Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;49:87-100
DOI: 10.1159/000492846
Published online: 22 August, 2018
Accepted: 13 August, 2018

Original Paper

Long Non-Coding RNA MEG3 Functions as a Competing Endogenous RNA to Regulate HOXA11 Expression by Sponging miR-181a in Multiple Myeloma

Xuxing Shen[a] Hua Bai[a] Huayuan Zhu[a] Qing Yan[a] Ye Yang[b] Wenjun Yu[a] Qinglin Shi[a] Juejin Wang[c] Jianyong Li[a] Lijuan Chen[a]

[a]Department of Hematology, First Affiliated Hospital of Nanjing Medical University, Jiangsu Province Hospital, Nanjing, [b]Basic Medical College, Nanjing University of Chinese Medicine, Nanjing, [c]Key Laboratory of Cardiovascular Disease and Molecular Intervention, Department of Physiology, Nanjing Medical University, Nanjing, China

Key Words
Meg3 • LncRNA • MiR-181a • Multiple myeloma

Abstract
Background/Aims: Long non-coding RNA maternally expressed gene 3 (MEG3) has been reported to play an essential role in cancer progression and metastasis. However, the overall biological role and regulatory mechanism of MEG3 in multiple myeloma (MM) development and progression remains largely ill-defined. ***Methods:*** MEG3 and miR-181a expression of MM patients were analyzed by publicly available MM data sets. Cell counting kit-8 and flow cytometry analysis were used to identify the function of MEG3 on MM *in vitro*. Additionally, we conducted tumor formation experiments in mice models to explain the role of MEG3 on MM *in vivo*. Then, several mechanism experiments, including dual-luciferase reporter assay and RNA immunoprecipitation were performed to evaluate the emulative relationship between MEG3 and miR-181a. ***Results:*** In this research, we found that MEG3 was downregulated in MM patients, which was linked with tumor progression. In addition, we demonstrated that miR-181a was overexpressed in MM patients in consistent with its cancer-promoting function. Importantly, several mechanism experiments revealed that MEG3, acting as an endogenous competitive RNA, could contend with miR-181a to inhibit tumor progression. Furthermore, as the target mRNA of miR-181a, homeobox gene A11(HOXA11) could be positively regulated by MEG3 through sponging miR-181a competitively *in vitro*. ***Conclusion:*** Our present work supplies the first discovery of a MEG3/miR-181a/HOXA11 regulatory network in MM and highlights that MEG3 may serve as a promising target for MM therapy in the future.

X. Shen, H. Bai and H. Zhu contributed equally to this work.

Lijuan Chen Department of Hematology, the First Affiliated Hospital of Nanjing Medical University, Jiangsu Province Hospital
300 Guangzhou Road, Nanjing 210029 (China)
E-Mail Chenljb@126.com

Introduction

Multiple myeloma (MM), so far an incurable disease, is characterized by the abnormal proliferation and aberrant accumulation of malignant plasma cells in the bone marrow [1]. As a frequent hematological malignancy, second only to non-Hodgkin lymphoma, MM is remarkable for the expansion of monoclonal immunoglobulin, bone marrow plasmacytosis, renal failure, bone lesion and immunodeficiency [2]. In spite of the use of innovative therapeutic strategies for MM, patient survival rate is still very dismal. Mainly due to the high rates of drug resistance and disease recurrence after treatment, it is urgent to find a brand-new breakthrough for MM.

Increasing evidence has demonstrated that MM is caused by a serious of complex polygenic events, and a variety of non-coding RNAs (ncRNAs), including short microRNAs(miRNAs) and long non-coding RNAs (lncRNAs), which are important regulatory molecules in oncogenic and tumor-suppressor pathways despite the lack of coding capabilities [3]. The dysregulation of lncRNAs exhibits in many cancers and is associated with tumorigenesis, metastasis, disease diagnosis and prognosis [4, 5]. As we all know, miRNAs are a category of small non-coding RNAs in the length of nearly 23 nucleotides [6]. MiRNAs equip to bind the 3'UTR of certain mRNAs to enforce miRNA-induced expression suppression. Recent studies have reported that lncRNAs, functioning as competitive endogenous RNAs, could serve as miRNA 'sponge-like' material indirectly modulating target gene expression by sharing the common miRNA response elements (MREs), which influenced post-transcriptional regulation by inhibiting available miRNA activity [7, 8]. For instance, miR-331-3p could be antagonized by HOTAIR acting as a competing endogenous RNA for HER2 in gastric cancer [9]. What's more, LncRNA H19 modulates gallbladder cancer cell proliferation by regulating miR-194-5p target of AKT2[10]. In addition, miR-204 inhibited by LncRNA UCA1 leads to up-regulated translational repression of its target transcript ATF2 in prostate cancer [11].

Maternally expressed gene3 (MEG3), located at chromosome 14q32, is the first identified tumor suppressive lncRNA so far [12]. Studies have shown that MEG3 possesses tumor suppressor function and is able to induce cell apoptosis though both p53-dependent and p53-independent signaling pathways [13-16]. The expression of MEG3 was found decreased in a wide variety types of cancer, such as urothelial carcinoma, colorectal cancer and cervical cancer，revealing its tumor-suppressive veil [17-19]. Existing studies have shown that MEG3 expression could also be silenced by the epigenetic modification of DNA methylation [20, 21]. The promoter hypermethylation of MEG3 was also found in most MM patients and linked with disease stage and subtype [22]. In addition, a study using bone marrow mesenchymal stromal cells(BMSC) as the research model demonstrated that up-regulated MEG3 enhanced osteogenic differentiation partly by promoting BMP4 transcription [23]. In 2013, a report regarded MEG3 as an explicit ceRNA in brain tumor [24]. Except of its competing role in brain tumor, MEG3 could also be an important ceRNA in other cancers and disorders [25, 26]. Previous studies have demonstrated that miR-181a as a member of the miR-181family, which exerts as a tumor-promotor gene, is upregulated in many tumors, including chondrosarcoma and gastric cancer [27, 28]. Based on the previous theory, we are final successful to find MEG3 could function as a ceRNA for miR-181a in MM, which implies its potential as an important tumor biomarker source [29].

Hox genes play crucial roles in modulating morphological change and cell differentiation, which was described as a subgroup of the highly conserved homeobox superfamily [30]. In humans, HOXA11 genes are separated into four rickles(named A,B,C,D), located on four parted chromosomes [31]. Although there is still some controversy regarding the role of HOX family in carcinogenesis, HOXA11 has been reported as a tumor-suppressive gene in many cancers [32, 33]. Previous studies have shown that miR-181 could target HOXA11 to change its post-transcrptional expression [34]. In the current study we aimed to investigate the co-regulation crosstalk among MEG3, miR-181a and HOXA11.

Materials and Methods

Microarray data analysis

The mRNA expression of MEG3 and HOXA11 in NP, MGUS(GSE5900), and MM(GSE2658) plasma cells were obtained from the Affymetrix U133 Plus2.0 microarray (Affymetrix, Santa Clara, CA, USA), which were performed as previously described. Data containing miRNA-181a expression comparison were available from GSE17306. Statistical microarray data analysis results were relied on GraphPad Prism5.

Cell culture

Human MM cells ARP-1 and LP-1 were obtained from the American Type Culture Collection (ATCC) cultured in the RPMI-1640 media (KeyGEN Biotech, Nanjing, China) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, USA) under standard conditions (37°C, 5%CO_2). The cell culture medium was changed every two days averagely.

