Tumor-Associated Macrophages Secret Exosomal miR-155 to Promote Metastasis of Non-Small-Cell Lung Cancer

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Research

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Abstract

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

Methods: The role of macrophages in NSCLC was elucidated by gene set enrichment analysis via The Cancer Genome Atlas database (TCGA) database, and we further verified it through Quantitative real-time PCR and immunohistochemical staining. Exosomes from TAMs were extracted and co-cultured with A549 cells,the biological functions of miR-155 were evaluated through miRNAs sequencing, transwell assays,western blotting,fluorescence labeling,luciferase reporter assay, and animal experiments.

Results: We found that M2 TAMs are abundant in metastatic tissues of NSCLC patients and exosomes secreted by M2 TAMs promote epithelial mesenchymal transition(EMT) and migration of A549 cells.Mechanistically,we demonstrated that miR-155 is the biomolecule in exosomes secreted by M2 TAMs and targets 3'-untranslated regions (UTRs) of RASSF4 to promote NSCLC metastasis.

Conclusions: MiR-155 is the key functional molecule in M2 TAMs-released exosomes that promote EMT of NSCLC cells through targeting RASSF4. Our study suggests that miR-155 in TAMs and exosomes may serve as a novel therapeutic target in the treatment of lung cancer.

Background

According to global cancer statistics in 2018, lung cancer accounts for ~ 12% of newly diagnosed cancer cases and ~ 18% of total cancer deaths [1]. About 85% of diagnosed lung cancers is non-small-cell lung cancers (NSCLCs), which have 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) are the pivotal orchestrators as a class of immune cells [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 are positively related with the survival time of NSCLC patients [19, 20]. In contrast, M2 TAMs are found to promote NSCLC metastasis through activating the epithelial-mesenchymal transition (EMT) of NSCLC cells and the invasion of lung cancer cells [20, 21]. 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 [22, 23]. 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 the gene expression [24]. For instance, gastric cancer cells secret exosomes carrying miR-21 into peritoneal mesothelial cells to promote peritoneal metastasis [22]. 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 [25]. Moreover, M2 TAMs also can release exosomes carrying miR-501 to promote the progression of pancreatic cancer [26]. Nevertheless, whether M2 TAMs promote NSCLC metastasis through exosomes and miRNAs still remains unclear.

Ras association domain family member 4 (RASSF4), a member of the RASSF family, abnormally expresses in human cancers and involves in carcinogenesis process. Moreover, it had a suppression effect on biological process. For instance, in osteosarcoma cells, RASSF4 overexpression markedly inhibits the proliferation, invasion and EMT [28]. In NSCLCs, it is reported that RASSF4 is down-regulated in NSCLC tissues and effectively inhibits cancer cell proliferation and invasion [29]. These studies suggested that RASSF4 serves as an important tumor suppressor in the development of cancer.

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 RASSF4.

Materials And Methods

Cell lines and Cell culture

The A549 and THP-1 cells were obtained from Chinese Academy of Sciences, Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). A549/Luc cells (A549 cells stably express luciferase) were constructed by Synthgene (Nanjing, China). THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium and A549 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) (Gibco, Rockville, USA) supplemented with 10% FBS (fetal bovine serum) (Gibco, Rockville, USA) and 100 μg/ml streptomycin and penicillin (Gibco, Rockville, USA) in a humidified atmosphere at 37˚C. After 7 days in culture, THP-1 cells were treated with 100 ng/mL phorbol 12-myristate-13-acetate (PMA) to differentiate into macrophages for 48 h. Next, 100 ng/mL lipopolysaccharide (LPS) and 20 ng/mL interferon-γ (IFN-γ) were adopted to treat THP-1 cells for 24 h, polarizing them into M1 macrophages.

Following treatment with 20 ng/mL interleukin-4 (IL-4) for 72 h, the cells were polarized into M2 macrophages.

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 the 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 [30]. The expression levels of TNF-α, IRF5, IRF4, Arg-1 and miR-155 were analyzed by quantitative real-time PCR with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene or U6 as a standard control. Primers of TNF-α, IRF5, IRF4, Arg-1, miR-155, miR-19b-1-5p, miR-12206-5p, miR-3091-3p, miR-12180-3p, U6 and GAPDH were as follows: TNF-α (Forward: 5’-CCTCTCTCTAATCAGCCCTCTG-3’; Reverse: 5’-GAGGACCTGGGAGTAGATGAG-3’); IRF5 (Forward: 5’-GGGCTTCAATGGGTCAACG-3’; Reverse: 5’-GCCTTCGGTGTATTTCCCTG-3’); IRF4 (Forward: 5’-GCTGATCGACCAGATCGACAG-3’; Reverse: 5’-CGGTTGTAGTCCTGCTTGC-3’); Arg-1 (Forward: 5’-GTGGAAACTTGCATGGACAAC-3’; Reverse: 5’-AATCCTGGCACATCGGGAATC-3’); miR-155 (Forward: 5’-GGAGGTTAATGCTAATCGTGATAG-3; Reverse: 5’-GTGCAGGGTCCGAGGT-3’); miR-19b-1-5p (Forward: 5’-GCGAGTTTTGCAGGTTTGCA-3; Reverse: 5’-AGTGCAGGGTCCGAGGTATT-3’); miR-12206-5p (Forward: 5’-GCGCGTACTATGCCTGGAAG-3; Reverse: 5’-AGTGCAGGGTCCGAGGTATT-3’); miR-3091-3p (Forward: 5’-GCGGGCCTGACCAGTCT-3; Reverse: 5’-AGTGCAGGGTCCGAGGTATT-3’); miR-12180-3p (Forward: 5’-GCGCGAGGAGCTGTGGA-3; Reverse: 5’-AGTGCAGGGTCCGAGGTATT-3’); U6 (Forward: 5’-TCGGCAGCACATATACTAA-3’; Reverse: 5’-CGCTTCACGAATTTGCGTGT-3’); GAPDH (Forward: 5’-GACCTCAACTACATGGTT-3’; Reverse: 5’-AACCATGTAGTTGAGG-3’). These primers were synthesized and purified by RiboBio (Guangzhou, China).

