Tumor-associated macrophages secret exosomal miR-155 to promote metastasis of non-small-cell lung cancer

Xiang Li  
Jiangsu Province people's Hospital and the First Affiliated Hospital of Nanjing Medical University

Yaojun Ni  
Jiangsu Province People's Hospital and the First Affiliated Hospital of Nanjing Medical University

Zhipeng Chen  
Jiangsu Province People's Hospital and the First Affiliated Hospital of Nanjing Medical University

Quan Zhu  
Jiangsu Province People's Hospital and the First Affiliated Hospital of Nanjing Medical University

Weibing Wu  
Jiangsu Province People's Hospital and the First Affiliated Hospital of Nanjing Medical University

Zhicheng He  
Jiangsu Province People's Hospital and the First Affiliated Hospital of Nanjing Medical University

Yang Xia  
Jiangsu Province People's Hospital and the First Affiliated Hospital of Nanjing Medical Univeristy

Tong Zhou  
University of Nevada School of Medicine

Liang Chen  
Jiangsu Province People's Hospital and the First Affiliated Hospital of Nanjing Medical University

Xiao Xiao  
Nanjing University

Xueying Xie  
Southeast University

Jun Wang  
wangjun_01@126.com  
Jiangsu province people's hospital and the first affiliated hospital of nanjing medical university

Research

**Keywords:** non-small-cell lung cancer, miR-155

**Posted Date:** April 10th, 2020

**DOI:** https://doi.org/10.21203/rs.3.rs-22030/v1
Abstract

Background: Understanding the molecular basis underlying metastasis of non-small-cell lung cancer (NSCLC) may provide new therapeutic modality for treatment of NSCLC. However, the mechanisms by which tumor-associated macrophages (TAMs) affect NSCLC metastasis still remain undefined.

Method: Phenotype of TAMs was identified by flow cytometry. The migration of the tumor cells was detected by transwell assay. Transmission electron microscopy (TEM) and PKH-67 was used to identified and label the exosome. Expression of miR-155 was measured by qRT-PCR. Luciferase analysis, western blot, and rescue assay were used to investigate potential mechanisms of miR-155.

Results: We discovered a novel regulatory pathway involved in NSCLC metastasis. We found that M2 TAMs were the main TAMs in metastatic tissues of NSCLC patients and exosomes derived from M2 TAMs were able to promote epithelial-mesenchymal transition (EMT) and migration of NSCLC cells. We demonstrated that miR-155 was abundant in M2 TAMs and exosomes secreted by M2 TAMs. Moreover, we also verified that miR-155 was the key functional biomolecule in exosomes secreted by M2 TAMs. Furthermore, we confirmed that deletion of miR-155 in M2 TAMs could significantly prevent NSCLC metastasis. Overall,

Conclusions: we revealed a new regulatory pathway that is M2 TAMs secret exosomal miR-155 to promote NSCLC metastasis. Our findings may provide a practical target for treatment of NSCLC.

Background

According to global cancer statistics 2018, lung cancer accounts for ~ 12% of newly diagnosed cancer cases and ~ 18% of total cancer deaths[1]. About 85% of diagnosed lung cancers are non-small-cell lung cancers (NSCLCs), which has a low 5-year survival rate (< 15%).[2, 3] Although timely treatments such as surgery, chemotherapy and radiotherapy can prolong the overall survival of patients, NSCLC is still the most fatal disease. The causes for lung cancer death vary and metastasis is a predominant factor.[4] Therefore, understanding the molecular basis of NSCLC metastasis is critical for proposing new therapeutic approaches for its treatment.[5, 6] Several classes of biomolecules, such as microRNAs (miRNAs), matrix metalloproteinases and transcription regulatory protein BACH1, have been well studied in the metastasis of NSCLC.[5, 7–11] Although tumor microenvironment is also known to mediate NSCLC metastasis, the role of key components of tumor microenvironment in NSCLC metastasis has not been fully elucidated.[12, 13]

Tumor microenvironment consists of cancer cells and stromal cells that include macrophages, endothelial cells and fibroblasts.[14–16] Moreover, products of these cells, such as cytokines, growth factors and enzymes, are also present in the tumor microenvironment. Among these components in tumor microenvironment, tumor-associated macrophages (TAMs) that are a class of immune cells are pivotal orchestrators.[17] TAMs have two populations, M1 and M2 types. It has been found that M1 TAMs have an anti-tumor function, whereas M2 TAMs can promote tumor metastasis.[18] For NSCLC, M1 TAMs
can suppress the angiogenesis and is positively related with the survival time of NSCLC patients.