Cell transfection and reagents

MiRNA-181a negative control/mimics/inhibitor (GenePharma, Shanghai, China) and pcDNA-negative control/MEG3 plasmid (GenePharma, Shanghai, China) were delivered into cells using Lipofectamine 3000 reagent (Invitrogen, USA) according to the manufacturer's specifications. The sequences of miR-181a mimics, miR-181a inhibitor and miR-181a negative control are as following: miR-NC sense 5'-UUCUCCGAACGUGUCACGUTT-3' antisense 5'-ACGUGACACGUUCGGAGAATT-3; miR-181a mimics sense 5'-AACAUUCAACGCUGUCGGUGAGU-3' antisense 5'-UCACCGACAGCGUUGAAUGUUUU-3' miR-181a inhibitor 5'- ACUCACCGACAGCGUUGAAUGUU-3'

Protein isolation and Western blotting assay

Cells were dissolved in RIPA buffer (KeyGEN Biotech, Nanjing, China) plus protease inhibitor cocktails (Biotool, Shanghai, China) at the ratio of 100:1. After high speedy centrifugation, the cellular protein lysates were blended with 5× loading buffer. Prepared protein samples were separated by 10% SDS-PAGE then transferred onto 0.22 μm PVDF membranes (Millipore, USA) and incubated with respective antibodies. The mentioned antibodies were: anti-HOXA11 antibody (Proteintech, Wuhan, China), β-actin (Proteintech, Wuhan, China) and the secondary antibodies (Vazyme Biotech, Nanjing, China).

RNA isolation and Real-time PCR

Total cellular and tumor tissue RNA were extracted using RNA Trizol (Invitrogen, USA) according to the manufacturer's instructions. Newly synthesized cDNA samples for RT-PCR were achieved complied with the previous report. The primers we used were listed (5'-3'): hsa-miR-181a RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACTCACCG.hsa-miR-181a forward: ACACTCCAGCTGGGAACATTCAACGCTGTCG. U6 RT: AACGCTTCACGAATTTGCGT. U6 forward: CTCGCTTCGGCAGCACA.URP: TGGTGTCGTGGAGTCG. HOXA11 forward: GGGATGCATGGAGATAGCCC. HOXA11 reverse: CTGCAGGCTCCAAGAGTGAA. GAPDH forward: TTTGGTATCGTGGAAGGAC. GAPDH reverse: AAAGGTGGAGGAGTGGGT.MEG3 forward: CTGCCCATCTACACCTCACG. MEG3 reverse: CTCTCCGCCGTCTGCGCTAGGGGCT.

Cell viability

In brief, ARP-1 and LP-1 cells were seeded in 96-well plates per 2×10^3 cells/well with respective transfection reagents. Then cell viability was evaluated at 24, 48, 72 and 96 hours with Cell Counting Kit-8 (Selleck, Shanghai, China), followed by the manufacturer's instructions. Absorbance at 450 nm using Elx800 Reader was then been recorded (Bio-Tek Instruments Inc, USA).

Flow cytometry

Both ARP-1 and LP-1 cell lines were transfected with related reagents as mentioned before for 48 hours respectively. Then, the cells were harvested and double stained with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide(PI) according to the manufacturer's instructions (Vazyme Biotech, Nanjing, China). Cell analysis was conducted by FACS Caliburflow cytometer (BD Biosciences, USA) applying the FlowJo7.6 analysis software tool. For cell cycle flow cytometry, transfected cells were suspended by pre-

cooling ethyl alcohol overnight then prepared for FACS Caliburflow cytometer (BD Biosciences, USA) and similarly analyzed by FlowJo7.6.

Dual-Luciferase reporter assay

ARP-1 and LP-1 cell lines were cultured in 24-well plates with MEG3-WT or MEG3-MU and added with 50 nM miR-181a mimics or miR-NC (GenePharma, Shanghai, China) as well as 20ng SV40 and 800ng luciferase reporter vector (Realgene, Shanghai, China). After 48 hours of transfection, the luciferase activities were performed using the Dual-Luciferase® Reporter Assay System (Promega, USA).

RNA immunoprecipitation (RIP) assay

A Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) was used according to the product specification. Briefly, cells were washed with pre-cooling PBS and then resuspended with lysis buffer. Added the supernatant of the lysis mixture with immunoprecipitation buffer containing ago2 antibody (Abcam, USA) or IgG antibody (Abcam, USA) overnight. At last, the concentration of RNA was purified by digestion of proteinase K, phenol extraction and ethanol precipitation step by step and final to analyze via RT-PCR.

Tumor formation experiments in mice models

NOD–SCID mice were purchased from Charles River Laboratories (Beijing, China). The transfected ARP-1 cells (0.5×10^6 cells in 100 μl PBS) were injected subcutaneously into the hindquarters of each 4-week-old NOD–SCID mice. All the mice were observed every 2 days for at least 7 weeks. The tumor growth was assessed with volume as $a \times b^2/2$, where a is the widest length and b is the perpendicular diameter. Until the appointed day, tumor tissue samples derived from the NOD–SCID mice were took out for hematoxylin and eosin (H&E) and stained for Ki67 antibody. The remaining tumor tissue samples were dissolved by RNA Trizol or protein lysate for further detecting HOXA11 mRNA and protein expression.

Ethics statement

All procedures involving the mice were approved by the local animal ethics committee at Nanjing Medical University.

Statistical analysis

Data were represented as mean ± SD and all the experiment results were analyzed by GraphPad Prism 5.0 (CA, USA). Images of MEG3 expression correlating with OS and EFS were obtained by Kaplan-Meier method, and the log-rank test was used for group comparison. Significance was determined using the two tailed Student's t- test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Results

The expression of MEG3 is decreased and correlated with prognosis in MM patients

We first evaluated the expression of MEG3 in MM samples by interrogating microarray data sets (GSE5900 and GSE2658). Our data shows that significant down-regulation of MEG3 is obvious in 351 MM patients compared with NP and MGUS (Fig. 1A). Moreover, we investigated the delicate correlation between MEG3 expression and event-free survival (EFS) and overall survival (OS). As we can see from the diagram, patients with high MEG3 expression exhibited a trend for better event-free survival (median 72 months for MEG3 high versus 42 months for MEG3 low, Fig. 1B) and better overall survival (Fig. 1C). These data indicate that the expression of MEG3 is downregulated in MM patients and correlated with poor prognosis.

Over-expression of MEG3 promotes cell apoptosis and inhibits cell proliferation in vitro

Following up on the clinical observations described above, we therefore speculated that aberrant MEG3 expression might be associated with tumor growth in MM. We first measured MEG3 expression in several MM cell lines (data not shown) and discovered that

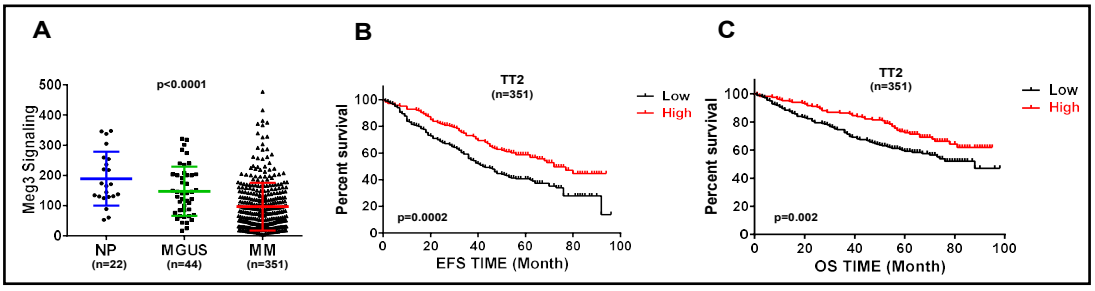

Fig. 1. MEG3 expression in MM patients. A. The analysis of MEG3 expression in plasma cells in MM samples (n = 351), normal plasma cells (NP) (n = 22) and monoclonal gammopathy of undetermined significance(MGUS) (n = 44) by interrogating microarray data sets (GSE5900 and GSE2658). P<0.0001 B-C. Kaplan-Meier analyses of the associations between MEG3 expression level and event-free survival (EFS) (P=0.0002) and overall survival (OS) (P=0.002) of patients with MM in TT2 cohort. The log-rank test was used to calculate P-values.