Cell migration and invasion assays

The cell invasion and migration assays were performed by 24-well Transwell cell culture chambers with 8-μm sized pores with or without precoated Matrigel (BD Biosciences, San Jose, CA). Specifically, A549 cells, A549 cells co-cultured with M2 macrophages treated with or without GW4869 and A549 cells co-cultured with exosomes from M1/M2 macrophages or M2 macrophages transfected with different plasmids, at a density of 5×10^4 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 DMEM medium (10% FBS). After incubation for 24 h, the cells remaining in the upper chamber were removed, 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 from M1 or M2 macrophages were isolated by density gradient ultracentrifugation according to previously reported protocol [31]. Briefly, cell culture medium was collected and centrifuged at 1,000g for 10 min, 2,000g for 20 min, 4,000g for 30 min and 10,000g for 1 h to obtain the supernatant. The exosomes were collected by centrifuging the supernatant at 100,000g for 2 h at 4 °C. The size stribution and concentration of exosomes were analyzed 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.

miRNA profiling

The aberrant miRNAs expressions of M1-exosome and M2-exosome were analyzed by miRNAs sequencing. Briefly, total RNA was isolated from M1-exosome and M2-exosome using TRIzol reagent (Invitrogen, MA, USA). 250 ng total RNA of M1-exosome and M2-exosome were extracted to prepare the small RNA sequencing library by using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (NEB, USA). The libraries were finally sequenced and the Solexa CHASTITY quantity filtered reads were harvested as Clean Reads. For data analysis, differentially expressed miRNA profiles between M1-exosome and M2-exosome groups were compared, fold changes were calculated to identify significant differentially expressed miRNAs and hierarchical clustering was performed. The selected miRNAs were verified by qRT-PCR.

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 exosomes, cells or tissues. 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); 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

Cy3-labeled miR-155 mimics were purchased (GenePharma, China). The A549 cells were co-cultured with exosomes isolated from M2 macrophages transfected with Cy3-labeled miR-155 to further observe the transfer of miRNA. Then, the cells were washed with PBS and 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 purchased. For the in vivo lung metastases model, exosomes purifified from M1/M2 macrophages or M2 macrophages transfected with 1×10^9 ifu of miR-155 inhibitor lentivirus were respectively injected into the peritoneum. Four days post-injection, 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

To evaluate the role of macrophages in the metastasis of NSCLC, we firstly utilized gene set enrichment analysis via The Cancer Genome Atlas (TCGA) database and observed that CD68, a macrophage marker, was significantly increased in 122 patients with the metastasis of NSCLC in Fig.1A. To further validate the distribution of macrophages in NSCLC, we analyzed the expression of CD68 by immunohistochemical staining and found that the higher density of TAMs in NSCLC metastasis tissues than that in localized tissues in Fig.1B and 1C, suggesting TAMs recruitment related to the metastasis of NSCLC. Next, we estimated the two subpopulations of macrophages including macrophage M1 and M2 in NSCLC patients.

The results of qRT-PCR in Fig.1D showed that the transcription expressions of M1 macrophages marker such as tumor necrosis factor alpha (TNF-α) and interferon regulatory factor 5 (IRF5) had a significant decrease in all localized 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 tissues compared with localized tissues. These data indicated that M2 TAMs probably involved in the metastasis of NSCLC.

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

The results in Fig.1 showed that M2 macrophages were more enriched in metastatic NSCLC patients than those without metastasis. To evaluate whether M2 TAMs involved in the metastasis of NSCLC, we co-cultured A549 cells with M2 macrophages in a transwell insert. In Fig.2A and 2B, we found that the migration capacity of A549 cells was enhanced in A549 cells co-cultured with M2 macrophages group compared with the control, suggesting that M2 macrophages can promote the migration potential of A549 cells. The macrophages have been extensively reported to regulate a series of biological processes of NSCLC through exosomes. To evaluate whether the effect of M2 macrophages on A549 cells was through exosomes, we treated M2 macrophages with GW4869 (an inhibitor of exosome release). For inhibition of exosome generation, M2 macrophages were cultured in media containing 10 μM GW4869. In Fig.2A and 2B, following GW4869 treatment, M2 macrophages failed to promote the migration of A549 cells; however, the migrating number of control group pretreated by GW4869 had no significant changes, suggesting that exosomes derived from M2 macrophages play an essential role in the migration of A549 cells.

