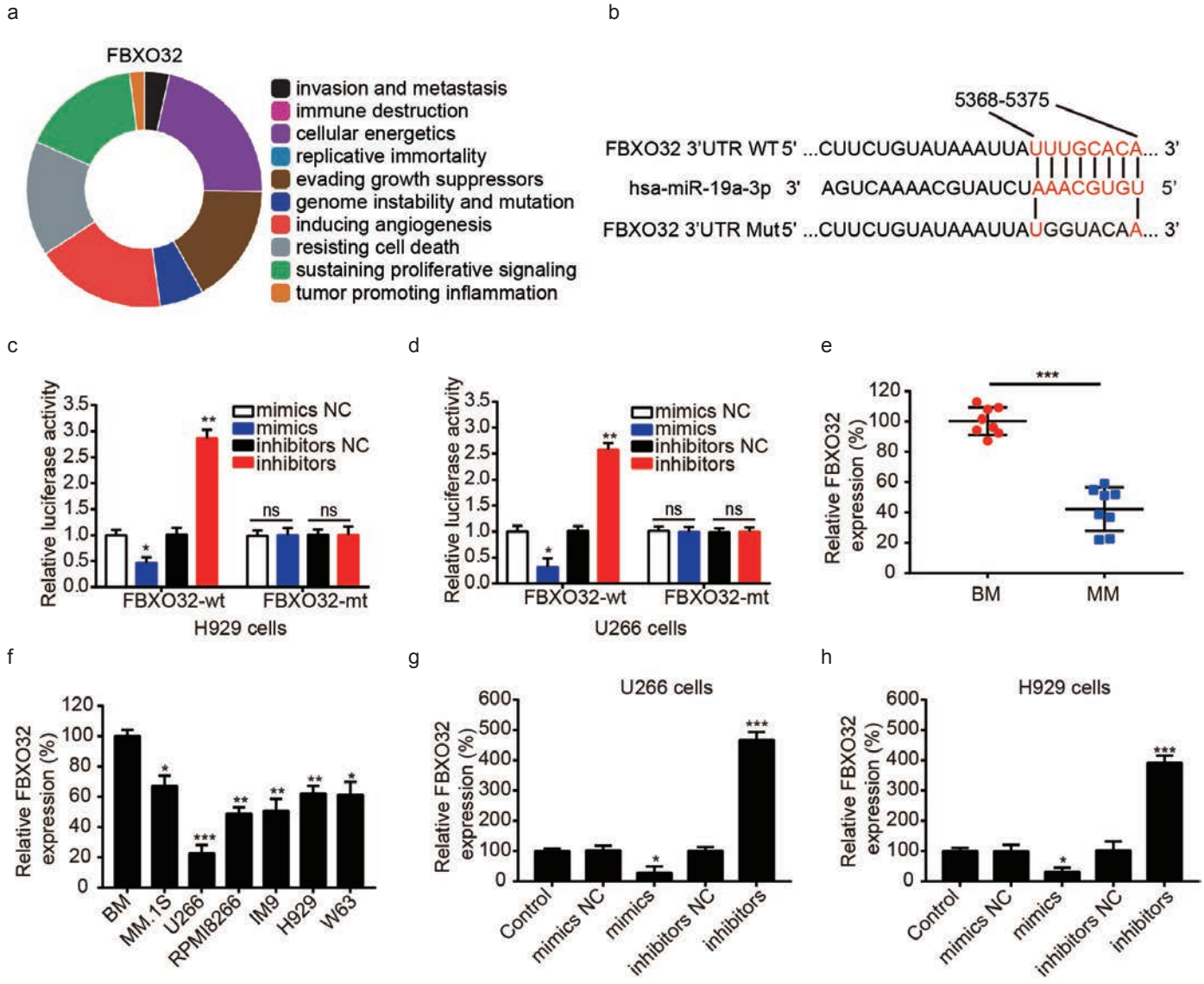

FIG. 4. a-h. FBXO32 acted as a direct target of miR-19a-3p in multiple myeloma (MM) cell lines. Association between FBXO32 and hallmarks of cancer from Cancer Hallmarks Analytics Tool (CHAT) (a). miR-19a-3p and its predicted binding sequences in the 3'-UTRs of FBXO32 Using TargetScan analysis tool (b). The dual-luciferase report system was used to detect the effect of miR-19a-3p on FBXO32 in H929 cells and U266 cells (c and d). Relative FBXO32 expression levels between the bone marrow (BM) and MM (e). Relative FBXO32 expression levels between BM and multiple myeloma cell lines (f). miR-19a-3p negatively regulated the expression of FBXO32 (g and h). *p<0.05; **p<0.01; ***p<0.001; each bar represented mean±standard deviation from 3 independent experiments

miR-19a-3p regulated cell viability and apoptosis through FBXO32 in multiple myeloma cells