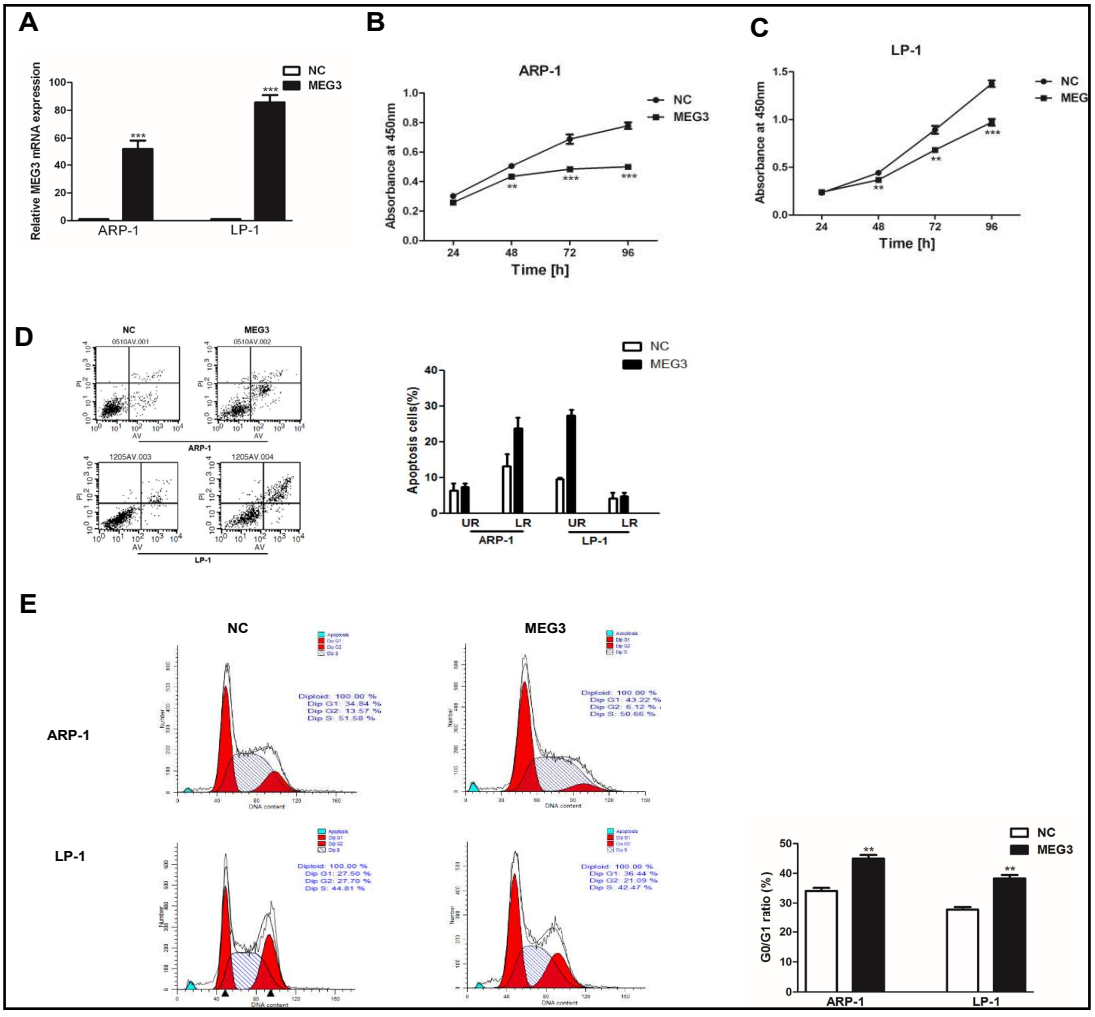

Fig. 2. MEG3 overexpression affect MM cells activity in vitro. A. MEG3 overexpression efficiency on MM cell lines ARP-1 and LP-1 transfected with pcDNA-MEG3 and pcDNA-NC. B-C. CCK-8 assays was used to determine the proliferation effect of MEG3 on ARP-1 and LP-1 cells. D. Flow cytometry showed the effect of MEG3 on cell apoptosis in ARP-1 and LP-1 cells. E. Cell cycle flow cytometry assay was performed to unfold the proliferation effect of pcDNA-MEG3 and pcDNA-NC transfected ARP-1 and LP-1 cells. All those in vitro experiments results were obtained after 48h transfection. **P<0.01, ***P<0.001.

the expression level of MEG3 was lower in ARP-1 and LP-1. After 48h transfection of pcDNA-MEG3 and pcDNA-NC, the over-expression efficiency was obtained by RT-PCR nearly 50-fold in ARP-1 and 100-fold in LP-1 cells (Fig. 2A). Then, cell-counting kit-8 (CCK-8) assays was performed and a dramatic decline of cell viability was detected in ARP-1 and LP-1 cells after treatment with pcDNA-MEG3, compared with pcDNA-NC treated group (Fig. 2B and 2C). Then we used flow cytometry to investigate the effect of MEG3 on cell apoptosis and cell cycle after 48h transfection. The results indicated that up-regulated MEG3 expression significantly promoted cell apoptosis (Fig. 2D). In the cell cycle flow cytometry assay, the number of cells in the G0/G1 stages was greater in the pcDNA-MEG3 transfected group than in the pcDNA-NC transfected group (Fig. 2E). All these above prove that lncRNA-MEG3 promotes cell apoptosis and inhibits cell proliferation in MM cell lines.

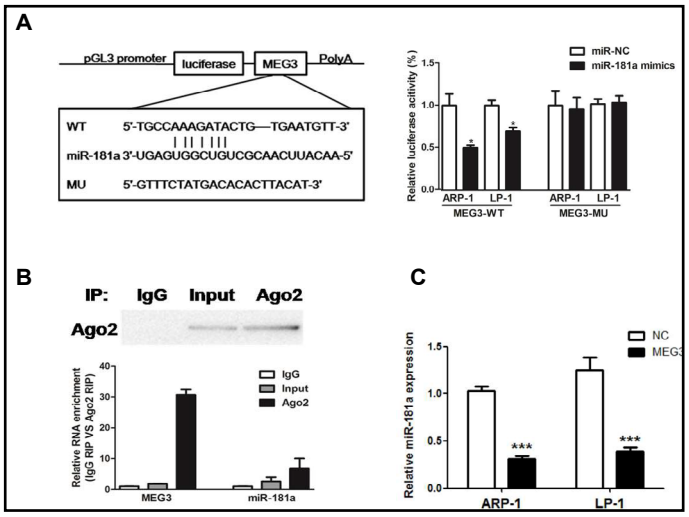

Fig. 3. MEG3 binds with miR-181a in MM cells. A. The predicted miR-181a binding sites in the region of MEG3 and the corresponding mutant sequence was shown. Luciferase reporter assay showed miR-181a overexpression decreased the fluorescence activity in ARP-1 and LP-1 transfected with the MEG3-WT vector. B. Connection between MEG3 and miR-181a was proved by RNA binding protein immunoprecipitation assay. C. Relative expression of miR-181a in ARP-1 and LP-1 cells transfected with pcDNA-MEG3. *P<0.05, **P<0.01, ***P<0.001.