Subsequently, a large number of exosomes were found in the supernatant of M2 macrophages culture by transmission electron microscope. And the shape and size of exosomes were solid with typical structure of two-layer membrane and exosomes had 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 exosomes compared with cell lysis, confirming the successful extraction of exosomes. To examine whether M2 macrophages derived exosomes (M2 exosomes) can be taken up by A549 cell, we pre-labeled M2 exosomes with PKH67. In Fig.2G, 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 taken up by A549 cells. Furthermore, after 48 h of co-culture of A549 cells with M1 exosome or M2 exosome in vitro, transwell assay and western blot analysis were applied to examine whether exosomes generated from M2 macrophages were sufficient to induce the cell migration and invasion. The results in Fig.2H and 2I revealed that the invasion and migration ability of A549 cells co-cultured with exosomes secreted from M2 macrophages were strengthen compared with that co-cultured with M1 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 markedly promote EMT, migration and invasion of A549 cells.

MiR-155 is enriched in M2 macrophages-derived exosomes

Emerging evidences indicated that miRNAs which involved in cell-cell communications are frequently encapsulated in exosomes, and implement their biological functions in the recipient cells. To explore the mechanisms by which M2-exosome produced a marked effect in NSCLC, we generated miRNA profiles of M1-exosome and M2-exosome by miRNA microarray analysis. The comparison of the expression levels of the miRNAs in the M1-exosome and M2-exosome groups is depicted in Fig.3A. Among the miRNAs, miR-155 was the most abundant in M2-exosome group in Fig.3A and 3B. In further analysis, in Fig.3C, miR-155 was most markedly upregulated (miR-155, miR-19b-1-5p, miR-3091-3p, miR-12206-5p and miR-12180-3p) in M2-exosome group. Next, we confirmed that the miR-155 expression was much higher in 183 patients with the metastasis of NSCLC as compared to localized persons by LUAD dataset analysis in Fig.3D. In addition, the NSCLC metastasis tissues showed higher levels of miR-155 than localized tissues in Fig.3E. In summary, these data suggested that miR-155 derived from M2 macrophage exosomes may play an important role in NSCLC metastasis.

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

The results in Fig.3 showed that miR-155 is enriched in M2 macrophages-derived exosomes and M2-exosomes promote the migration of A549 cells in Fig.2. Emerging evidences showed that exosomes deliver proteins, miRNAs or mRNAs into target cells to mediate a series of physiological processes. Therefore, we hypothesized that M2-exosomes might induce the increased migration of recipient A549 cells through the transfer of functional miR-155. As shown in Fig.4A and 4B, we further confirmed that miR-155 expression was much higher in M2 cell lysis and M2 exosome as compared to M1 macrophages. To further determine whether the M2-exosomes transfer of miR-155, M2 macrophages were transiently transfected with Cy3-labeled miR-155. The purified M2-exosomes were added to A549 cells culture medium and the fluorescence signals could be detected in Fig.4C, suggesting that M2 macrophage-derived exosomes mediate miR-155 shuttling. In Fig.4D-4F, we found that the M2-exosomes with miR-155 overexpression promoted the migration of A549 cells, increased the protein expression level of vimentin and inhibited the E-cadherin expression, while the opposite pattern could be observed in M2/miR-155-inhibitor exosomes group. Taken together, these data suggested that miR-155 carried by exosomes derived from M2 macrophages mediated a series of biological processes of NSCLC.

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

In NSCLCs, it is reported that RASSF4 is down-regulated in NSCLC tissues and effectively inhibits cancer cell proliferation and invasion. The prediction of TargetScan Release 7.0 database showed that RASSF4 in 3’-untranslated regions (UTRs) possesses putative binding sites for miR-155 in Fig.5A. 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.5B, the luciferase activity transfected with RASSF4-WT significantly decreased compared with negative control. The response of RASSF4 to miR-155 overexpression or miR-155 inhibition was examined by western blot analysis. As shown in Fig.5C and 5D, 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 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, the promoted expressions of RASSF4 and E-cadherin by miR-155 knockdown were alleviated by RASSF4 inhibition. In addition, the effects of miR-155 knockdown on A549 cells, including the protein levels of RASSF4, vimentin and E-cadherin and migration abilities, could be reversed by the knockdown of RASSF4 in Fig. 5E-5H. Collectively, our findings confirmed that RASSF4 is the direct target of miR-155.

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, M1 exosome, M2 exosome and M2/miR-155 knockdown exosome were injected respectively after intravenous injection of A549/Luc cells into mice to construct lung metastasis model. In Fig.6A, M2-derived exosomes markedly contributed to the lung dissemination. And luciferase activity of M2 exosome group was significantly higher than that of M1 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 lung tissues of each group in Fig.6B showed that M2-derived exosomes with the knockdown of miR-155 markedly contributed to alleviate the metastasis of NSCLC resulting from M2 exosomes. In addition, in Fig.6C, the transcription expression of miR-155 in M2 exosome group was significantly up-regulated by approximately 18-fold compared to M1 exosome group. However, the transcription levels of miR-155 in M2 exosome group were attenuated when miR-155 was inhibited. In Fig. 6D and 6E, western blot analysis from tumor tissues of each group revealed that the protein levels of RASSF4 in M2/miR-155 knockdown exosome group were significantly up-regulated compared with M2-exosome group. 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 [32]. 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 [33, 34]. The modes of action of stromal cells include 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 [20, 21]. 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 [35–37]. 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 [38]. The roles of miRNAs in NSCLC metastasis have also been discovered [10, 39]. Therefore, targeting certain miRNAs involved in NSCLC has been considered as an efficient therapeutic approach [40].

