HMGB1 regulates SNAI1 during NSCLC metastasis, both directly, through transcriptional activation, and indirectly, in a RSF1-IT2-dependent manner

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High-mobility group protein B1 (HMGB1) has important functions in cancer cell proliferation and metastasis. However, the mechanisms of HMGB1 function in non-small-cell lung cancer (NSCLC) remain unclear. This study aimed to investigate the underlying mechanism of HMGB1-dependent tumor cell proliferation and NSCLC metastasis. Firstly, we found high HMGB1 expression in NSCLC and showed that HMGB1 promoted proliferation, migration, and invasion of NSCLC cells. HMGB1 could bind to SNAI1 promoter and activate the expression of SNAI1. In addition, HMGB1 could transcriptionally regulate the lncRNA RSF1-IT2. RSF1-IT2 was found to function as ceRNA, sponging miR-129-5p, which targets SNAI1. Notably, HMGB1 was also identified as a target of miR-129-5p, which indicates the establishment of a positive feedback loop. Consequently, high expression of RSF1-IT2 and SNAI1 was found to closely correlate with tumor progression in both HMGB1-overexpressing xenograft nude mice and patients with NSCLC. Taken together, our findings provide new insights into molecular mechanisms of HMGB1-dependent tumor metastasis. Components of the HMGB1–RSF1-IT2–miR-129-5p–SNAI1 pathway may have a potential as prognostic and therapeutic targets in NSCLC.

1. Introduction

Lung cancer is the most common malignant tumor with high morbidity and mortality worldwide, 80–85% of which is pathologically diagnosed with non-small-cell lung cancer (NSCLC) including squamous cell carcinoma, adenocarcinoma, and large cell carcinoma (Asamura et al., 2015; Ettinger et al., 2016). Although there are diverse treatments for NSCLC such as surgery, radiotherapy, chemotherapy, and targeted therapy, the overall 5-year survival rate is only 18.2% (Feng et al., 2016). Tumor metastasis is the leading cause of death in NSCLC patients, and it is regulated by many complex factors (Borghaei et al., 2015). Therefore, exploring the molecular mechanism of NSCLC metastasis is conducive to find efficient strategies to inhibit tumor growth and improve the therapeutic effect.

Abbreviations
CCK-8, Cell Counting Kit-8; EMT, epithelial-mesenchymal transition; HMGB1, high-mobility group protein B1; IRS, immunoreactive score; lncRNA, long noncoding RNA; NSCLC, non-small-cell lung cancer; NTs, noncancerous tissues; RAGE, receptor for advanced glycation end products; RSF1-IT2, remodeling and spacing factor 1-intronic transcript 2; TLR, toll-like receptor.
High-mobility group protein B1 (HMGB1), a ubiquitous nuclear protein, plays a significant role in regulating transcription, replication, repair, and genetic stability (Wu et al., 2018b; Wu and Yang, 2018). Intracellular HMGB1 mainly acts in the nucleus and regulates gene expression as an architectural chromatin-binding protein, while extracellular HMGB1 can bind receptor for advanced glycation end products (RAGE) and toll-like receptor (TLR) inducing multiple biological effects (Luan et al., 2018). Recent studies have shown that HMGB1 is highly expressed in various human carcinomas (Chuangui et al., 2012; Wu and Yang, 2018). Moreover, high HMGB1 expression is significantly associated with advanced stages, distant metastasis, and poor prognosis in lung cancer patients (Liu et al., 2017; Postmus et al., 2013; Wu et al., 2018a). It is indicated that HMGB1 can act as an effective therapeutic target to offer a new way for the treatment of lung cancer.

Long noncoding RNAs (lncRNAs) are a class of non-protein coding transcripts with a length greater than 200 nucleotides. Recently, lncRNAs have attracted more attention because of its important role in transcription, translation, epigenetic modification, and cell cycle regulation (Xuan et al., 2019; Yao and Wang, 2019). Emerging evidence revealed that lncRNAs were abnormally expressed in various cancer types and affected tumor metastasis (Yao and Wang, 2019). Remodeling and spacing factor 1 (RSF1), which is one of chromatin-remodeling factors, has been demonstrated to be highly expressed and associated with poor prognosis in NSCLC, renal cancer, and ovarian cancer (Wu et al., 2017; Yang et al., 2014; Zhang et al., 2018). RSF1-intronic transcript 2 (RSF1-IT2), derived from an intron within the RSF1 gene, has been mapped to chromosome 11 region 77717712–77718741 reverse strand according to the NCBI. RSF1-IT2 was located in 11q14 with 2 exons, and its role in cancers remained largely unknown.

Previous studies have shown that HMGB1 accelerates tumor growth and metastasis (Chuangui et al., 2012; Lv et al., 2016; Wang et al., 2015). However, the molecular mechanisms and downstream effector pathways of HMGB1 in NSCLC are still unclear. Moreover, the role of RSF1-IT2 in cancers and the mechanism of its regulation by HMGB1 have not been reported. The present study focused on the investigation of HMGB1-induced tumor metastasis and regulated RSF1-IT2, hoping to further clarify the signaling pathway of HMGB1 and provide novel therapeutic target for the treatment of NSCLC.

2. Materials and methods

2.1. Tissue samples and cell lines

The tissue specimens were consisted of 122 NSCLC tissues and 120 noncancerous tissues (NTs). All patients were primary pathologically diagnosed with NSCLC at Affiliated Xuzhou Municipal Hospital of Xuzhou Medical University between July 2007 and June 2011, and followed up for 5 years. The study methodologies conformed to the standards set by the Declaration of Helsinki.

H1299 and H460 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). H1299 and H460 cells were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% bovine blood serum (Sijiqing Laboratories, Hangzhou, Zhejiang, China) at 37 °C in a humidified atmosphere with 5% CO2.