In contrast, M2 TAMs is found to promote NSCLC metastasis through activating the epithelial-mesenchymal transition (EMT) of NSCLC cells and lung cancer cell invasion. However, the detailed mechanisms by which M2 TAMs promote NSCLC metastasis still remain elusive.

Exosomes are small vesicles secreted by parental cells into extracellular microenvironment to mediate cross-talk between parental cells and target cells. Almost all cell types have the capacity to release exosomes and the size of exosomes is ~ 30–150 nm in diameter. To mediate cell-cell communication, exosomes deliver proteins, miRNAs or mRNAs into target cells to change gene expression. For instance, gastric cancer cells secret exosomes carrying miR-21 into peritoneal mesothelial cells to promote peritoneal metastasis. For the cross-talk between cancer cells and TAMs, exosomes carrying miRNAs are also key players. Taking an example, ovarian cancer cells secret exosomes containing miR-222 to induce M2 polarization of TAMs. Moreover, M2 TAMs also can release exosomes carrying miR-501 to promote progression of pancreatic cancer. Nevertheless, whether M2 TAMs promote NSCLC metastasis through exosomes and miRNAs still remains unclear.

Here, we report that M2 TAMs promote EMT of NSCLC cells through secreting exosomes. miR-155 is the key functional molecule in M2 TAMs-released exosomes that promote EMT of NSCLC cells through targeting Ras association domain family member 4 (RASSF4).

**Materials And Methods**

**Cell lines and Cell culture**

The A549 cells were obtained from Chinese Academy of Sciences, Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). A549/Luc cells (A549 cells stably expressing luciferase) were constructed by Synthgene (Nanjing, China). Cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) (Gibco, Rockville, USA) supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml streptomycin and penicillin (Gibco, Rockville, USA) in a humidified atmosphere at 37°C.

**Immunohistochemistry Assay**

The immunohistochemistry assay was performed according to Yin et al [26]. The sections were incubated with the primary antibody of CD68 (Ab955, 1: 100, Abcam, Cambridge, UK) at 4 °C overnight and horseradish peroxidase labeled goat anti-mouse IgG antibody (A205719, 1: 200, Abcam, Cambridge, UK) at room temperature for 1 h. The Color reaction was performed with diaminobenzidine chromogen solution (Dako, Carpinteria, USA). Brown-yellow particles represented the positive expression of CD68 protein and blue particles represented the nucleus stained by hematoxylin (Sigma, USA).

**Rna Extraction And Quantitative Real-time Pcr Analysis**
The extraction and reverse transcription of total RNA were performed according to the previous report [27]. The expression levels of TNF-α, IRF5, IRF4, Arg-1 and miR-155 were analysed by quantitative real-time PCR with the GAPDH gene as a standard control. Primers of TNF-α, IRF5, IRF4, Arg-1 and miR-155 were as follows: TNF-α (Forward: 5'- CCTCTCTCTAATCGGCCTCTG-3'; Reverse: 5'-GAGGACCTGGGAGTAGATGAG-3'); IRF5 (Forward: 5'-GGGCTTCAATCGGCCTCTG-3'; Reverse: 5'-GCCTCGGTATATTCCCTG-3'); IRF4 (Forward: 5'-GCTGATCGACCAGATGAG-3'; Reverse: 5'-CGGTTGTAGCCCTGACG-3'); Arg-1 (Forward: 5'-GTGGAAACTTGCTGCATGGCAG-3'; Reverse: 5'-AATCCCTGGCAGACCTGGGAAT-3'); miR-155 (Forward: 5'-GGAGGTTAATGCTAATCAG-3'; Reverse: 5'-GTCGCCGCTGGTCAGG-3')