FBXO32 was significantly overexpressed in U266 and H929 cells ($p<0.01$) (Figure 5a). CCK-8 assays revealed that FBXO32 significantly inhibits cell viability ($p<0.05$) (Figure 5b). Flow cytometry showed that FBOX32 significantly promotes cell apoptosis ($p<0.01$) (Figure 5. c, d). Furthermore, we found that FBXO32 significantly reverses the function of miR-19a-3p promoting cell viability ($p<0.05$) (Figure 5e). In addition, FBXO32 significantly reversed the function of miR-19a-3p inhibiting cell apoptosis ($p<0.05$) (Figure 5. f, g). WB revealed that miR-19a-3p significantly inhibits cleaved caspase 3 expression and increases *Bcl-2* expression, which was also reversed by FBXO32 overexpression ($p<0.05$) (Figure 5h).

DISCUSSION

MM remains an incurable hematological malignancy given the clinical use of chemotherapeutics, glucocorticoids, and novel treatments (25). Hence, it is essential to identify new biomarkers in tumorigenesis and progression, which contribute to realizing the pathogenesis and find novel treatment for MM. Recently, studies have demonstrated aberrant miRNA expression in carcinoma tis-

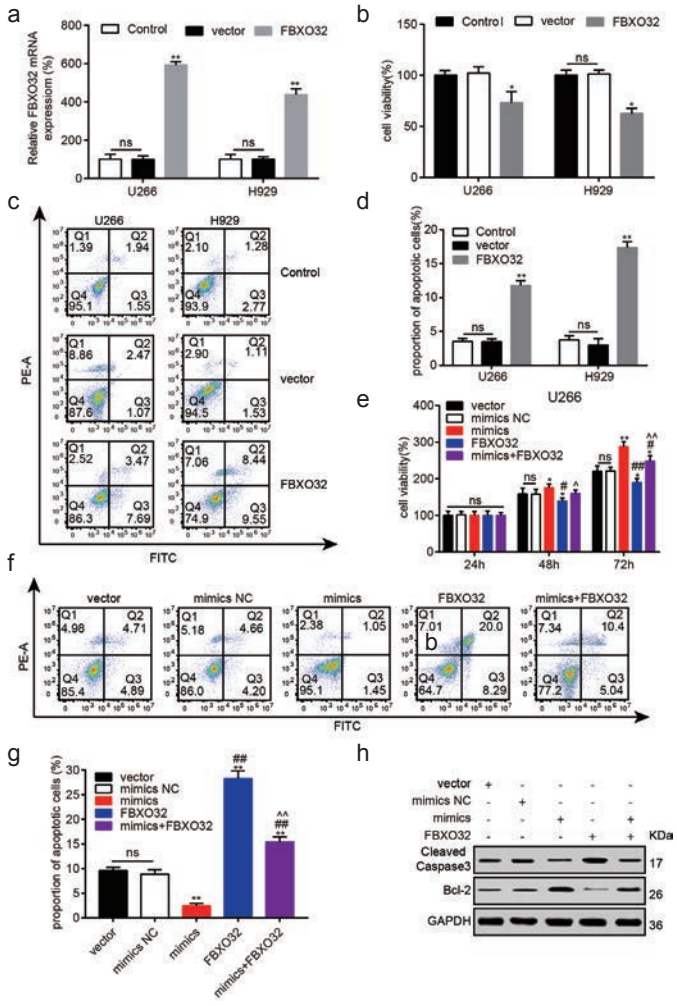

FIG. 5. a-h. miR-19a-3p regulates cell viability and cell apoptosis via FBXO32 in multiple myeloma (MM) cell lines. FBXO32 was overexpressed in U266 and H929 cells (a). Overexpressed FBXO32 significantly inhibited cell viability in U266 and H929 cells (b). Overexpressed FBXO32 significantly promoted cell apoptosis in U266 and H929 cells (c and d). FBXO32 rescued the effect of miR-19a-3p promoting cell viability (e). FBXO32 saved the result of miR-19a-3p inhibiting cell apoptosis (f and g). WB detected the expression of cleaved caspase 3 and BCL-2 (h). *p<0.05 vs vector group and mimics group, #p<0.05 vs mimics group, ^p<0.05 vs FBXO3 group; each bar represented mean±standard deviation from 3 independent experiments.