Inhibiting miRNA-enriched exosomes from M2 TAMs has been found to be useful for treating cancer metastasis [41, 42]. 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 [43]. 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.

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.

Conclusions

In this study, 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, we revealed a new regulatory pathway that was M2 TAMs secreted exosomal miR-155 to promote NSCLC metastasis. Our findings may provide a practical target for treatment of NSCLC.

Abbreviations

NSCLC
non-small-cell lung cancer ;TAMs:tumor-associated macrophages; EMT:epithelial mesenchymal transition;miRNAs:microRNAs;RASSF4:Ras association domain family member 4;TNF-α:tumor necrosis

factor alpha;IRF5:interferon regulatory factor 5;IRF4:interferon regulatory factor 4;UTRs:untranslated regions.

Declarations

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Not applicable.

Authors' contributions

XL, LC, and JW conceived this study. XX, YX, and ZH performed the data analysis. JW, WW, QZ, LC, YX, and ZH 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.

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Availability of data and materials

The dataset used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Nanjing Medical University of 1st affiliated hospital. The Laboratory Animal Care and Use Committees of the hospital approved all experimental procedures.

Consent for publication

All authors agree to submit the article for publication.

Competing interests

The authors declare no conflict of interest.

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Figures

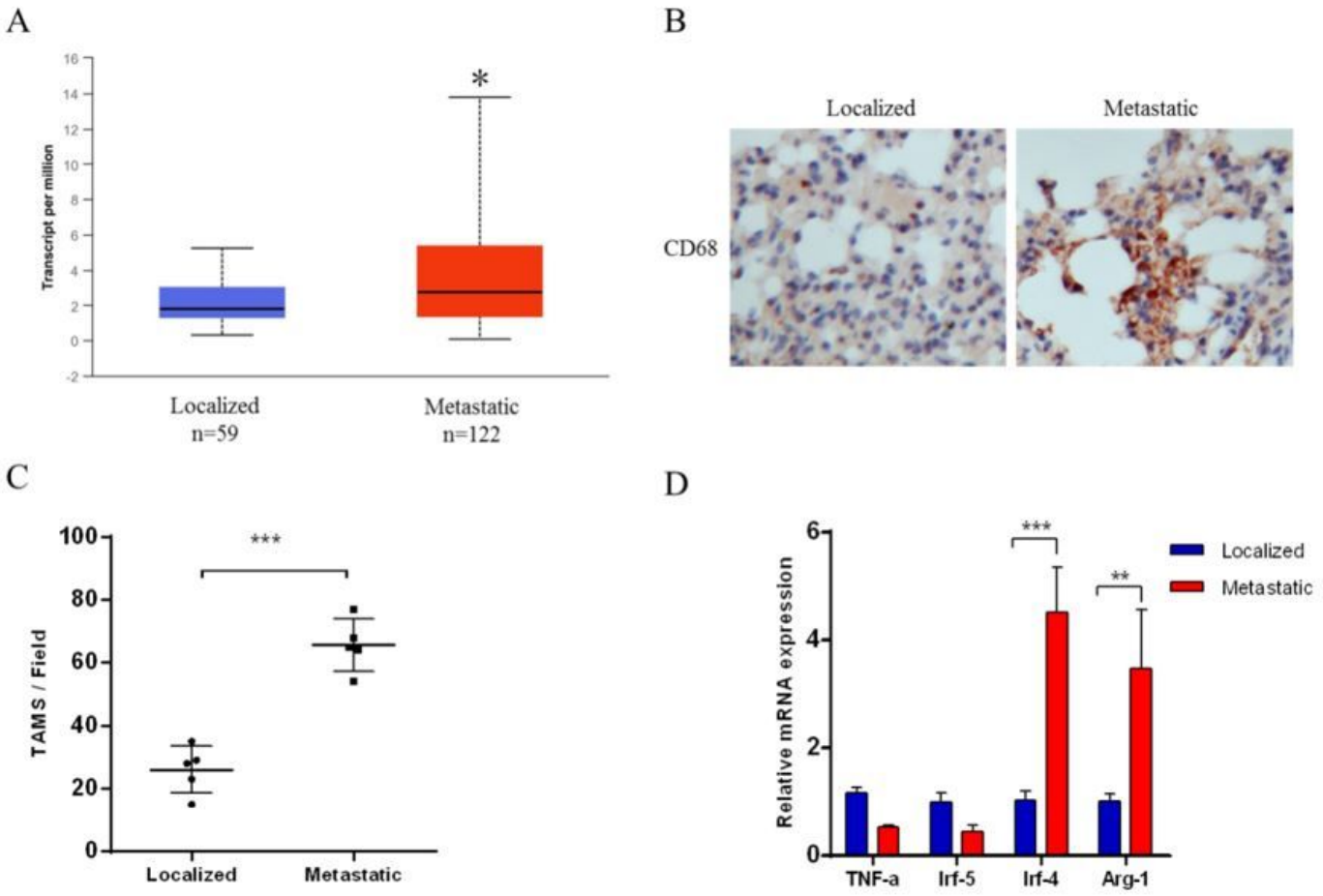

Figure 1

M2 TAMs are abundant in metastatic tissues of NSCLC patients. A. Expression of CD68 in NSCLC patients (n=59) and localized persons (n=122) from the analysis of the TCGA database. B-C. Immunohistochemical staining and quantitative analysis of CD68 expression in localized tissues and metastatic NSCLC tissues; C. Quantitative real-time PCR analysis of TNF-α, IRF5, IRF4 and Arg-1 expression in localized tissues and metastatic NSCLC tissues. 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)