**Cell Migration And Invasion Assays**

The cell invasion and migration assays were performed by Chamber matrigel invasion 24-well DI kit (BD Biosciences, San Jose, CA). Specifically, A549 cells as the control, A549 cells co-cultured with M2 macrophages treated with or without 5 µM GW4869 and A549 cells co-cultured with exosome from M0/M2 macrophages or M2 macrophages transfected with different plasmids, at a density of 2 × 10^4 cells/ml, were re-suspended with 200 µl DMEM medium (serum-free) and seeded into the upper chamber, while the lower chamber was placed with 600 µl complete DMEM medium (10% FBS). For cell invasion, A549 cells cocultured with exosome of M0 or M2 macrophages were added to the lower chamber, and the medium with 10% FBS was placed in the upper chamber. After incubation for 24 h, the invaded or migrated A549 cells were fixed with the methanol (100%), stained with crystal violet (0.1 mg/mL) and counted under a microscope.

**Isolation, Identification And Labeling Of Exosomes**

The exosomes were isolated by density gradient ultracentrifugation according to previously reported protocol [28]. Briefly, cell culture medium was collected and centrifuged at 1,000 g for 10 min, 2,000 g for 20 min, 4,000 g for 30 min and 10,000 g for 1 h, with the supernatant being retained each time. The exosomes were collected by centrifuging the samples at 100,000 g for at least 2 h at 4 ºC. Size distribution and concentration of exosomes were analysed at a flow rate of 0.03 ml per min using a Zetasizer Nano ZS (Malvern Instrument, UK) and NanoSight NS300 (Westborough, MA, USA), respectively. Purified exosomes were labeled with the PKH-67 green fluorescent linker Mini Kit (Sigma, USA) according to the manufacturer's instructions.

**Western Blot Analysis**

The radio-immunoprecipitation assay (RIPA) lysis buffer (Solarbio, Shanghai, China) with 0.5% phenylmethanesulfonyl fluoride (PMSF) (Solarbio, Shanghai, China) was used to extract the total protein of exosome, cell or tissue. The protein concentration was quantified by the BCA protein quantification Kit (Sigma, USA). The primary antibodies in this study were purchased from Abcam: rabbit anti-CD63
(ab68418, 1: 500), CD81 (ab109201, 1: 200), RASSF4 (ab243709, 1: 1000) and TSG101 (ab30871, 1: 500) and mouse anti-E-cadherin (ab11512, 1: 1000), vimentin (ab8978, 1: 1000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab8245, 1: 5000). The horseradish peroxidase labeled goat anti-rabbit IgG antibody (ab205718, 1: 10000) and goat anti-mouse antibody (ab6789, 1: 10000) were available as the secondary antibodies. Image J software was used to quantify each protein band.

Detection Of Cy3-labeled Mir-155 Exosome Transfer

The A549 cells were co-cultured with Cy3-labeled miR-155 exosome or free Cy3-labeled miR-155 for 24 h to confirm the transfer of miR-155 by exosomes. Then, the cells were washed with PBS and were incubated with Hoechest33342 at room temperature. Images were obtained by a confocal microscope.

Luciferase Reporter Assay

The 3'-UTR segments of RASSF4 in wild type and mutant were synthesized and inserted into a firefly luciferase reporter construct. Luciferase activity in this study was measured by the Dual Luciferase Reporter Assay System (Promega, USA) according to the protocol.

Animal Studies

6-week-old male athymic BALB/c nude mice were used. For the in vivo lung metastases model, A549/Luc cells were injected into the tail vein of representative mice (n = 5 per group). The luciferase signal intensity from days 0 to 28 is on equivalent scales in the models. Bioluminescent flux (photons/s/cm$^2$/steradian) was determined for the lung metastases. Metastatic progression was monitored and imaged using an IVIS-100 system (Caliper Life Sciences, MA, USA) 10 min after intraperitoneal injection of luciferin (300 mg/kg i.v.) in 80 µl of saline. After 28 days, mice were sacrificed and tissues were separated for further experiments. Animal care and euthanasia were carried out with the approval of the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University.

Hematoxylin & eosin (HE) staining

The dewaxed sections were firstly incubated with hematoxylin to stain the nucleus for 5 min, then 1% ethanol-hydrochloric acid for 30 s and eosin solution for 3 min. Finally, the sections were dehydrated in graded alcohol following by clearing in xylene.