2.2. Transfection

siRNAs were purchased from GenePharma, Shanghai, China. The sequences of all oligos used for transfections were as follows: of si-HMGB1, sense, 5'-GCAUAAGAAGAACCCATT-3', of si-SNAI1, sense, 5'-GGCCUUCACUGCAAAUAUTT-3', of si-RSF1-IT2, sense, 5'-CAAGCTTTGATATTTTAAGGA-GATTCATTTTTG-3'. The cells were transfected with siRNA using siLentFect Lipid Reagent (Bio-Rad, Hercules, CA, USA). The pcDNA3.1, pcDNA3.1-HMGB1 plasmids, and pcDNA3.1-RSF1-IT2 plasmids were purchased from Guangzhou FulenGen Co. (Guangzhou, Guangdong, China), which were transfected using X-tremeGENE HP DNA Transfection Reagent (Roche, Indianapolis, IN, USA). The LV-Vector-H1299 and LV-HMGB1-H1299 cells were established by infected with lentiviruses (GenePharma).

2.3. Transwell assay

Migration or invasion assays were performed with the transwell inserts (Corning Incorporated, New York, NY, USA) with or without Matrigel (BD Biosciences, San Jose, CA, USA). Cells were immobilized for 15 min with 4% paraformaldehyde (Vicmed, Xuzhou, China) and stained with crystal violet staining (Vicmed). The results were taken with Nikon digital camera (Pan et al., 2017).
Fig. 1. HMGB1 expression is high in NSCLC tissues and serves as a potential prognostic indicator for NSCLC. (A) Representative images of HMGB1 immunohistochemical staining in NSCLC patients. Magnification ×100, ×200, ×400. (B, C) Association between HMGB1 expression and clinicopathological features in NSCLC tissues (*P < 0.05, **P < 0.01, ***P < 0.001, NS, no significance, χ² test). (D) High HMGB1 expression correlated with worse overall survival over a 5-year period in 122 NSCLC patients (HMGB1\textsuperscript{High} n = 82, HMGB1\textsuperscript{Low} n = 40, P < 0.05, log-rank test).
HMGB1 regulates SNAI1 during NSCLC metastasis

2.5. Cell proliferation assay

After transfection, H1299 and H460 cells (4 × 10^5) were cultured in 96-well microplate (Corning Incorporated) in triplicate. Then, 10 µL Cell Counting Kit-8 (CCK-8; Vimed) solution was added to each well at 24, 48, 72, and 96 h, respectively. Absorbance was measured at 450 nm by a multifunctional enzyme-linked analyzer (BioTek Instruments, Winooski, VT, USA).

2.6. EdU staining

H1299 and H460 cells were cultured in 24-well plate and transfected with si-HMGB1 or pcDNA3.1-HMGB1. After transfection, the cells were incubated with 3.7% neutral methanol for 15 min, and then 0.1% Triton X-100 for 15 min and 10 µm EdU for 30 min using keyFlour488 Click-It EdU imaging detection kit (KeyGEN Biotech, Nanjing, China). The results were captured with a Nikon fluorescence inversion microscope (Pan et al., 2017).

2.7. Cell apoptosis assay

After transfection, cells were washed twice with ice-cold PBS, then incubated with 200 µL 1 × binding buffer containing 5 µL Annexin V-FITC, and then with 300 µL 1 × binding buffer containing 5 µL propidium iodide (PI) for 10 min at room temperature in the dark using the Annexin V-FITC Kit (FITC Annexin V Apoptosis Detection Kit I; BD). After incubation, cells were visualized under a fluorescence microscope.

2.8. Immunohistochemistry and RNAscope

Tissues were cut into 4-mm sections, and then deparaffinized and rehydrated. Endogenous peroxidase

Table 1. Cox univariate regression analysis of the risk factors for death in lung cancer patients.

| Pathology character | HR    | 95% CI | P value |
|---------------------|-------|--------|---------|
| Age (year)          | 1.555 | 0.896–2.696 | 0.116   |
| Gender              | 0.545 | 0.31–0.957  | 0.035*  |
| TNM stage (I–II vs III–IV) | 1.906 | 1.199–1.892 | 0.000*  |
| pT status (T1-2 vs T3-4) | 3.892 | 1.806–8.386 | 0.001*  |
| pN status (N0 vs N+) | 1.042 | 0.736–1.474 | 0.818   |
| Metastasis (M0 vs M1) | 2.696 | 0.957–7.850 | 0.116   |
| HMGB1 staining (− vs +) | 1.996 | 1.058–3.766 | 0.033*  |

*P < 0.05.

Table 2. Cox multivariate regression analysis of independent risk factors for death in lung cancer patients.

| Pathology character | HR    | 95% CI | P value |
|---------------------|-------|--------|---------|
| Gender              | 0.466 | 0.257–0.844 | 0.012*  |
| TNM stage (I–II vs III–IV) | 4.388 | 1.367–14.084 | 0.967   |
| pT status (T1-2 vs T3-4) | 2.919 | 1.026–8.305 | 0.045*  |
| HMGB1 staining (− vs +) | 2.143 | 1.074–4.274 | 0.031*  |

*P < 0.05.

2.4. Wound healing assay

After transfection, H1299 and H460 cells pretreated with mitomycin C (10 µg/mL) were harvested and seeded into 6-well plates. A linear scratch/wound was created on cell monolayers using a pipette tip. Photomicrographs were taken of live cells at ×100 magnification with a Nikon digital camera (Nikon, Toyko, Japan), and the distance migrated was observed within an appropriate time.