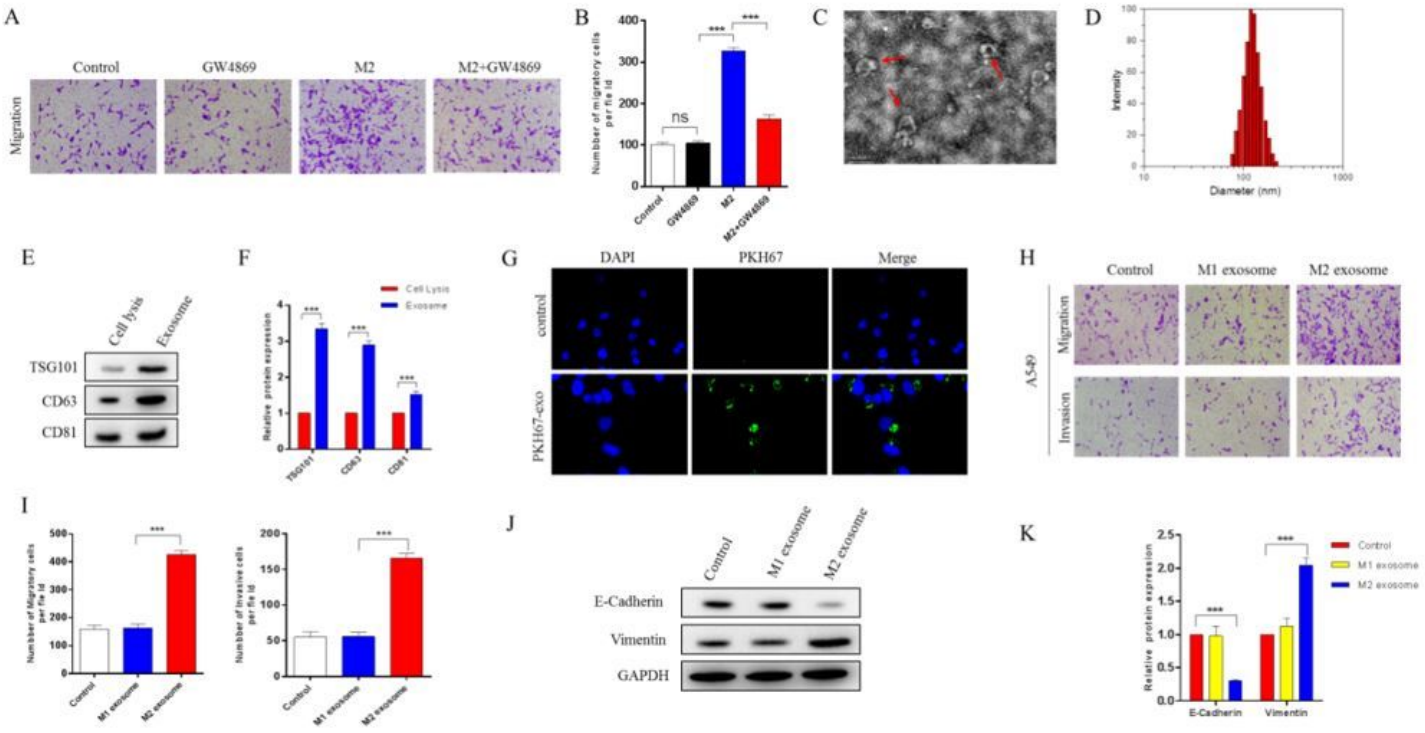

Figure 2

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 exosome was identified by transmission electron microscope. D. The size of exosome was detected by nanoparticle tracking analysis. E-F. The protein levels of exosomal markers TSG101, CD63 and CD81 in exosomes and cell lysis were analyzed by western blot. G. The fluorescence signal from A549 cells co-cultured with PKH67-labeled M2 exosomes or control was detected. H-I. The migration and invasive abilities of A549 cells after M1 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 M1 exosome or M2 exosome treatment were analyzed 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)