Statistical analysis

All statistical analyses were performed by GraphPad Prism 6.0 software. The Student’s t-test was used to analyze significant differences in this study. The error bars indicate the standard deviation from the mean.
of triplicate measurements. Asterisks indicate significant differences (*P < 0.05; **P < 0.01; ***P < 0.001) compared with the corresponding control.

Results

M2 TAMs are abundant in metastatic tissues of NSCLC patients

Initially, we observed that CD68, a macrophage marker, was significantly increased in NSCLC tissues of metastatic patients compared with normal lung tissues through the immunohistochemical analysis in Fig. 1A and 1B, suggesting TAMs recruitment related to the metastasis of NSCLC. Further analysis by qRT-PCR in Fig. 1C, the transcription expressions of M1 marker such as tumor necrosis factor alpha (TNF-α) and interferon regulatory factor 5 (IRF5) had no significant changes in all NSCLC samples in this study; however, the signatures of M2 macrophages like interferon regulatory factor 4 (IRF4) and arginase-1 (Arg-1) were up-regulated in metastatic NSCLC tissues compared with localized tissues. Interestingly, we observed that the tissues derived from the metastatic patients showed the high expression of miR-155 in Fig. 1D.

Exosomes secreted by M2 TAMs promote EMT and migration of A549 cells

The macrophages have been extensively reported to regulate a series of biological processes of NSCLC through miRNAs carried by exosomes. To evaluate whether the metastasis effect of macrophages on NSCLC cells was through exosomes, we treated A549 cells with M2 macrophages or an inhibitor of exosome release GW4869. In Fig. 2A and 2B, we found that the migrating number of A549 cells was significantly promoted through M2 macrophages compared with the control. In addition, the migration ability promoted by M2 macrophages were attenuated when A549 cells simultaneously treated with GW4869; however, the migrating number of control cells pretreated by 5 µM of GW4869 had no significant changes, suggesting that exosomes derived from M2 macrophage cells play an essential role in the metastasis of non-small-cell lung cancer.

Subsequently, a large of exosomes were found in the supernatant of M2 macrophage cell culture by transmission electron microscope and the shape and size of exosomes was solid with typical structure of two-layer membrane and an average diameter of 100 nm as shown in Fig. 2C and 2D. Western blot analysis in Fig. 2E and 2F showed that the protein levels of exosomal markers TSG101, CD63 and CD81 had a significant increase in exosome compared with cell lysis, confirming the successful extraction of exosomes. Next, A549 cells co-cultured with PKH67-labeled M2 exosomes for 48 h exhibited the green fluorescence, confirming the exosomes derived from M2 macrophages can be uptaken by A549 cells. Furthermore, after 48 h of co-culture of A549 cells with M0 exosome or M2 exosome in vitro, transwell assay and western blot analysis were applied to examine whether exosomes generated form M2
macrophages affected the cell migration and invasion. The results in Fig. 2H and 2I revealed that invasiveness and migration ability of A549 cells co-cultured with exosomes secreted from M2 macrophages were strengthen compared with that co-cultured with M0 exosomes. Moreover, the protein expression levels of epithelial cell marker (E-cadherin) decreased and the expression levels of mesenchymal cell marker (vimentin) increased in A549 cells co-cultured with M2 exosomes in Fig. 2J and 2K, which clarified that exosomes secreted from M2 macrophages can promote EMT, migration and invasion of A549 cells.

**miR-155 is the key biomolecule in exosomes secreted by M2 TAMs**

We previously found that miR-155 was highly expressed in NSCLC tissue of metastatic patients in Fig. 1D. As shown in Fig. 2A and 2B, we further confirmed that the miR-155 expression was much higher in M2 cell lysis and M2 exosome as compared to M0 cell lysis and M0 exosome, respectively. To determine whether the elevated expression of miR-155 was resulted from the exosomes of M2 macrophages, we transiently transfected the Cy3-labeled miR-155 exosome or negative control to A549 cells. The fluorescence signals could be detected in A549 cells incubated with M2 exosomes isolated from Cy3-labeled miR-155 transfected cells in Fig. 3C. In addition, we found that the exosome with miR-155 overexpression promoted the migration of A549 cells in Fig. 3D and 3E, as well as the levels of vimentin, while inhibiting E-cadherin expression in Fig. 3F. Taken together, these data suggest that miR-155 carried by exosomes derived from M2 macrophages mediated a series of biological processes of NSCLC cells.