Fig. 2. HMGB1 promotes NSCLC invasion and migration through the up-regulation of SNAI1 expression. (A) Representative images of EdU staining after transfection of siHMGB1 or pcDNA3.1-HMGB1. The EdU-positive cells were measured and shown as a bar graph (scale bar, 100 µm). (B) The cell proliferation was detected by CCK-8 assays after transfection of siHMGB1 or pcDNA3.1-HMGB1, at 24, 48, and 72 h. (C) Western blot analysis of HMGB1, cleaved caspase-3, cleaved PARP in H1299 and H460 cells after transfection with siHMGB1 or pcDNA3.1-HMGB1. (D) Annexin V-FITC binding assay was used to observe apoptotic cells by fluorescence microscope in NSCLC cells transfected with siHMGB1 or pcDNA3.1-HMGB1 and their negative control (scale bar, 100 µm). (E) Representative images of the wound healing distance of shift after transfection with siHMGB1 or pcDNA3.1-HMGB1 in H1299 and H460 cells. (F) The cell migration and invasion of H1299 and H460 cells after transfection with siHMGB1 or pcDNA3.1-HMGB1 were detected by transwell assays. (G) Western blot analysis of HMGB1, Twist, SNAI1, Vimentin, β-catenin, N-cadherin, E-cadherin, and Fibronectin in H1299 and H460 cells after transfection with siHMGB1 or pcDNA3.1-HMGB1. (H) Luciferase reporter assay was carried out in H1299 and H460 cells. The relative luciferase activities of cotransfection of the SNAI1, Twist, and Vimentin with HMGB1 plasmid against pcDNA3.1(+)pGL3-SNAI1, pcDNA3.1(+)pGL3-Twist, and pcDNA3.1(+)pGL3-Vimentin group. (I) Soluble chromatins of H1299 and H460 cells were prepared and subjected to ChIP assay using anti-HMGB1 antibody as described in the section of ‘Materials and methods’. (J) The cell migration and invasion of LV-Vector-H1299 and LV-HMGB1-H1299 cells after transfection with siNC and si-SNAI1 were detected by transwell assays. All experiments were performed three times (n = 3), the data were presented as mean ± SD. Student’s t test was used to determine statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
HMGB1 regulates SNAI1 during NSCLC metastasis

X. Wu et al.
activity was blocked with 0.3% hydrogen peroxide for 15 min. After blocking with 5% normal goat serum for 30 min, slides were incubated with the primary antibodies overnight at 4 °C followed by secondary antibody for 15 min. The slides were then counterstained with hematoxylin. The staining intensity was scored 0–3 (0 = negative; 1 = weak; 2 = moderate; 3 = strong). The percentage of positive stained cells was scored 0–4: 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%), or 4 (76–100%). The immunoreactive score (IRS) was calculated by multiplying the scores of staining intensity and the percentage of positive cells. IRS of 0–1 was defined as low expression, and IRS of 2–12 was defined as high expression (Lv et al., 2019).

The RNAscope probe was designed and synthesized by Advanced Cell Diagnostics Company (Newark, CA, USA). RSF1-IT2 and miR-129-5p expression were detected using an RNAscope 2.5 High Definition (HD)-BROWN Assay Kit (Advanced Cell Diagnostics). The signals were scored based on the average number of dots per cell: 0 (0–1 dots/10 cells), 1 (1–3 dots/cell), 2 (4–10 dots/cell), 3 (>10 dots/cell with dots in clusters), and 4 (>15 dots/cell and >10% of the dots presented in clusters). Based on the scores, staining pattern was defined as low (score: 0–1) and high (score: 2–4) (Jin et al., 2019).

2.9. Western blot

After performing specific treatments, cells were washed with ice-cold PBS twice and lysed in lysis buffer (Beyotime, Shanghai, China). Proteins were separated on SDS/PAGE and transferred to nitrocellulose filter membrane. Membranes were incubated in turn with antibodies overnight at 4 °C followed by secondary antibody for 15 min. The slides were then counterstained with hematoxylin. The staining intensity was scored 0–3 (0 = negative; 1 = weak; 2 = moderate; 3 = strong). The percentage of positive stained cells was scored 0–4: 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%), or 4 (76–100%). The immunoreactive score (IRS) was calculated by multiplying the scores of staining intensity and the percentage of positive cells. IRS of 0–1 was defined as low expression, and IRS of 2–12 was defined as high expression (Lv et al., 2019).

2.10. Luciferase reporter assay

Cells were split into 48-well plates, and each well was transfected with plasmids following the manufacturer’s procedures. After 24 h, whole-cell lysate was collected and their luciferase activities were evaluated using Dual-Luciferase Kit (Promega, Shanghai, China). Reactions were measured using an Orion Microplate Luminometer (Berthold Detection System, Bad Wildbad, Germany).

2.11. Chromatin immunoprecipitation assay

The EZ ChIP kit (Upstate, NY, USA) was used. Firstly, the cells are treated with ultrasound to disrupt chromatin DNA fragments in the cells to 200–500 bp. The sonicated cells were centrifuged, and the supernatants were aspirated. The supernatants were inverted with anti-HMGB1 or normal rabbit IgG at 4 °C overnight. The cross-linked DNA-protein was heated at 65 °C overnight and then subjected to PCR analysis (Zuo et al., 2014).

2.12. Quantitative real-time RT-PCR

Total RNA from H1299 and H460 cells was extracted using TRIzol reagent and used as a template for cDNA synthesis. Lnc RNAs, miRNAs, and mRNAs were reverse-transcribed by using PrimeScript RT Master Mix (Takara, Dalian, China). RT-PCR was performed with ABI StepOne (Carlsbad, CA, USA) following the instructions. The relative quantification of miR-129-5p was carried out by the 2−ΔΔCt method, where U6 was used as the control gene. The expression levels of HMGB1, RSF1-IT2, and SNAI1 were normalized to the levels of internal control GAPDH. Primers for the individual genes were as follows: GAPDH forward, 5′-CAAGGTACCTCAGTGA-CAACCTTG-3′; GAPDH reverse, 5′-GTCCAC-CACCCCTGGCTGTA-3′; U6 forward, 5′-GCTTCGCTGCAGAT-3′; U6 reverse, 5′-GCTTCGCTGCAGAT-3′.
HMGB1 regulates SNAI1 during NSCLC metastasis

X. Wu et al.
GGCACGACATATACTAAAT-3'; U6 reverse, 5'-CGCTTTCCAGATTTGCGTCT-3'; HMGB1 forward, 5'-ATGCTTCACTTACCTCAGGA-3'; HMGB1 reverse, 5'-ATTTTTTCCTTCTTATACG-3'; RSF1-IT2 forward, 5'-CCAGGCTGGAGCCTGG-3'; RSF1-IT2 reverse, 5'-AGGCTGAGGAGGCCGAGAATCGG-3'; miR-129-5p forward, 5'-CGCTTTTTGCGGTCTGG-3'; miR-129-5p reverse, 5'-AGTGCAGGGTCCGAGGTATT-3'; SNAI1 forward, 5'-GGTCTTCTGCCTTCTTCTCTC-3'; SNAI1 reverse, 5'-CCAGGCTGAGGTATCTTCTTGC-3'.