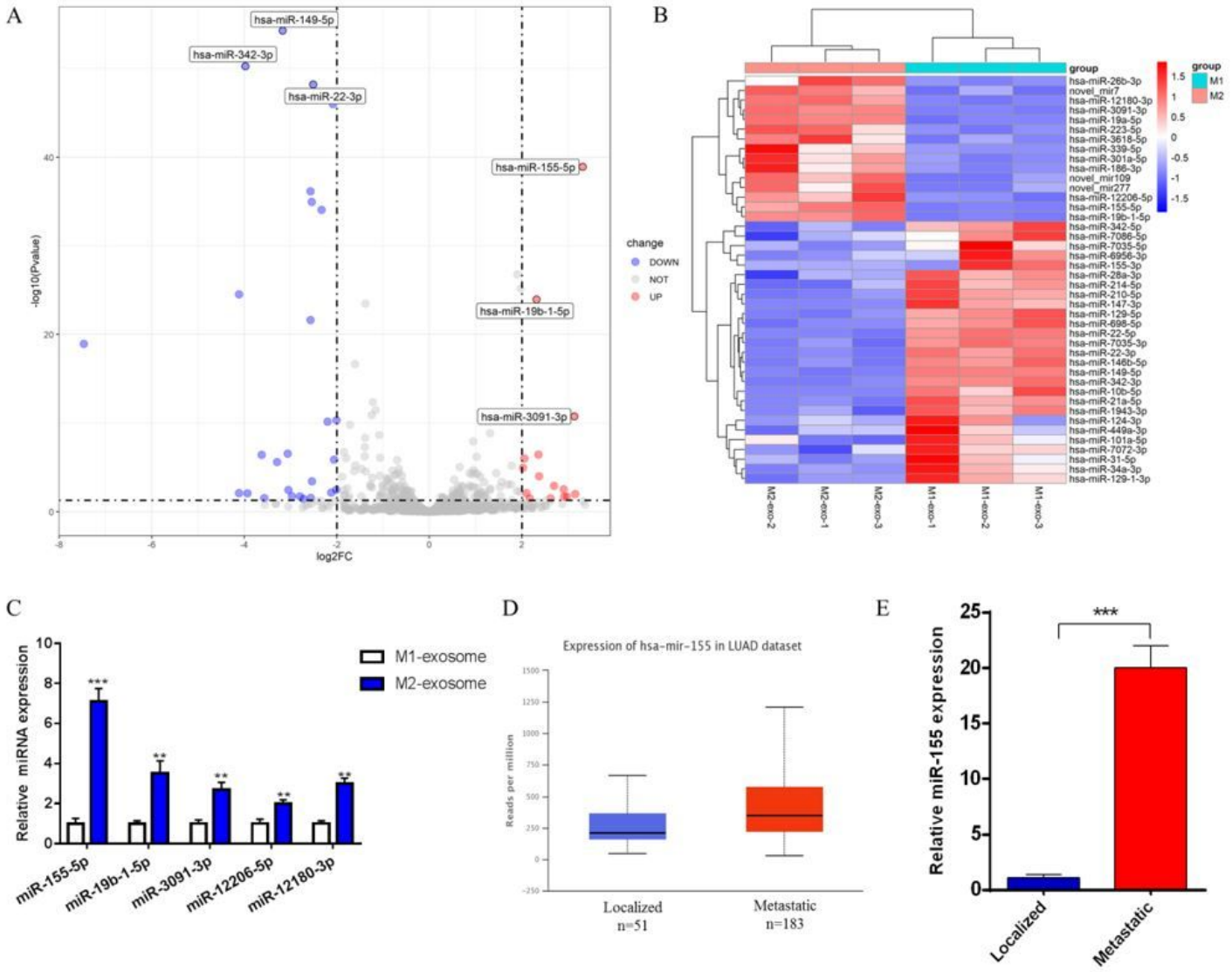

Figure 3

MiR-155 is enriched in M2 macrophages-derived exosomes. A. Volcano plot of the differentially expressed miRNAs as assessed by a microarray analysis in M1 and M2 exosomes. B. Microarray analysis of exosomal miRNAs in M1 and M2 TAMs were presented in a heatmap. C. The transcription expression of miR-155, miR-19b-1-5p, miR-3091-3p, miR-12206-5p and miR-12180-3p in M1-exosome and M2-exosome groups. D. Expression of miR-155 in metastatic NSCLC patients (n=51) and localized persons (n=183) from the analysis of the TCGA database. E. Quantitative real-time PCR analysis of miR-155 in localized tissues and metastatic NSCLC tissues. 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)