**The direct target of exosomal miR-155 in NSCLC is RASSF4**

The prediction of TargetScan Release 7.0 database showed RASSF4 in 3’-untranslated regions (UTRs) possesses putative binding sites for miR-155 in Fig. 4A. Subsequently, the luciferase reporter plasmids containing the WT (wild type) or mutation-type of 3’-UTR of RASSF4 were successfully transfected into A549 cells. As shown in Fig. 4B, the luciferase activity transfected with WT RASSF4 is significantly decreased compared with the treated with NC-plasmid. The response of RASSF4 to miR-155 overexpression or miR-155 inhibition was examined by Western blotting analysis. As shown in Fig. 4E and 4F, the protein levels of RASSF4 and epithelial cell marker (E-cadherin) were markedly down-regulated and the expression levels of vimentin increased when miR-155 was overexpressed. Western blot analysis of epithelial cell marker, mesenchymal cell marker and RASSF4 and transwell assay revealed that the significant down-regulation in the expression of RASSF4 and E-cadherin, up-expression of vimentin and the increase of invasion abilities resulting from miR-155 overexpression can be reversed when RASSF4 was overexpressed. Correspondingly, promoted expression of RASSF4 and E-cadherin by miR-155 knockdown in A549 cells were alleviated by down-regulated RASSF4. In addition, the down-expressed RASSF4 could reverse the effects of miR-155 knockdown on A549 cells with inhibited protein levels of vimentin and decrease of invasion abilities in Fig. 4E-4H. Collectively, our findings confirmed that RASSF4 is the direct target of exosomal miR-155 in NSCLC.
M2 TAMs secret exosomal miR-155 to promote NSCLC metastasis in vivo

To assess the effect of miR-155 carried by exosomes from M2 macrophages on lung metastasis in vivo, M0 exosome, M2 exosome and M2/miR-155 knockdown exosome were injected intravenously respectively after intravenous injection of A549/Luc cells into mice to construct lung metastasis model. In Fig. 5A, luciferase activity of M2 exosome group was significantly higher than that of M0 exosome group in 21 days and 28 days, while the high expression of luciferase activity in M2 exosome group could be effectively decreased through the knockdown of miR-155. Next, HE staining from tumor tissues of each group in Fig. 5B showed that M2-derived exosomes with the knockdown of miR-155 markedly contributed to alleviate the metastasis of NSCLC resulting from M2 exosome. In addition, the transcription expression of miR-155 in M2 exosome group was significantly up-regulated by approximately 18-fold compared to M0 exosome group or M2/miR-155 knockdown exosome group in Fig. 5C. Western blot analysis from tumor tissues of each group in Fig. 5D revealed that the protein levels of RASSF4 and E-cadherin were significantly down-regulated in M2/miR-155 knockdown exosome group compared to M2 exosome group, while the opposite pattern of vimentin be observed. In addition, these results in Fig. 5E revealed that the exosomes from M2 macrophages suppresses RASSF4 protein expression. In general, our findings reveal that NSCLC metastasis can be promoted by exosomal miR-155 secreted from M2 TAMs in vivo.

Discussion

Due to the metastasis to bone, brain and liver, poor prognosis is common in lung cancer patients, resulting in high mortality. Although various biomolecules have been found to mediate lung cancer metastasis, knowledge regarding to roles of tumor microenvironment in lung cancer metastasis has largely lagged behind. Stromal cells including TAMs in the tumor microenvironment are either induced or directly interacting with cancer cells to promote the progression and metastasis of lung cancer. The modes of action of stromal cells including the secretion of extracellular components, such as growth factors, chemokines and exosomes. However, due to the extreme complexity of these processes, the molecular mechanisms underlying lung metastasis has not been fully understood.