MEG3 binds to miR-181a in MM cells

To verify whether MEG3 could function as a competing endogenous RNA for a certain miRNA, we used the bioinformatics tool to hunt for candidate miRNAs. Only those with highly ranking target sites and supernal expression in MM specimens were suitable for further analysis and it predicts the emulative relationship between MEG3 and miR-181a. Herein, we focused on the miR-181a as a matching miRNA for further investigating the interaction between MEG3 and miR-181a. As shown in Fig. 3A, luciferase reporter assay demonstrated that overexpression of miR-181a could impair the increased fluorescence activity in ARP-1 and LP-1 after transfected with the MEG3-WT vector, while had no obvious impact on the activity of cells transfected with MEG3-MU vector. MiRNAs have been reported to be subsistent in the cytoplasm in the form of miRNA ribonucleoprotein complexes (miRNPs). Ago2, as an important component of miRNPs constituent proteins, took part in composing RNA induced silencing complex (RISC)[35]. Similar results were obtained from RNA binding protein immunoprecipitation assay (RIP) where Ago2 antibody enriched immunoprecipitation emerged more abundant MEG3 and miR-181a RNA compared with the control group (Fig. 3B), which indicated that MEG3 could directly bind to miR-181a through an Ago2-dependent manner. To further elucidate whether miR-181a could regulated by MEG3 we compared miR-181a expression in pcDNA-NC and pcDNA-MEG3 transfected groups and the results showed that overexpression of MEG3 reduced the expression of miR-181a in MM cells as a consequence of releasing less free miR-181a (Fig. 3C). Together, these data indicated that miR-181a can directly bind to MEG3 in a usual way.

MiRNA-181a functions as an oncogenic molecular in MM

As the important regulators of mRNA, miRNAs have been well-recognized as important molecular in cancer progression [6]. Intriguingly, on the contrary to MEG3, our subsequent studies revealed increased expression of miR-181a in MM patients, which was in line with our prediction (Fig. 4A). On the basis of previous experimental results, we conjectured that miR-181a may play an oncogenic role in MM. To prove this, we first ascertain the transfection efficiency of miR-181a mimics and inhibitor in MM cells (Fig. 4B). Accordingly, the CCK-8 proliferation assay manifested that overexpression of miR-181a exerted more evident cell proliferation, while silencing miR-181a expression inhibited cell growth (Fig. 4C). As expected, reduced expression of miR-181a identically led to significant increasing apoptosis level both in ARP-1 and LP-1 cells as shown in flow cytometry assay (Fig. 4D). Therefore, these findings suggest the cancer-friendly roles of miR-181a in MM cells objected to its ceRNA MEG3.

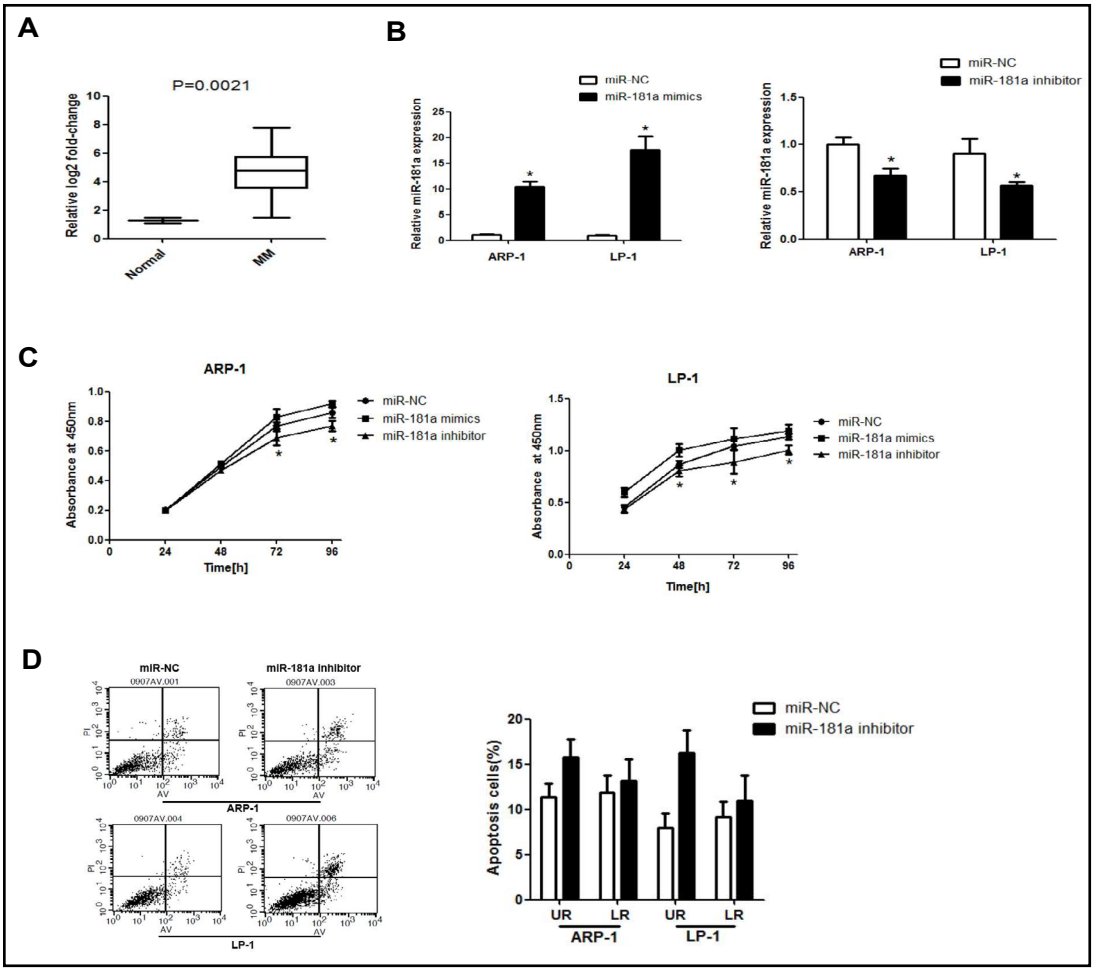

Fig. 4. miRNA-181a functions as an oncogenic molecular in MM. A. Microarray analysis from GEO database numbered GSE17306 showed that miR-181a was highly expressed in MM patients. P=0.0021 B. Relative expression levels of miR-181a in MM cell lines ARP-1 and LP-1 after transfected with miR-181a mimics or inhibitor. C.CCK-8 proliferation assay manifested that overexpression of miR-181a exerted more evident cell proliferation, while silencing miR-181a expression inhibited cell growth. D. Flow cytometry was performed to investigate the effect of miR-181a on cell apoptosis in ARP-1 and LP-1 cells. All those in vitro experiments results were obtained after 48h transfection. *P<0.05, **P<0.01.

MiR-181a reverses the tumor-suppressive effects of MEG3 in MM cells

In order to investigate the biological behaviors of MM cells co-regulated by MEG3 and miR-181a, we set up a control group to perform rescue experiments on cell apoptosis and proliferation. The CCK-8 assay carried out in wells showed that cell proliferation was reduced in MEG3 overexpressed cells, whereas miR-181a mimics reversed the reduction of proliferation caused by MEG3 (Fig. 5A). Furthermore, when MEG3 and miR-181a mimics were co-transfected in the ARP-1 and LP-1 cells, miR-181a mimics could significantly reduce the apoptosis-promoting role of MEG3 in MM cells (Fig. 5B). Similarly, cell cycle profile reflected the consistent results that miR-181a mimics were able to rescue the inhibiting function of MEG3 (Fig. 5C). Based on the results, we confirmed that the miR-181a mimics reversed the tumor-promoting effects of MEG3 on MM cell lines.