2.13. Bioinformatics analysis

Firstly, the mircode (http://www.mircode.org/) website was used for prediction of lncRNA and miRNA targets. Then, miRNA-mRNA pairs were established using online analysis tools, namely TargetScan (http://www.targetscan.org) and mircode (http://www.mircode.org/).

2.14. Xenograft tumor model in nude mice

Four- to five-week-old female BALB/c nude mice were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China) and quarantined for a week before tumor implantation. The NSCLC xenograft model was established by tail intravenous injection of 2 × 10^6 H1299 cells (transfected with LV-Vector or LV-HMGB1). The mice were put to death after 4 weeks of rearing (n = 12 for each group). Lungs were surgically retrieved from mice, and tumor nodules at lung surface were counted.

2.15. Statistical analysis

Two-tailed Student's t-test was performed to calculate significance in an interval of 95% confidence level. Statistical differences between the means for the different groups were evaluated with Instat 5.0 (GraphPad Software, San Diego, CA, USA) using one-way analysis of variance (ANOVA). The Pearson correlation coefficient was used for correlation analysis. All values are shown as mean ± SD, and a value of P < 0.05 was considered statistically significant. The association between HMGB1 staining and the clinicopathological parameters of the NSCLC patients, including ages, pT status, pN status, and TNM stage, was evaluated by chi-square test. The Kaplan–Meier method and log-rank test were used to evaluate the correlation between HMGB1 expression and patient survival.

3. Results

3.1. HMGB1 expression is high in NSCLC tissues and serves as a potential prognostic indicator for NSCLC

We firstly detected the expression levels of HMGB1 by immunohistochemistry in tissue microarray slides containing 122 NSCLC tissues and 120 paired adjacent NTs. NSCLC tissues exhibited various degrees of HMGB1 expression (Fig. 1A). High HMGB1 expression staining was observed in 82 of 122 (67.4%) NSCLC tissues and 10 of 122 (8.3%) adjacent NTs. Increasing HMGB1 expression was significantly correlated with advanced TNM stages (P = 0.000) and metastasis (P = 0.026). However, we did not find a significant association of HMGB1 expression with patient’s age or sex (Fig. 1B,C). Through Kaplan–Meier and log-rank test analyses in 122 NSCLC patients, high HMGB1 expression was correlated with worse overall survival time (P < 0.05, Fig. 1D). Furthermore, we found that TNM stage (I–II vs III–IV, P = 0.001), pT status (T1–2 vs T3–4, P = 0.001), pN status (N0 vs N+, P = 0.022), and HMGB1 staining (− vs +, P = 0.023) were the risk factors for death in NSCLC patients (Table 1), wherein that TNM stage (P = 0.0014), pT status (P = 0.005) and pN status (P = 0.001) were the independent risk factors for death in NSCLC patients by using Cox multivariate regression analysis (Table 2).

3.2. HMGB1 promotes NSCLC invasion and migration through the up-regulation of SNAI1 expression

Next, we performed loss-of-function and gain-of-function experiments to explore the biological role of HMGB1 in NSCLC cells. The results indicated that HMGB1 accelerated tumor proliferation and inhibited apoptosis in NSCLC cells (Fig. 2A–D). After that, we observed that HMGB1 remarkably promoted cell migration and invasion in H1299 and H460 cells (Fig. 2E,F). To figure out the mechanism of HMGB1 promoting metastasis, the expression of the epithelial–mesenchymal transition (EMT) markers was analyzed by western blot. It revealed that SNAI1, Twist, and Vimentin were significantly increased after overexpressing HMGB1 and attenuated after silencing HMGB1 (Fig. 2G). Moreover, cotransfection of the SNAI1 promoter with HMGB1 plasmid resulted in multiplied promoter activity and HMGB1 could directly bind the SNAI1 promoter region (Fig. 2H,I). Besides, SNAI1 knockdown could reverse the induction of tumor...
Fig. 4. MiR-129-5p targets both SNAI1 and HMGB1. (A) qRT-PCR showed that miR-129-5p inhibitor up-regulated SNAI1 expression and miR-129-5p mimics decreased SNAI1 expression (n = 3, mean ± SD, t-test). (B) Western blot showed SNAI1 increased in miR-129-5p inhibitor group and decreased in miR-129-5p mimics group in H1299 and H460 cells. β-actin is the internal control. (C) The potential binding sites between SNAI1 and miR-129-5p. (D) WT-SNAI1 or MUT-SNAI1 was cotransfected into H1299 cells with miR-129-5p mimics or their corresponding negative controls (n = 3, mean ± SD, t-test). (E) qPCR showed that miR-129-5p inhibitor up-regulated HMGB1 expression and miR-129-5p mimics decrease HMGB1 expression (n = 3, mean ± SD, t-test). (F) Western blot showed HMGB1 increased in miR-129-5p inhibitor group and decreased in miR-129-5p mimics group in H1299 and H460 cells. β-actin was the internal control. (G) The potential binding sites between HMGB1 and miR-129-5p. (H) WT-HMGB1 or MUT-HMGB1 was cotransfected into H1299 cells with miR-129-5p mimics or their corresponding negative controls (n = 3, mean ± SD, t-test) (*P < 0.05, **P < 0.01, ***P < 0.001).
invasion by HMGB1 in NSCLC cells (Fig. 2J). These results manifested that HMGB1 promoted NSCLC migration and invasion through up-regulating SNAI1 expression at the transcriptional level.