sues and cells, which may indicate significant molecular and clinical implications of miRNA in tumorigenesis and progression (26).

Previous studies reported that miR-19a-3p was increased in many cancers (including MM, hepatocellular carcinoma, and breast cancer) (5, 27, 28). Currently, our results presented that miR-19a-3p was overexpressed in MM cells and patients with MM (Figure 1. b, Cc). Interestingly, the significance level in patients with MM ($p<0.001$) was higher than in MM cells ($p<0.01$). Although the significance level does not account for the difference in expression, it is an interesting situation to consider. On the one hand, it may be caused by the lack of clinical samples. The miRNA expression levels of different individuals were significantly different. In contrast, these cell lines are derived from patients with MM, but screened. Cell lines are not equivalent to primary cells. Further studies revealed that miR-19a-3p suppressed cell apoptosis and promoted cell viability in U266 and H929 cells (Figure 2. a, c). These results showed that miR-19a-3p is an oncogene in MM cells. miRNAs alter gene expression by degrading or repressing target mRNAs. Subsequently, we predicted miR-19a-3p target genes in MM cells. Bioinformatics analysis ultimately extracted 5 core subnetworks (Figure 3f), which provides the research direction. Our previous studies screened these potential regulatory molecules. Eventually, we found that FBXO32 may be regulated by miR-19a-3p.

FBXO32 is a novel E3 ligase, which is one of the 4 subunits of the ligase complex of ubiquitin proteins (13). Recent evidence revealed the function of FBXO32 in tumorigenesis (29). FBXO32 was reported to be decreased in cancers, and FBXO32 induced apoptosis and increased cisplatin chemosensitivity (14-16, 29). Our results demonstrated that FBXO32 is down-regulated in MM cells and tissues (Figure 4. e, f). Besides, miR-19a-3p negatively regulated FBXO32 mRNA expression (Figure 4. g, h) in U266 and H929 cells. Luciferase assay verified that miR-19a-3p regulates gene expression by degrading FBXO32 mRNA (Figure 4. c, d).

Next, FBXO32 was overexpressed in U266 and H929 cells, and the results revealed that FBXO32 significantly suppressed cell viability and increased cell apoptosis (Figure 5. b, c). To verify that miR-19a-3p actually regulates a variety of biological functions by regulating FBXO32 expression in MM, both mimics and FBXO32 overexpression plasmid were transfected in U266 cells, so as to detect cell viability, cell apoptosis, and apoptosis-associated protein expression. Our findings demonstrated that FBXO32 overexpression reverses the functions of miR-19a-3p increasing cell viability and suppressing apoptosis (Figure 5. e, f). In addition, FBXO32 overexpression (Figure 5h) abolishes the influences of miR-19a-3p suppressing cleaved caspase 3 and increasing BCL-2.

Based on the abovementioned results, we preliminarily revealed that miR-19a-3p directly regulates FBXO32. It would be significant to discover detailed mechanism in MM in the future. More studies are still necessary to confirm these findings, which will provide strong evidence supporting the function of miR-19a-3p and FBXO32 in MM occurrence and development.

Our current work confirmed the expression levels of miR-19a-3p in MM cells and patients with MM. miR-19a-3p significantly promoted cell viability and suppressed cell apoptosis. Moreover, FBXO32 was a target gene of miR-19a-3p. FBXO32 was down-regulated and significantly suppressed cell viability and promoted cell apoptosis in MM cells. Rescue assay further suggested that miR-19a-3p plays a role of oncogene by regulating FBXO32 expression in MM cells.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of the Jinshan Hospital of Fudan University (JIEC-2018-04).

Patient Consent for Publication: Written informed consent was obtained from the all patients.