Previous reports have indicated that M2 TAMs can promote NSCLC metastasis. Nevertheless, the exact mechanisms by which M2 TAMs promote NSCLC metastasis remain undefined. In this paper, we demonstrated that M2 TAMs secrets exosomal miR-155 to promote EMT and migration of NSCLC cells. miR-155 is previously identified as an oncogenic miRNA in various cancers, such as breast cancer, ovarian cancer and lung cancer. In our work, we observed that miR-155 was up-regulated in both M2 TAMs and exosomes secreted by M2 TAMs. Moreover, the function of exosomes secreted by M2 TAMs in NSCLC metastasis was mediated by miR-155, which modulated RASSF4 in NSCLC cells. miRNAs are important gene regulators, which control various physiological and pathological processes.
roles of miRNAs in NSCLC metastasis has also been discovered.[10, 36] Therefore, targeting certain miRNAs involved in NSCLC has been considered as an efficient therapeutic approach.[37]

Inhibiting miRNA-enriched exosomes from M2 TAMs has been found to be useful for treating cancer metastasis.[38, 39] In our work, we found that inhibiting exosomal miR-155 could alleviate NSCLC metastasis activated by exosomes derived from M2 TAMs. Therefore, targeting exosomal miR-155 from M2 TAMs may be a potential therapeutic strategy for NSCLC metastasis. Moreover, previous researches have also indicated that miRNA can control polarization of TAMs.[40] Whether miR-155 is involved in the M2 polarization of TAMs deserve further investigation, which is now underway in our group. The use of therapeutic drugs to target miR-155 in TAMs may also promote M1 polarization of TAMs to inhibit tumor growth.

**Conclusion**

Overall, this study showed that M2 TAMs were the main population of TAMs in metastatic tissues of NSCLC and promoted NSCLC metastasis. The cross-talk between M2 TAMs and lung cancer cells was mediated by exosomes that carry miR-155. miR-155, which was abundant in both M2 TAMs and exosomes from M2 TAMs, promoted EMT and migration of NSCLC through targeting RASSF4. Inhibition of miR-155 in M2 TAMs could prevent NSCLC metastasis. These findings suggest that miR-155 in TAMs and exosomes may serve as a novel therapeutic target in the treatment of lung cancer.

**Declarations**

*Ethics approval and consent to participate*

All procedures involving human participants were performed in accordance with the ethical standards of the Institutional and National Research Committee, and with the Declaration of Helsinki. Ethical approval was obtained from the Ethical Committee of Jiangsu Province People's Hospital and the First Affiliated Hospital of Nanjing Medical University. Verbal informed consent was obtained from all patients included in the present study.

*Consent for publication*

All authors have agreed to publish this manuscript.

*Availability of data and material*

The datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

*Competing interests*

The authors declare that they have no competing interests.
Funding

This work was supported in part by Clinical Research Cultivation Program (2017CX010), and Social Development Foundation of Jiangsu Province - Clinical Frontier Technology (BE2018746). This work was also supported by the start-up grants provided by the University of Nevada to TZ.

Authors' contributions

XL, LC, and JW conceived this study. XX, YX, and TZ performed the data analysis. JW, WW, QZ, LC, YX, and TZ interpret the results. JW, XL, YN, ZC, and XX wrote the manuscript. All authors contributed to the final version of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

References


**Figures**
M2 TAMs are abundant in metastatic tissues of NSCLC patients. A-B. Immunohistochemical analysis of CD68 expression and quantification of TAMs recruitment in NSCLC tissues from metastatic patients and normal persons; C. Quantitative real-time PCR analysis of TNF-α, IRF5, IRF4 and Arg-1 expression in NSCLC tissues from metastatic patients and normal persons, respectively; D. Quantitative real-time PCR analysis of miR-155 expression in NSCLC tissues from metastatic patients and normal persons. Error bars represent standard deviations and asterisks show significant differences from corresponding control according to Student’s t test (**P < 0.01, ***P < 0.001)
Figure 2