3.3 HMGB1-induced RSF1-IT2 promotes NSCLC cell migration and invasion through sponging miR-129-5p

As a nonhistone DNA-binding protein, HMGB1 could affect lncRNA expression in lung cancer progression. Therefore, we selected the top 30 up-regulated lncRNAs in NSCLC using TCGA database and performed qPCR to ulteriorly verify whether HMGB1 regulated lncRNA level in cancer metastasis (Figs S1 and S2). Intriguingly, it was found that HMGB1 could increase the expression of RSF1-IT2 through binding RSF1-IT2 promoter (Fig. 3A,B, Fig. S3). Thus, we inferred that RSF1-IT2 could function as an oncogene to accelerate NSCLC cell proliferation and invasion. It was indicated that RSF1-IT2 overexpression was effective in promoting NSCLC cell growth and invasion (Fig. 3I,J). Together with these results, RSF1-IT2 increased SNAI1 expression at the levels of RNA and protein (Fig. 3E,F). To certify the mechanism of RSF1-IT2 promoting cell invasion, we used MIRCODE (http://www.mircode.org/) software to search for the predicted potential target miRNAs of RSF1-IT2. The data revealed that miR-129-5p had putative RSF1-IT2 binding sites and was down-regulated by RSF1-IT2, indicating RSF1-IT2 could act as a ceRNA for miR-129-5p thus facilitating metastasis in NSCLC (Fig. 3G,H, Fig. S4). Furthermore, miR-129-5p mimics attenuated the effect of RSF1-IT2 on tumor growth and invasion (Fig. 3I,J). Together with these observations, RSF1-IT2 induced NSCLC cell proliferation, migration, and invasion through sponging miR-129-5p.

3.4 MiR-129-5p targets both SNAI1 and HMGB1

Since RSF1-IT2 can sponge miR-129-5p, the interactions between miR-129-5p and SNAI1 were explored. After being treated with miR-129-5p mimics, SNAI1 expression was evidently reduced, and knockdown of miR-129-5p up-regulated SNAI1 (Fig. 4A,B). Through blasting the binding site of miR-129-5p and SNAI1, we constructed luciferase vectors of mutant SNAI1 3'UTR and wild-type. The results proved that the luciferase activities were significantly decreased after transfected with wild-type SNAI1 3' UTR vector together with miR-129-5p mimics, but not mutant SNAI1 3'UTR (Fig. 4C,D). Interestingly, we found miR-129-5p also targeted HMGB1 through miRcode (http://www.mircode.org/) software. Similarly, miR-129-5p could inhibit the expression of HMGB1 (Fig. 4E,F). The luciferase activity reflected the interaction between wild-type HMGB1 3' UTR and miR-129-5p mimics (Fig. 4G,H). In a word, the above data demonstrated the negative regulatory effect of miR-129-5p on SNAI1 and HMGB1 expression.

3.5 RSF1-IT2 and SNAI1 are up-regulated, whereas miR-129-5p is down-regulated in NSCLC tissues

Next, we investigated RSF1-IT2, SNAI1, and miR-129-5p expression in 122 NSCLC tissues. RSF1-IT2 high expression was observed in 49 of 82 (59.8%) HMGB1 highly expressed NSCLC tissues (Fig. 5A,B). RSF1-IT2 expression was elevated in NSCLC tissues, and high RSF1-IT2 expression was associated with advanced TNM stages, metastasis, and poor prognosis (Fig. 5C,D). SNAI1 was also expressed in different levels through immunohistochemistry in 122 NSCLC tissues. SNAI1 expression was positively correlated with HMGB1 expression (Fig. 5E,F). Meanwhile,
HMGB1 regulates SNAI1 during NSCLC metastasis

X. Wu et al.
SNAI1 high expression was related to advanced TNM stages, metastasis, and poor prognosis in NSCLC (Fig. 5G,H). Additionally, miR-129-5p was negatively correlated with HMGB1 expression and down-regulated in most NSCLC tissues compared with adjacent NTs (Fig. 5I–K). The reduced expression of miR-129-

Fig. 6. HMGB1 promotes expression of RSF1-IT2 and SNAI1, as well as NSCLC metastasis in vivo. (A) Fluorescence microscope and western blot detect the infection efficiency of lentivirus in H1299 cells (magnification ×100, scale bar 100 μm). (B) Representative images of lungs with metastatic nodules 4 weeks after injection of LV-Vector-H1299 or LV-HMGB1-H1299 cells (n = 12, mean ± SD, t-test, ***P < 0.001). (C) Representative images of HE staining in lung nodules of tumor models (magnification ×200, ×400, scale bar 50 μm). (D) Western blot analyzed the protein levels of HMGB1 and SNAI1 in harvested tumor tissues. (E) qRT-PCR investigated the RSF1-IT2, miR129-5p, and SNAI1 expression in harvested tumor tissues (n = 3, mean ± SD, t-test, *P < 0.05). (F) Representative images of HMGB1, SNAI1, E-cadherin immunohistochemical staining with HMGB1, and SNAI1 antibody in LV-Vector and LV-HMGB1 groups (magnification ×200, scale bar 50 μm).
HMGB1 regulates SNAI1 during NSCLC metastasis

X. Wu et al.

5p was linked with advanced TNM stages, metastasis, and poor prognosis (Fig. 5K,L).