Author Contributions: Design - D.Z., Y.L.; Analysis and/or Interpretation - Y.Ma, Y.Meng.; Writing Manuscript - Y.L.; S.G.

Data-sharing Statement: N/A.

Conflict of Interest: The authors have no conflicts of interest to declare.

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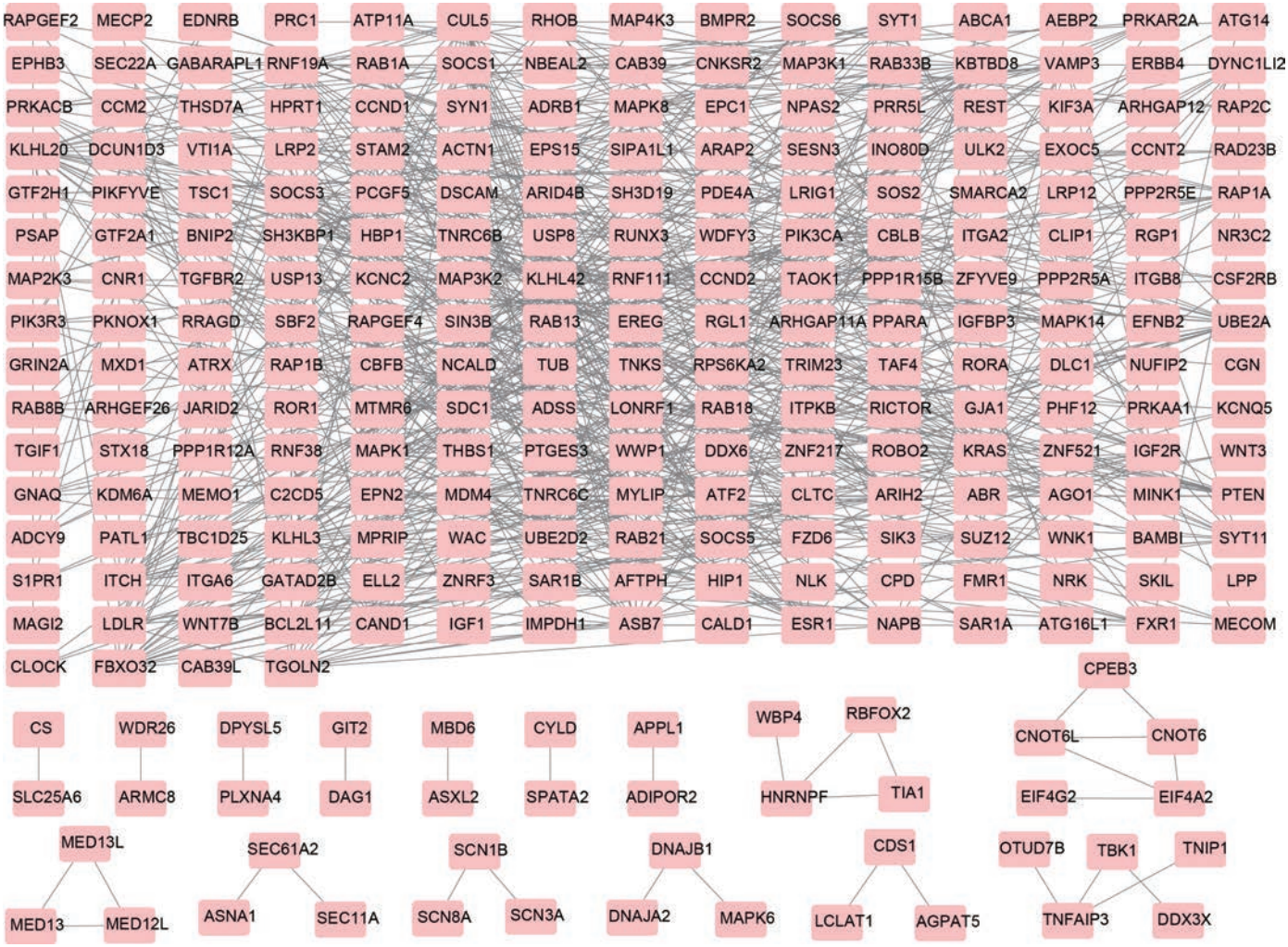

FIG. S1. Protein interaction network of miR-19a-3p target genes was analyzed at STRING analysis tools and visualized by Cytoscape software.