Exosomes secreted by M2 TAMs promote EMT and migration of A549 cells. A-B. The migration abilities of A549 cells treated with GW4869, M2 macrophages or M2 macrophages + GW4869 were measured by transwell assay. C. The structure of exosomes was identified by transmission electron microscope. D. The size of exosomes was detected by nanoparticle tracking analysis. E-F. The protein levels of exosomal markers TSG101, CD63 and CD81 in exosome and cell lysis were analysed by Western blot. G. The fluorescence from A549 cells co-cultured with PKH67-labeled M2 exosomes or without co-culture (control) were detected. H-I. The migration and invasive ability of A549 cells after M0 exosome or M2 exosome treatment in vitro was examined by transwell assay. J-K. The protein expression levels of epithelial cell marker (E-cadherin) and mesenchymal cell marker (vimentin) in A549 cells after M0 exosome or M2 exosome treatment were analysed by Western blot. Error bars represent standard deviations and asterisks show significant differences from corresponding control according to Student's t test (***P < 0.001)
miR-155 is the key biomolecule in exosomes secreted by M2 TAMs. A. Quantitative real-time PCR analysis of miR-155 expression in M0 and M2 cell lysis, respectively. B. Quantitative real-time PCR analysis of miR-155 expression in M0 and M2 exosome, respectively. C. The fluorescence signals were detected in A549 cells incubated with M2 exosomes isolated from Cy3-labeled miR-155 transfected cells or free Cy3-labeled miR-155. D-E. The migration abilities were measured by transwell assay in A549 cells treated with M2/NC exosome, M2/miR-155 overexpression exosome or M2/miR-155 knockdown exosome. F. The protein expression levels of E-cadherin and vimentin were analysed by Western blot in A549 cells treated with M2/NC exosome, M2/miR-155 overexpression exosome or M2/miR-155 knockdown exosome. Error bars represent standard deviations and asterisks show significant differences from corresponding control according to Student's t test (***P < 0.001)
Figure 4

The direct target of exosomal miR-155 in NSCLC is RASSF4. A. The putative binding sites of RASSF4 for miR-155 by TargetScan Release 7.0 database. B. The luciferase activity in A549 transfected with wild-type or mutated 3'UTR of RASSF4 were measured. C-D. The protein expression levels of RASSF4, E-cadherin and vimentin in A549 cells transfected with the plasmids of negative control (NC), miR-155 overexpression (miR-155 OE) or miR-155 knockdown (miR-155 KD) were analysed by Western blot. E-F. The protein expression levels of RASSF4, E-cadherin and vimentin were analysed by Western blot in A549 cells transfected with the plasmids of negative control (NC), miR-155 overexpression (miR-155 OE), miR-155 overexpression and RASSF4 overexpression (miR-155 OE + RASSF4 OE), miR-155 knockdown (miR-155 KD) or miR-155 knockdown and RASSF4 knockdown (miR-155 KD + RASSF4 KD). G-H. The migration abilities were measured by transwell assay in A549 cells transfected with the plasmids of negative control (NC), miR-155 overexpression (miR-155 OE), miR-155 overexpression and RASSF4 overexpression (miR-155 OE + RASSF4 OE), miR-155 knockdown (miR-155 KD) and miR-155 knockdown or RASSF4 knockdown (miR-155 KD + RASSF4 KD). Error bars represent standard deviations and asterisks show significant differences from corresponding control according to Student’s t test (***P < 0.001)
Figure 5

M2 TAMs secret exosomal miR-155 to promote NSCLC metastasis in vivo. A. The bioluminescence images in different groups (M0 exosome group, M2 exosome group and M2/miR-155 knockdown exosome group) were captured. B. Bright Field images and HE staining for lung tissues in different groups was analysed. The red arrow indicated the location of the metastases. C. Quantitative real-time PCR analysis of miR-155 expression in M0 exosome group, M2 exosome group and M2/miR-155 knockdown exosome group, respectively. D. The protein expression levels of RASSF4, E-cadherin and vimentin were analysed by Western blot in A549 cells co-cultured with exosomes from M0 macrophages, M2 macrophages or M2 macrophages transfected with the plasmids of miR-155 knockdown (miR-155 KD). E. The protein expression level of RASSF4 was analysed in control group, M1-exosome group and M2-exosome group. Error bars represent standard deviations and asterisks show significant differences from corresponding control according to Student’s t test (***P < 0.001)