3.6 HMGB1 promotes RSF1-IT2 and SNAI1 expression, as well as NSCLC metastasis in vivo

To investigate the effect of HMGB1 on NSCLC metastasis in vivo, xenograft cancer models were established by subcutaneously inoculating H1299 cells stably transfected with HMGB1 (Fig. 6A). Through observation, it was found that the mice injected with LV-HMGB1-H1299 cells exhibited more metastatic nodules (P < 0.001, Fig. 6B). Pathological report of lung nodules was consistent with metastatic tumor of NSCLC (Fig. 6C). Additionally, we observed that RSF1-IT2 and SNAI1 expression increased, whereas miR-129-5p expression decreased from harvested tumor tissues in the LV-HMGB1 group (Fig. 6D–F). These findings were identical to the in vitro results described above, which firmly validated that HMGB1 promoted RSF1-IT2 and SNAI1 expression as well as NSCLC metastasis in vivo.

4. Discussion

HMGB1, as a nonhistone chromosome-binding protein, could promote tumor invasion and metastasis (Shen et al., 2009; Swartz, 2014). In the present study, we identified HMGB1 expression was significantly increased in NSCLC tissues. HMGB1, a risk factor for death in NSCLC patients, was closely related to advanced TNM stages and poor prognosis. It has been widely studied a series of biological behaviors (Cao et al., 2017; Ge et al., 2019). Our data suggested that RSF1-IT2 could competitively bind miR-129-5p and reversely regulate its expression. Thus, we implied that RSF1-IT2 promoted tumor metastasis through sponging miR-129-5p. During our study of how the HMGB1-RSF1-IT2-miR-129-5p exerted its influence on lung cancer, an interesting positive loop revealed itself. RSF1-IT2 overexpression significantly rescued the silencing effect of miR-129-5p on SNAI1 protein expression. Moreover, miR-129-5p also targeted HMGB1 and regulated EMT process as a tumor suppressor (Li et al., 2017). The above results supported the conclusion that HMGB1 regulated RSF1-IT2 thus reducing the available miR-129-5p to target SNAI1. HMG1 was also targeted by miR-129-5p in turn, which just formed a positive feedback loop (Fig. 7).

The lncRNA RSF1-IT2 was derived from RSF1 gene, and its role in NSCLC had not been reported. RSF1 was highly expressed in various tumors and identified as an independent prognostic marker in prostate cancer (He et al., 2019; Höflmayer et al., 2019). Besides, a previous report analyzed TCGA datasets to systematically identify lncRNAs related to gastric cancer. It was demonstrated that 1294
Intronic non-coding RNAs including RSF1-IT2 were differentially expressed in gastric cancer. And RSF1-IT2 was independently associated with overall survival of gastric cancer (Han et al., 2017). On the basis of the above studies, the biological functions of RSF1-IT2 in NSCLC were further investigated. In our study, RSF1-IT2 promoted NSCLC cell proliferation and miR-129-5p mimics abrogated RSF1-IT2-mediated increase in cell proliferation. Collectively, RSF1-IT2 promoted tumor growth and metastasis at least in part via the miR-129-5p axis. Importantly, high expression of RSF1-IT2 was found closely correlated with tumor progression in both HMGB1-overexpressed xenograft nude mice and NSCLC patients. These results demonstrated the poorly known RSF1-IT2 might act as a useful diagnostic biomarker for NSCLC patients.

The present study had confirmed that HMGB1 was a tumor-promoting factor; however, increasing evidence showed that HMGB1 played dual effects on tumor. On the one hand, HMGB1 could promote the maturation of dendritic cells and enhanced the reactivity to lymph node chemokines, thereby stimulating the occurrence of immune response to produce antitumor effect (Liu et al., 2011). On the other hand, HMGB1 also promoted the tumor angiogenesis thus slowing down the apoptosis of cancer cells (Wu et al., 2018b). Besides, HMGB1 resulted in the resistance of tumor cells to chemoradiotherapy through the release of Beclin1 protein inducing autophagy (Jin and Choi, 2012; Kong et al., 2015). Some studies have shown that the bidirectional effect of HMGB1 on antitumor immunity was closely related to its extracellular concentration and reductive state of molecules. High concentration and reductive state of HMGB1 mainly induced autophagy and promoted tumor immune escape, which was related to tumor metastasis (Kusume et al., 2009; Tang et al., 2010a). Further studies are needed to elucidate the mechanism and signaling pathways of HMGB1 and how to activate its own antitumor immunity effect, which will be of great help to the antitumor treatment of HMGB1.

5. Conclusions

Our study revealed that HMGB1 enhanced the proliferation, migration, and invasion of NSCLC. We demonstrated that HMGB1 up-regulated SNAI1 expression in a direct transcriptional activation and RSF1-IT2-dependent manner during NSCLC metastasis. RSF1-IT2 reduced the available miR-129-5p to target SNAI1, and HMGB1 was also targeted by miR-129-5p in turn. We also firstly reported RSF1-IT2 played key parts in tumor progression. The findings in this study provided new insights into the understanding of the molecular mechanism involved in tumor metastasis and served a potential prognosis marker and therapeutic target for NSCLC.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

XW, YC, and WG performed the experiments and wrote the paper; WG, TL, and HD analyzed and interpreted the data; WW, MX, and GM partially participated in the experiments; XW and DP designed and supervised the project. All authors read and approved the final manuscript.
Ethics statement

This study was performed under a protocol approved by the Review Board of the Affiliated Xuzhou Municipal Hospital of Xuzhou Medical University, and all examinations were performed after obtaining written informed consents. Animal experiments were in conformance with the Institutional Animal Care and Use Committee of Xuzhou Medical University.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

References

Angelopoulou E, Piperi C, Adamopoulos C and Papavassiliou AG (2016) Pivotal role of high-mobility group box 1 (HMGB1) signaling pathways in glioma development and progression. J Mol Med (Berl) 94, 867–874.

Argast GM, Krueger JS, Thomson S, Sujka-Kwok I, Carey K, Silva S, O’Connor M, Mercado P, Mulford IJ and Young GD (2011) Inducible expression of TGF, snail and Zeb1 recapitulates EMT in vitro and in vivo in a NSCLC model. Clin Exp Metastasis 28, 593–614.