MEG3 regulated HOXA11, a target mRNA of miR-181a

For the purpose of finding out genes sharing the crosstalk between MEG3 and miR-181a, we sought some possible targets on TargetScan (http://www.targetscan.org/vert_71/), Miranda(http://34.236.212.39/microrna/home.do), miRDB4 (http://www.mirdb.org/mirdb/download.html) and DIANA(http://www.microrna.gr/micro). As we can see from the Venn diagram (Fig. 6A), we finally predicted 3 targets (CDX2, HOXA1, HOXA11). Based on the ceRNA competition principle, we concluded that the authentic target mRNA should be upregulated by MEG3 overexpression and downregulated by miR-181a mimics. To verify this relationship, we performed RT-PCR and focus on HOXA11 for further study due to only HOXA11 meet these conditions (Fig. 6B). Additionally, we also found that HOXA11 mRNA expression was increased by miR-181a inhibitor (Fig. 6C). Moreover, Western blot assays demonstrated the aligned results of HOXA11 protein level transformed by artificial miR-181a regents in MM cells (Fig. 6D). All this above was in consistent with the bivariate correlation analysis between MEG3 and HOXA11 expression from GSE2658 (Fig. 6E). To further study whether MEG3 could function as ceRNA to regulate HOXA11 expression by competing with miR-181a, we implemented western bolt assay to test HOXA11 protein level after transfecting MEG3 with or without miR-181a mimics. As shown in Fig. 6F and 6G, overexpression of MEG3 upregulated HOXA11 protein level while MEG3 combining with miR-181a mimics effectively reduced HOXA11 protein expression. Together, these data demonstrated that MEG3 could function as a ceRNA to relieve the suppression of HOXA11 expression by competitively binding miR-181a in MM cell lines.

Over-expression of MEG3 leads to tumor growth inhibition in vivo

Given the tumor-suppressive role of MEG3 *in vitro*, we supposed to find out its impact on tumor growth *in vivo*. We injected ARP-1 cells into NOD-SCID mice subcutaneously. Beforehand, we successfully induced expression-stable ARP-1 cells with lenti-viruses carrying overexpressed MEG3(MEG3OE) or empty-vector(MEG3EV). Throughout the growth of tumor, tumor lumps from the MEG3OE transfected mice developed slower than those from MEG3EV transfected mice (Fig. 7A). Up to 42 days, the mice were sacrificed and we measured the weight of each group. We found that xenograft tumors from MEG3OE transfected cells were obviously lighter than EV transfected lumps (0.49±0.21g vs 1.24±0.68g, Fig. 7B and 7C). Obtained tumor samples were dissociated for RT-PCR analysis and Western blo t assay and the results exerted that MEG3OE transfected lumps expressed higher HOXA11 mRNA and protein levels (Fig. 7D and 7E). Moreover, the immunohistochemistry results also showed that Ki67 protein levels in the tumor tissues generated from MEG3OE transfected cells were strongly reduced (Fig. 7F). Collectively, we concluded that upregulated MEG3 could inhibit tumor growth *in vivo*.

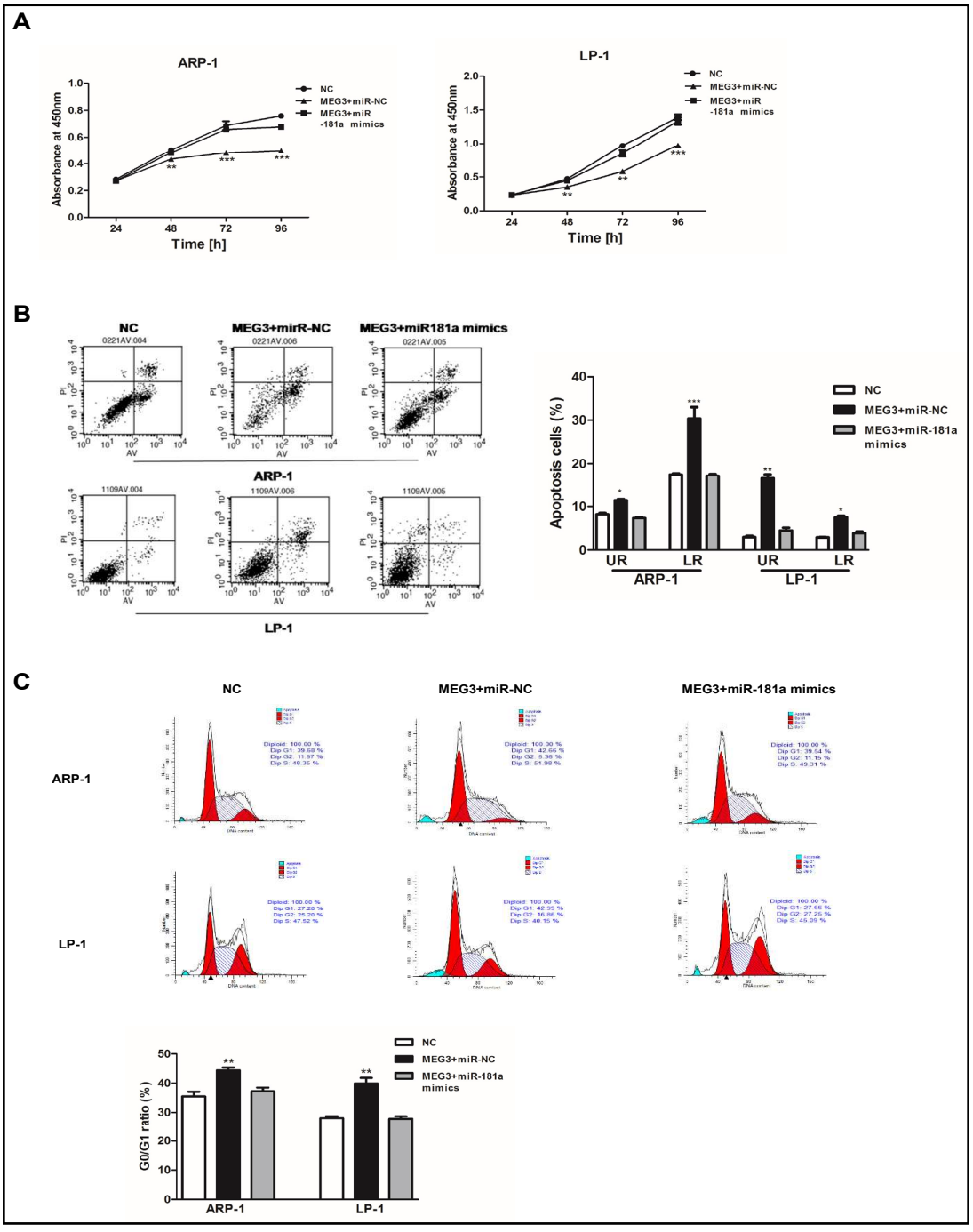

Fig. 5. miR-181a reverses the tumor-suppressive effects of MEG3 in MM cells. A. CCK-8 assay was performed to certify the proliferation of ARP-1 and LP-1 after co-transfected with MEG3 and miR-181a mimics. B. Flow cytometry was performed to detect the apoptosis effect of ARP-1 and LP-1 cells after co-transfected with MEG3 and miR-181a mimics. C. Cell cycle flow cytometry assay was performed to determine the proliferation effect after co-transfected with MEG3 and miR-181a mimics. All those in vitro experiments results were obtained after 48h transfection. *P<0.05, **P<0.01, ***P<0.001.