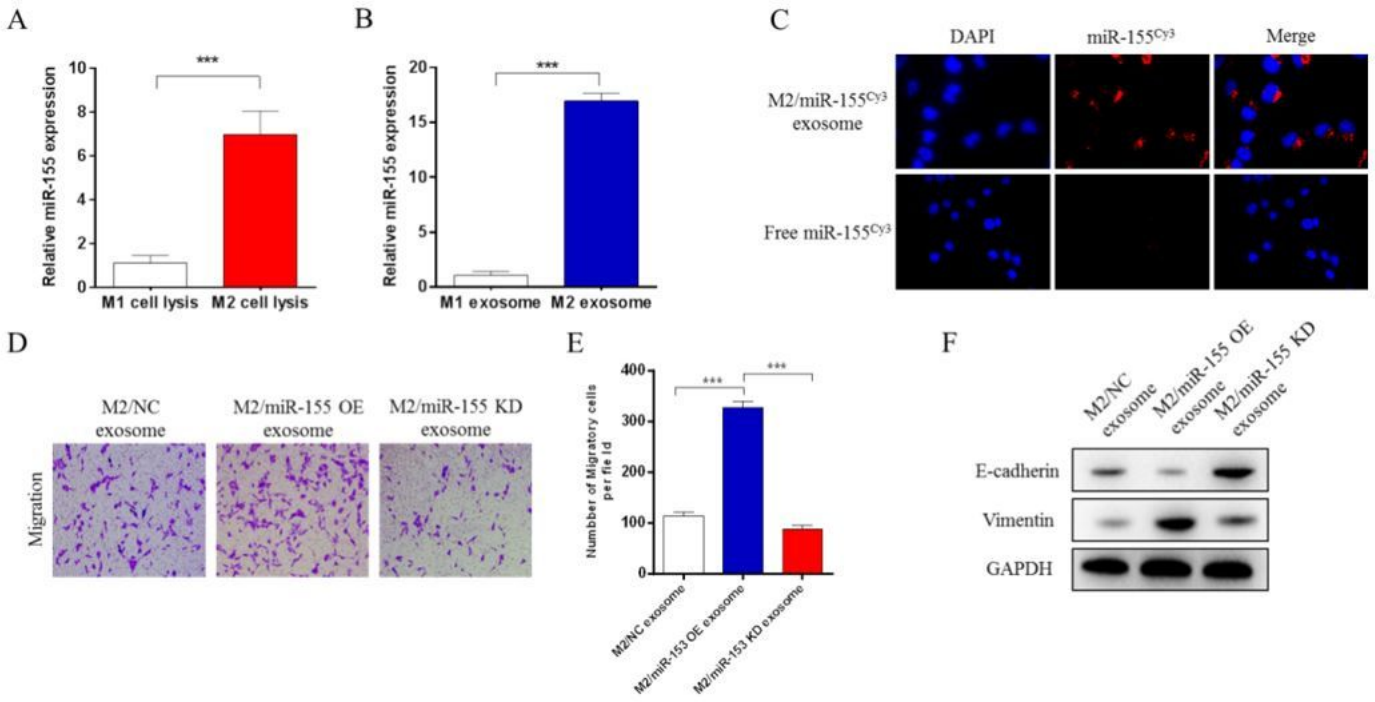

Figure 4

miR-155 is the key biomolecule in exosomes secreted by M2 TAMs. A. Quantitative real-time PCR analysis of miR-155 expression in M1 and M2 cell lysis, respectively. B. Quantitative real-time PCR analysis of miR-155 expression in M1 and M2 exosome, respectively. C. The fluorescence signals were detected in A549 cells incubated with M2 exosomes with 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 analyzed 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)