Asamura H, Chansky K, Crowley J, Goldstraw P, Rusch VW, Vansteenkiste JF, Watanabe H, Wu YL, Zielinski M, Ball D et al. (2015) The International Association for the Study of Lung Cancer Lung Cancer Staging Project: proposals for the revision of the N descriptors in the forthcoming 8th edition of the TNM classification for lung cancer. J Thorac Oncol 10, 1675–1684.

Avtsanski DB, Nagalingam A, Bonner MY, Arbiser JL, Saxena NK and Sharma D (2014) Honokiol inhibits epithelial-mesenchymal transition in breast cancer cells by targeting signal transducer and activator of transcription 3/Zeb1/E-cadherin axis. Mol Oncol 8, 565–580.

Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, Chow LQ, Vokes EE, Felip E, Golgado E et al. (2015) Nivolumab versus docetaxel in advanced nonsquamous non–small-cell lung cancer. N Engl J Med 373, 1027–1039.

Cao C, Zhang T, Zhang D, Xie L, Zou X, Lei L, Wu D and Liu F (2017) The long non-coding RNA, SNHG6-003, functions as a competing endogenous RNA to promote the progression of hepatocellular carcinoma. Oncogene 36, 1112–1122.

Chen Y, He F and Lin C (2012) The role of HMGB1 in cancer metastasis and its mechanism. China Oncol 22, 784–789.

Cheng M, Liu H, Zhang D, Liu Y, Wang C, Liu F and Chen J (2015) HMGB1 enhances the AGE-induced expression of CTGF and TGF-B via RAGE-dependent signaling in renal tubular epithelial cells. Am J Nephrol 41, 257–266.

Chuangui C, Peng T and Zhentao Y (2012) The expression of high mobility group box 1 is associated with lymph node metastasis and poor prognosis in esophageal squamous cell carcinoma. Pathol Oncol Res 18, 1021–1027.

Ettinger DS, Wood DE, Akerley W, Bazhenova LA, Borghaei H, Camidge DR, Cheney RT, Chirieac LR, D’Amico TA, Dilling TJ et al. (2016) NCCN guidelines insights: non-small cell lung cancer, version 4.2016. J Natl Compr Cancer Netw 14, 255–264.

Feng A, Tu Z and Yin B (2016) The effect of HMGB1 on the clinicopathological and prognostic features of non-small cell lung cancer. Oncotarget 15, 20507–20519.

Ge X, Li GY, Jiang L, Jia L, Zhang Z, Li X, Wang R, Zhou M, Zhou Y and Zeng Z (2019) Long noncoding RNA CAR10 promotes lung adenocarcinoma metastasis via miR-203/30/SNAI axis. Oncogene 38, 3061–3076.

Han W, Zhang Z, He B, Xu Y, Zhang J and Cao W (2017) Integrated analysis of long non-coding RNAs in human gastric cancer: an in silico study. PLoS ONE 12, e0183517.

He J, Fu L and Li Q (2019) Rsf-1 regulates malignant melanoma cell viability and chemoresistance via NF-kB/Bcl-2 signaling. Mol Med Rep 20, 3487–3498.

Heijink IH, van Oosterhout A and Kapus A (2010) Epidermal growth factor receptor signalling contributes to house dust mite-induced epithelial barrier dysfunction. Eur Respir J 36, 1016–1026.

Höffmayer D, Hamuda M, Schroeder C, Hube-Magg C, Simon R, Göbel C, Hinsch A, Weidemann S, Möller K and Izbicki JR (2019) High RSF1 protein expression is an independent prognostic feature in prostate cancer. Acta Oncol 5, 1–6.

Huang W, Zhao H, Dong H, Wu Y, Yao L, Zou F and Cai S (2016) High-mobility group box 1 impairs airway epithelial barrier function through the activation of the RAGE/ERK pathway. Int J Mol Med 37, 1189–1198.

Jin S, Yang X, Li J, Yang W, Ma H and Zhang Z (2019) p53-targeted lincRNA-p21 acts as a tumor suppressor by inhibiting JAK2/STAT3 signaling pathways in head and neck squamous cell carcinoma. Mol Cancer 18, 38.

Jin Y and Choi AM (2012) Cross talk between autophagy and apoptosis in pulmonary hypertension. Pulm Circ 2, 407–414.

Kong Q, Xu LH, Xu W, Fang JP and Xu HG (2015) HMGB1 translocation is involved in the transformation of autophagy complexes and promotes chemoresistance in leukaemia. Int J Oncol 47, 161–170.
Kroepil F, Fluegen G, Totikov Z, Baldus SE, Vay C, Schauer M, Topp SA, Esch JS, Knoefel WT and Stoecklein NH (2012) Down-regulation of CDH1 is associated with expression of SNAI1 in colorectal adenomas. *PLoS ONE* 7, e46665.

Kusume A, Sashairu T, Luo Y, Isobe M, Nakagawa N, Tatsumoto N, Fujii K, Ohmori H and Kuniyasu H (2009) Suppression of dendritic cells by HMGB1 is associated with lymph node metastasis of human colon cancer. *Pathobiology* 76, 155–162.

Li L and Li W (2015) Epithelial-mesenchymal transition in human cancer: comprehensive reprogramming of metabolism, epigenetics, and differentiation. *Pharmacol Ther* 3, 33–46.

Li XQ, Chen FS, Tan WF, Fang B, Zhang ZL and Ma H (2017) Elevated microRNA-129-5p level ameliorates neuroinflammation and blood-spinal cord barrier damage after ischemia-reperfusion by inhibiting HMGB1 and the TLR3-cytokine pathway. *J Neuroinflammation* 14, 205.