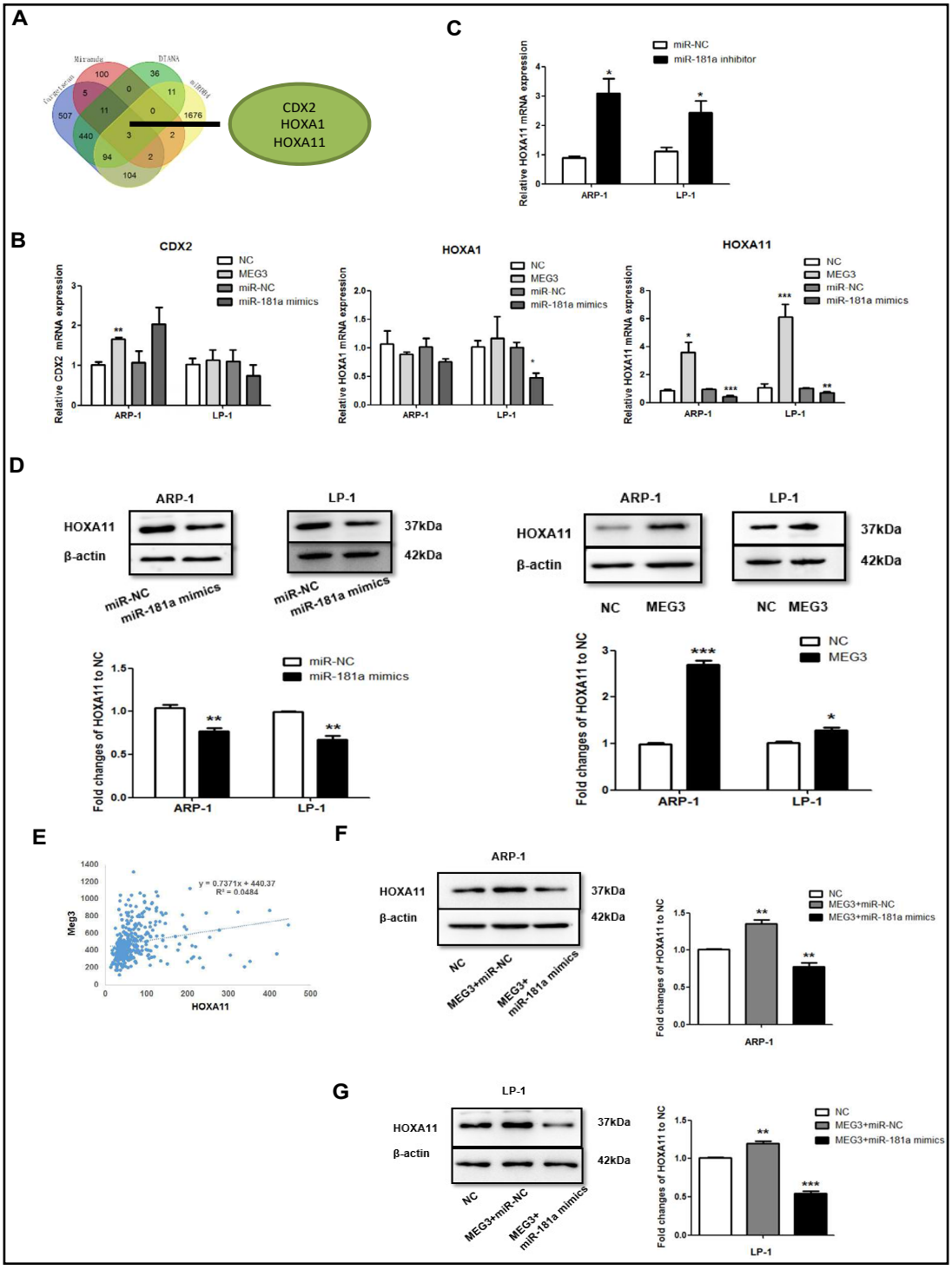

Fig. 6. MEG3 regulated HOXA11, a target mRNA of miR-181a. A. The predicted target gene was described in Venn diagram. Relevant data were from database TargetScan, Miranda, miRDB4 and DIANA. B. Relative expression of CDX2, HOXA1 and HOXA11 mRNA levels after transfected with MEG3 or miR-181a mimics. C. Relative expression of HOXA11 mRNA levels in ARP-1 and LP-1 cells after transfected with miR-181a inhibitor. D. Relative expression of HOXA11 protein levels in ARP-1 and LP-1 cells after transfected with MEG3 or miR-181a mimics. E. Bivariate correlation analysis between MEG3 and HOXA11 expression level. F-G. Relative expression of HOXA11 protein levels in ARP-1 and LP-1 cells after co-transfected with MEG3 and miR-181a mimics. *P<0.05, **P<0.01, ***P<0.001.

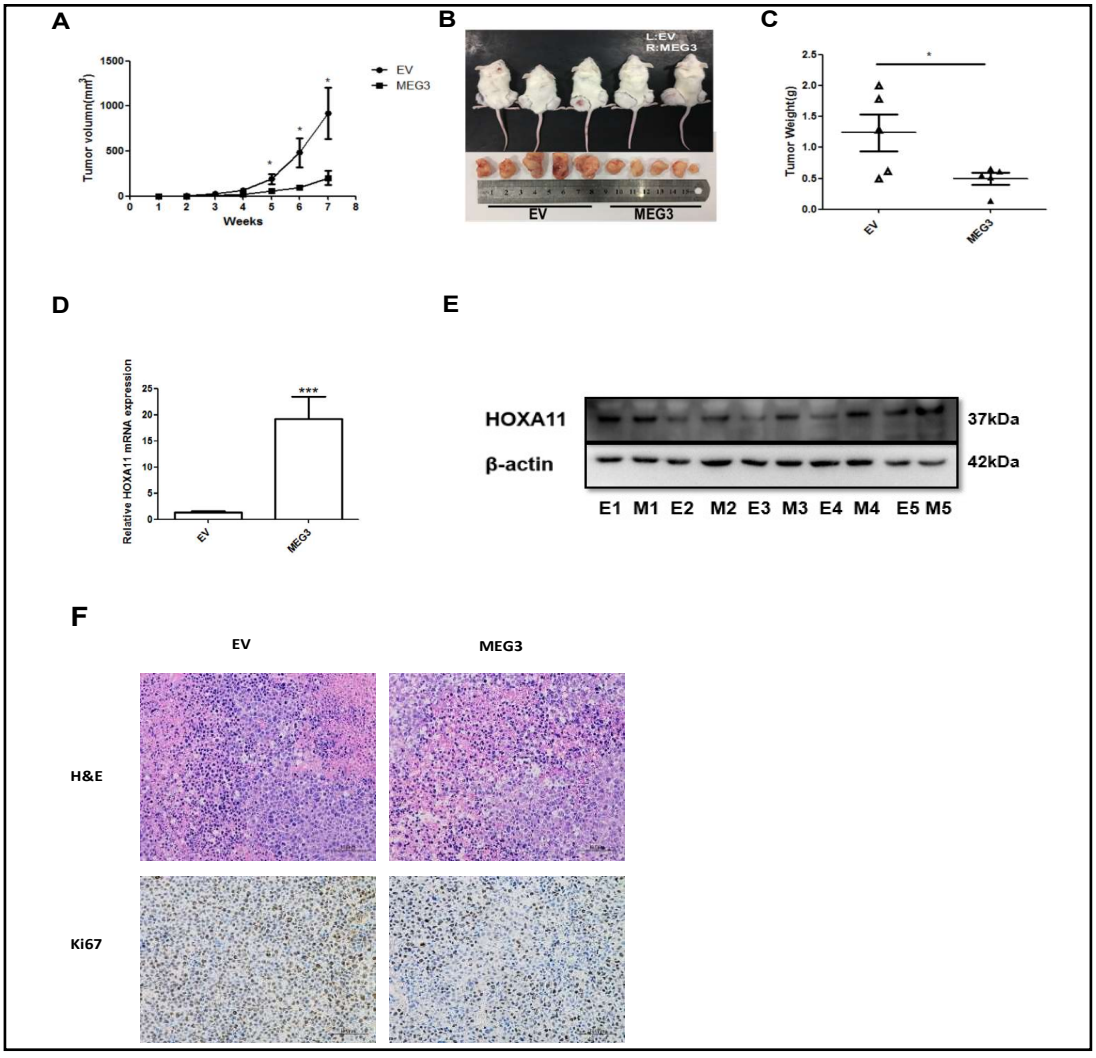

Fig. 7. Overexpression of MEG3 inhibits tumor growth in vivo. A. Tumor growth curve of experimental NOD-SCID mice models after injected with ARP-1 cells. Tumor volumes were calculated every week. B-C. Tumor weights of the mice at the end of 42 days are represented as means ± SD. For each mice, left tumor represents empty vector and right tumor represents MEG3 overexpression. D-E Obtained tumor samples were dissociated for RT-PCR analysis and Western blot assay (E: empty vector M:MEG3 overexpression) and the results exerted the HOXA11 expression. F. Representative Ki67 level in subcutaneous tumors of the mice model. Scale bars, 100μm. *P<0.05, **P<0.01, ***P<0.001.