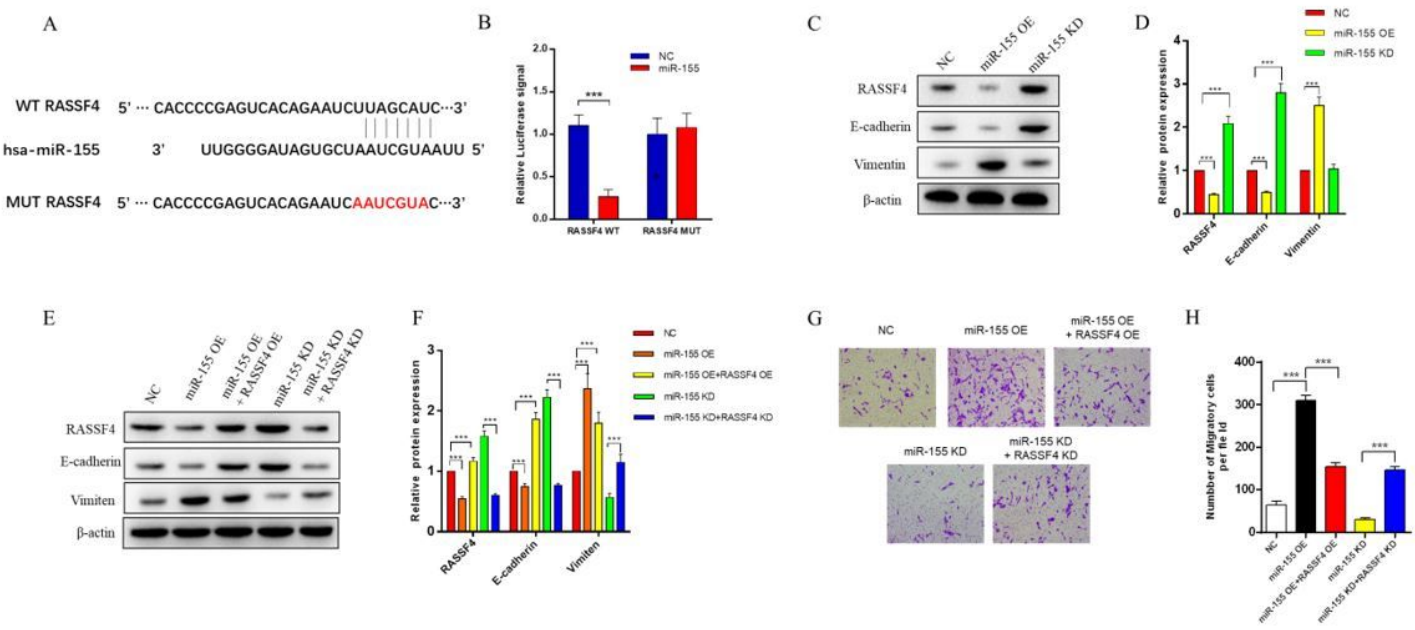

Figure 5

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 activities 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 negative control (NC), miR-155 mimic (miR-155 OE) or miR-155 inhibitor (miR-155 KD) were analyzed by western blot. E-F. The protein expression levels of RASSF4, E-cadherin and vimentin were analyzed by western blot in A549 cells transfected with negative control (NC), miR-155 mimic (miR-155 OE), miR-155 mimic and RASSF4 overexpression plasmid (miR-155 OE + RASSF4 OE), miR-155 inhibitor (miR-155 KD) or miR-155 inhibitor and RASSF4 knockdown plasmid (miR-155 KD + RASSF4 KD). G-H. The migration abilities were measured by transwell assay in A549 cells transfected with negative control (NC), miR-155 mimic (miR-155 OE), miR-155 mimic and RASSF4 overexpression plasmid (miR-155 OE + RASSF4 OE), miR-155 inhibitor (miR-155 KD) or miR-155 inhibitor and RASSF4 knockdown plasmid (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).

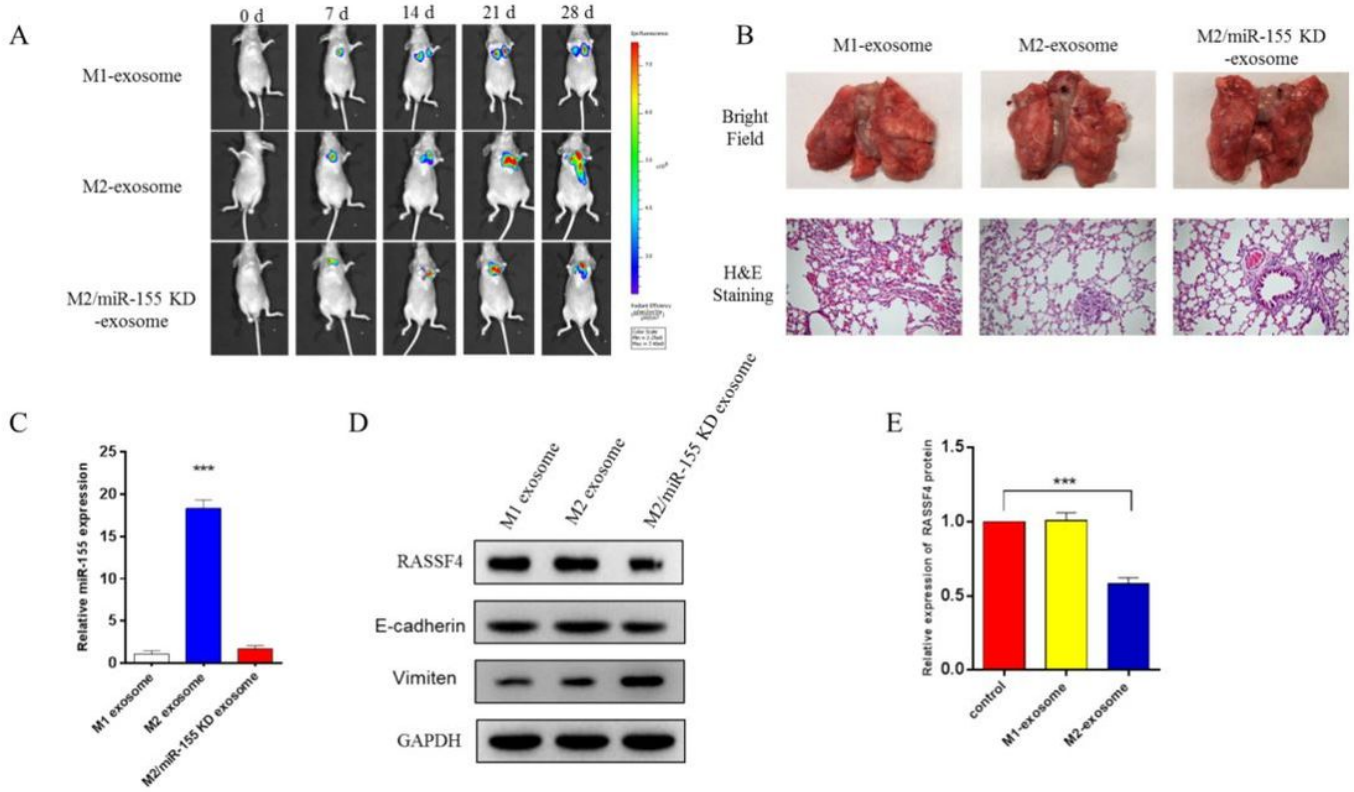

Figure 6

M2 TAMs secret exosomal miR-155 to promote NSCLC metastasis in vivo. A. The bioluminescence images in different groups (M1 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 analyzed. The red arrow indicated the location of the metastases. C. Quantitative real-time PCR analysis of miR-155 expression in M1 exosome group, M2 exosome group and M2/miR-155 knockdown exosome group, respectively. D-E. The protein expression levels of RASSF4 were analyzed in different groups (M1 exosome group, M2 exosome group and M2/miR-155 knockdown exosome group). 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)