Discussion

LncRNAs, emerging as pivotal regulatory molecules in tumor suppressing and oncogenic pathways, play important roles in MM pathogenesis [36]. According to our knowledge, LncRNA MEG3 promoter hypermethylation has been observed in patients with MM. However, how MEG3 function in MM pathogenesis remains obscure. In this study, we identified the tumor suppressive role of lncRNA MEG3 in MM and further revealed that MEG3 is able to contribute to positively upregulate the expression of HOXA11 via releasing less free miR-181a. Our research first provided a novel concept for lncRNA-miRNA-mRNA regulatory crosstalk, namely MEG3-miR-181a-HOXA11 in MM, which could be a new light on this brutal illness.

LncRNAs are common deregulated in various tumors and correlated with multiple physiological processes, such as proliferation, apoptosis, cell migration and so on. In this

study, we demonstrated that MEG3 functions as a tumor suppressor in MM. Over-expression of MEG3 inhibited proliferation and promoted apoptosis in ARP-1 and LP-1 cell lines, which implied its tumor suppressing effect. Besides, our *in vivo* data found MEG3OE mice presented smaller solid tumor mass and lower Ki67 expression than MEG3EV mice. Moreover, a mature data set showed that the levels of MEG3 were significantly down-regulated in MM patients, and the degradation of MEG3 was indeed correlated with shorter OS and EFS. All these *in vivo* data are in consistence with our *in vitro* results and further revealed its anti-tumor activity.

Although MEG3 has been shown to act as tumor suppressor role in MM, the precise molecular mechanisms remain largely unknown. ceRNAs are endogenous transcripts that messenger RNAs and lncRNAs through competing for MREs to form a positive correlation between each other's expression levels [37, 38]. For example, lncRNA UCA1 accelerates hepatocellular carcinoma progression in which it's inhibitory to miR-216b and modulating the levels of FGFR1 [39]. LncRNA MALAT1 functions as a ceRNA, which can directly bind to miR-200s, thereby modulating the ZEB2 expression in clear cell kidney carcinoma. Moreover, lncRNA MEG3 functions as ceRNA to regulate the gastric carcinogenesis through the MEG3-miR-181-Bcl2 axis [40]. So we conjectured that MEG3 may also act as a ceRNA of the target gene by competing for miRNA-181a binding sites and thereby regulate the mRNA expression of the target gene in MM. The preceding studies have reported that miR-181a is in high expression in several types of cancer and pertinent to the poor clinical outcome of patients [41-43]. Consistent with our findings, miR-181a is upregulated in MM, and is negatively correlated with MEG3 expression. To verify this hypothesis, we used the bioinformatics tools to analysis the mechanism of MEG3 as a ceRNA. Furthermore, Dual-Luciferase reporter assay combined with RNA binding protein immunoprecipitation assay provided further support for the interaction of MEG3-miR-181a activity. MEG3 could negatively control endogenous miR-181a and reverse the phenotype created by miR-181a. This result implied us to investigate the miRNA-associated target gene feature of MEG3 in MM nosogenesis.

HOXA11, a subtype of HOX gene family (cluster A, B, C, D)，is considered as a tumor suppressor. With the analysis of several bioinformatic tools, we discovered HOXA11 as the final object of the competition between MEG3 and miR-181a. To investigate whether HOXA11 was the target mRNA of miR-181a in MM, we especially focused on the expression of HOXA11 with miR-181a mimics treatment. Here, we showed that HOXA11 expression could also be reduced by miR-181a mimics treatment. To further confirm the effect of HOXA11 as a miRNA-181a "sponge" and verify whether MEG3 regulates HOXA11 expression through miR-181a, we observed that over-expression of MEG3 leads to increase HOXA11 protein levels, whereas over-expression of miR-181a restores HOXA11 synthesis to low levels in MM cells. Additionally, the expression of MEG3 was positively correlated with the expression of HOXA11 in MM cell lines as well as in publicly data sets. Taken together, these data affirmed that lncRNA MEG3 serves as a ceRNA to mediate HOXA11 expression for inhibiting MM cells proliferation and promoting apoptosis by miR-181a "sponge".

Our current study highlights that MEG3 acts as a tumor suppressor in MM through MEG3-miR-181a-HOXA11 pathway, which allows us to better understand the pathogenesis and development of MM and might shed a light for the evolution of MEG3-directed novel clinical treatment against MM. Novel therapeutic regimen target on LncRNA-miRNA- mRNA network may be a potential strategy to reverse multiple myeloma progression.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (81372540, 81670199), Jiangsu Province's Medical Elite Program (ZDRCA2016015).

Disclosure Statement

No conflict of interests exists.

References

1 Caers J, Vande broek I, De Raeve H, Michaux L, Trullemans F, Schots R, Van Camp B, Vanderkerken K: Multiple myeloma--an update on diagnosis and treatment. Eur J Haematol 2008;81:329-343.
2 Rollig C, Knop S, Bornhauser M: Multiple myeloma. Lancet 2015;385:2197-2208.
3 Amodio N, Di Martino MT, Neri A, Tagliaferri P, Tassone P: Non-coding RNA: a novel opportunity for the personalized treatment of multiple myeloma. Expert Opin Biol Ther 2013;13:S125-137.
4 Xu TP, Huang MD, Xia R, Liu XX, Sun M, Yin L, Chen WM, Han L, Zhang EB, Kong R, De W, Shu YQ: Decreased expression of the long non-coding RNA FENDRR is associated with poor prognosis in gastric cancer and FENDRR regulates gastric cancer cell metastasis by affecting fibronectin1 expression. J Hematol Oncol 2014;7:63.
5 Fan Y, Wang YF, Su HF, Fang N, Zou C, Li WF, Fei ZH: Decreased expression of the long noncoding RNA LINC00261 indicate poor prognosis in gastric cancer and suppress gastric cancer metastasis by affecting the epithelial-mesenchymal transition. J Hematol Oncol 2016;9:57.
6 Tang L, Chen HY, Hao NB, Tang B, Guo H, Yong X, Dong H, Yang SM: microRNA inhibitors: Natural and artificial sequestration of microRNA. Cancer Lett 2017;407:139-147.
7 Wang WT, Ye H, Wei PP, Han BW, He B, Chen ZH, Chen YQ: LncRNAs H19 and HULC, activated by oxidative stress, promote cell migration and invasion in cholangiocarcinoma through a ceRNA manner. J Hematol Oncol 2016;9:117.
8 Liang WC, Fu WM, Wong CW, Wang Y, Wang WM, Hu GX, Zhang L, Xiao LJ, Wan DC, Zhang JF, Waye MM: The lncRNA H19 promotes epithelial to mesenchymal transition by functioning as miRNA sponges in colorectal cancer. Oncotarget 2015;6:22513-22525.
9 Liu XH, Sun M, Nie FQ, Ge YB, Zhang EB, Yin DD, Kong R, Xia R, Lu KH, Li JH, De W, Wang KM, Wang ZX: Lnc RNA HOTAIR functions as a competing endogenous RNA to regulate HER2 expression by sponging miR-331-3p in gastric cancer. Mol Cancer 2014;13:1476-4598.
10 Wang SH, Wu XC, Zhang MD, Weng MZ, Zhou D, Quan ZW: Long noncoding RNA H19 contributes to gallbladder cancer cell proliferation by modulated miR-194-5p targeting AKT2. Tumour Biol 2016;37:9721-9730.
11 Zhang S, Dong X, Ji T, Chen G, Shan L: Long non-coding RNA UCA1 promotes cell progression by acting as a competing endogenous RNA of ATF2 in prostate cancer. Am J Transl Res 2017;9:366-375.
12 Zhou Y, Zhang X, Klibanski A: MEG3 noncoding RNA: a tumor suppressor. J Mol Endocrinol 2012;48:R45-53.
13 Benetatos L, Vartholomatos G, Hatzimichael E: MEG3 imprinted gene contribution in tumorigenesis. Int J Cancer 2011;129:773-779.
14 Lu KH, Li W, Liu XH, Sun M, Zhang ML, Wu WQ, Xie WP, Hou YY: Long non-coding RNA MEG3 inhibits NSCLC cells proliferation and induces apoptosis by affecting p53 expression. BMC Cancer 2013;13:461.
15 Zhou Y, Zhong Y, Wang Y, Zhang X, Batista DL, Gejman R, Ansell PJ, Zhao J, Weng C, Klibanski A: Activation of p53 by MEG3 non-coding RNA. J Biol Chem 2007;282:24731-24742.
16 Sun Z, Nie X, Sun S, Dong S, Yuan C, Li Y, Xiao B, Jie D, Liu Y: Long Non-Coding RNA MEG3 Downregulation Triggers Human Pulmonary Artery Smooth Muscle Cell Proliferation and Migration via the p53 Signaling Pathway. Cell Physiol Biochem 2017;42:2569-2581.
17 Greife A, Knievel J, Ribarska T, Niegisch G, Schulz WA: Concomitant downregulation of the imprinted genes DLK1 and MEG3 at 14q32.2 by epigenetic mechanisms in urothelial carcinoma. Clin Epigenetics 2014;6:29.
18 Yin DD, Liu ZJ, Zhang E, Kong R, Zhang ZH, Guo RH: Decreased expression of long noncoding RNA MEG3 affects cell proliferation and predicts a poor prognosis in patients with colorectal cancer. Tumour Biol 2015;36:4851-4859.
19 Wang Y, Liu Z, Yao B, Dou C, Xu M, Xue Y, Ding L, Jia Y, Zhang H, Li Q, Tu K, Jiao Y, Liu Q, Guo C: Long non-coding RNA TUSC7 acts a molecular sponge for miR-10a and suppresses EMT in hepatocellular carcinoma. Tumour Biol 2016;37:11429-11441.

20 Modali SD, Parekh VI, Kebebew E, Agarwal SK: Epigenetic regulation of the lncRNA MEG3 and its target c-MET in pancreatic neuroendocrine tumors. Mol Endocrinol 2015;29:224-237.
21 Lehner B, Kunz P, Saehr H, Fellenberg J: Epigenetic silencing of genes and microRNAs within the imprinted Dlk1-Dio3 region at human chromosome 14.32 in giant cell tumor of bone. BMC Cancer 2014;14:495.
22 Benetatos L, Dasoula A, Hatzimichael E, Georgiou I, Syrrou M, Bourantas KL: Promoter hypermethylation of the MEG3 (DLK1/MEG3) imprinted gene in multiple myeloma. Clin Lymphoma Myeloma 2008;8:171-175.
23 Zhuang W, Ge X, Yang S, Huang M, Zhuang W, Chen P, Zhang X, Fu J, Qu J, Li B: Upregulation of lncRNA MEG3 Promotes Osteogenic Differentiation of Mesenchymal Stem Cells From Multiple Myeloma Patients By Targeting BMP4 Transcription. Stem Cells 2015;33:1985-1997.
24 Shi X, Sun M, Liu H, Yao Y, Song Y: Long non-coding RNAs: a new frontier in the study of human diseases. Cancer Lett 2013;339:159-166.
25 Zhang YY, Feng HM: MEG3 Suppresses Human Pancreatic Neuroendocrine Tumor Cells Growth and Metastasis by Down-Regulation of Mir-183. Cell Physiol Biochem 2017;44:345-356.
26 Han L, Dong Z, Liu N, Xie F, Wang N: Maternally Expressed Gene 3 (MEG3) Enhances PC12 Cell Hypoxia Injury by Targeting MiR-147. Cell Physiol Biochem 2017;43:2457-2469.
27 Chen G, Shen ZL, Wang L, Lv CY, Huang XE, Zhou RP: Hsa-miR-181a-5p expression and effects on cell proliferation in gastric cancer. Asian Pac J Cancer Prev 2013;14:3871-3875.
28 Sun X, Charbonneau C, Wei L, Chen Q, Terek RM: miR-181a Targets RGS16 to Promote Chondrosarcoma Growth, Angiogenesis, and Metastasis. Mol Cancer Res 2015;13:1347-1357.
29 Qu J, Li M, Zhong W, Hu C: Competing endogenous RNA in cancer: a new pattern of gene expression regulation. Int J Clin Exp Med 2015;8:17110-17116.
30 Shah N, Sukumar S: The Hox genes and their roles in oncogenesis. Nat Rev Cancer 2010;10:361-371.
31 Bai Y, Fang N, Gu T, Kang Y, Wu J, Yang D, Zhang H, Suo Z, Ji S: HOXA11 gene is hypermethylation and aberrant expression in gastric cancer. Cancer Cell Int 2014;14:79.
32 Wang L, Cui Y, Sheng J, Yang Y, Kuang G, Fan Y, Jin J, Zhang Q: Epigenetic inactivation of HOXA11, a novel functional tumor suppressor for renal cell carcinoma, is associated with RCC TNM classification. Oncotarget 2017;8:21861-21870.
33 Cui Y, Gao D, Linghu E, Zhan Q, Chen R, Brock MV, Herman JG, Guo M: Epigenetic changes and functional study of HOXA11 in human gastric cancer. Epigenomics 2015;7:201-213.
34 Naguibneva I, Ameyar-Zazoua M, Polesskaya A, Ait-Si-Ali S, Groisman R, Souidi M, Cuvellier S, Harel-Bellan A: The microRNA miR-181 targets the homeobox protein Hox-A11 during mammalian myoblast differentiation. Nat Cell Biol 2006;8:278-284.
35 Sun M, Ding J, Li D, Yang G, Cheng Z, Zhu Q: NUDT21 regulates 3'-UTR length and microRNA-mediated gene silencing in hepatocellular carcinoma. Cancer Lett 2017;410:158-168.
36 Zhou M, Zhao H, Wang Z, Cheng L, Yang L, Shi H, Yang H, Sun J: Identification and validation of potential prognostic lncRNA biomarkers for predicting survival in patients with multiple myeloma. J Exp Clin Cancer Res 2015;34:102.
37 Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP: A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Nature 2010;465:1033-1038.
38 Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP: A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? Cell 2011;146:353-358.
39 Wang F, Ying HQ, He BS, Pan YQ, Deng QW, Sun HL, Chen J, Liu X, Wang SK: Upregulated lncRNA-UCA1 contributes to progression of hepatocellular carcinoma through inhibition of miR-216b and activation of FGFR1/ERK signaling pathway. Oncotarget 2015;6:7899-7917.
40 Peng W, Si S, Zhang Q, Li C, Zhao F, Wang F, Yu J, Ma R: Long non-coding RNA MEG3 functions as a competing endogenous RNA to regulate gastric cancer progression. J Exp Clin Cancer Res 2015;34:79.
41 Gao W, Yu Y, Cao H, Shen H, Li X, Pan S, Shu Y: Deregulated expression of miR-21, miR-143 and miR-181a in non small cell lung cancer is related to clinicopathologic characteristics or patient prognosis. Biomed Pharmacother 2010;64:399-408.
42 Jiao X, Zhao L, Ma M, Bai X, He M, Yan Y, Wang Y, Chen Q, Zhao X, Zhou M, Cui Z, Zheng Z, Wang E, Wei M: MiR-181a enhances drug sensitivity in mitoxantone-resistant breast cancer cells by targeting breast cancer resistance protein (BCRP/ABCG2). Breast Cancer Res Treat 2013;139:717-730.
43 Taylor MA, Sossey-Alaoui K, Thompson CL, Danielpour D, Schiemann WP: TGF-beta upregulates miR-181a expression to promote breast cancer metastasis. J Clin Invest 2013;123:150-163.