Liu PL, Liu WL, Chang JM, Chen YH, Liu YP, Kuo HF, Hsieh CC, Ding YS, Chen WW and Chong IW (2017) MicroRNA-200c inhibits epithelial-mesenchymal transition, invasion, and migration of lung cancer by targeting HMGB1. *PLoS ONE* 12, e0180844.

Liu QY, Yao YM, Yan YH, Dong N and Sheng ZY (2011) High mobility group box 1 protein suppresses T cell-mediated immunity via CD11c(low)CD45RB(high) dendritic cell differentiation. *Cytokine* 54, 205–211.

Luan Z, Hu B, Wu L, Jin S, Ma X, Zhang J and Wang A (2018) Unfractionated heparin alleviates human lung endothelial barrier dysfunction induced by high mobility group box 1 through regulation of P38-GSK3β-snail signaling pathway. *Cell Physiol Biochem* 46, 1907–1918.

Lv DJ, Song XL, Huang B, Yu YZ, Shu FP, Wang C, Chen H, Zhang HB and Zhao SC (2019) HMGB1 promotes prostate cancer development and metastasis by interacting with brahma-related gene 1 and activating the Akt signaling pathway. *Theranostics* 9, 5166–5182.

Lv W, Chen N, Lin Y, Ma H, Ruan Y, Li Z, Li X, Pan X and Tian X (2016) Macrophage migration inhibitory factor promotes breast cancer metastasis via activation of HMGB1/TLR4/NF kappa B axis. *Cancer Lett* 375, 245–255.

Mikami S, Katsume K, Oya M, Ishida M, Kosaka T, Mizuno R, Mukai M and Okada Y (2011) Expression of Snail and Slug in renal cell carcinoma: E-cadherin repressor Snail is associated with cancer invasion and prognosis. *Lab Invest* 91, 1443–1458.

Pan YJ, Wei LL, Wu XJ, Huo FC, Mou J and Pei DS (2017) MiR-106a-5p inhibits the cell migration and invasion of renal cell carcinoma through targeting PAK5. *Cell Death Dis* 8, e3155.

Postmus PE, Kerr KM, Oudkerk M, Senan S, Waller DA, Vansteenkiste J, Escrún C and Peters S (2013) Early and locally advanced non-small-cell lung cancer (NSCLC): ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 24, 89–98.

Salmena L, Poliseno L, Tay Y, Kats L and Pandolfo PP (2011) A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? *Cell* 146, 353–358.

Shen X, Hong L, Sun H, Shi M and Song Y (2009) The expression of high-mobility group protein box 1 correlates with the progression of non-small cell lung cancer. *Oncol Rep* 22, 535–539.

Su W and Bi M (2012) The expression of HMGB1/MMP9 and its clinical significance in non-small cell lung cancer. *J Clin Paln* 17, 1463–1465.

Swartz MA (2014) Immunomodulatory roles of lymphatic vessels in cancer progression. *Cancer Immunol Res* 2, 701–707.

Tang D, Kang R, Cheh CW, Livesey KM, Liang X, Schapiro NE, Benschop R, Sparvero LJ, Amoscoo AA, Tracey KJ et al. (2010a) HMGB1 release and redox regulates autophagy and apoptosis in cancer cells. *Oncogene* 29, 5299–5310.

Tang D, Kang R, Zeh HJ and Lotze MT (2010b) High-mobility group box 1 and cancer. *Biochim Biophys Acta* 1799, 131–140.

Wang X, Xiang L, Li H, Chen P, Feng Y, Zhang J, Yang N, Li F, Wang Y, Zhang Q et al. (2015) The role of HMGB1 signaling pathway in the development and progression of hepatocellular carcinoma: a review. *Int J Mol Sci* 16, 22527–22540.

Wolfson RK, Chiang ET and Garcia JG (2011) HMGB1 induces human lung endothelial cell cytoskeletal rearrangement and barrier disruption. *Microvasc Res* 81, 189–197.

Wu D, Liu J, Chen J, He H, Ma H and Lv X (2017) Overexpression of Rsf-1 correlates with poor survival and promotes invasion in non-small cell lung cancer. *Virchows Arch* 470, 553–560.

Wu D, Liu J, Chen J, He H, Ma H and Lv X (2018a) MiR-449a suppresses tumor growth, migration and invasion in non-small cell lung cancer by targeting HMGB1-mediated NF-κB signaling way. *Oncol Rep* 27, 227–235.

Wu L and Yang L (2018) The function and mechanism of HMGB1 in lung cancer and its potential therapeutic implications. *Oncol Lett* 15, 6799–6805.

Wu XJ, Chen YY, Gong CC and Pei DS (2018b) The role of high-mobility group protein box 1 in lung cancer. *J Cell Biochem* 119, 6354–6365.

Xuan W, Yu H and Zhang X (2019) Crosstalk between the lncRNA UCA1 and microRNAs in cancer. *FEBS Lett* 583, 1901–1914.

Yang YI, Ahn JH, Lee KT, Shih IM and Choi JH (2014) RSF1 is a positive regulator of NF-κB-induced gene...
expression required for ovarian cancer chemoresistance. 
*Cancer Res* 74, 2258–2269.

Yao RW and Wang Y (2019) Cellular functions of long noncoding RNAs. *Nat Cell Biol* 21, 542–551.

Zhang X, Xue D, Hao F, Xie L, He J, Gai J, Liu Y, Xu H, Li Q and Wang E (2018) Remodeling and spacing factor 1 overexpression is associated with poor prognosis in renal cell carcinoma. *Oncol Lett* 15, 3852–3857.

Zhang YC, Huo FC, Wei LL, Gong CC, Pan YJ, Mou J and Pei DS (2017) PAK5-mediated phosphorylation and nuclear translocation of NF-κB-p65 promotes breast cancer cell proliferation in vitro and in vivo. *J Exp Clin Cancer Res* 36, 146.

Zuo Z, Che X, Wang Y, Li B, Li J, Dai W, Lin CP and Huang C (2014) High mobility group Box-1 inhibits cancer cell motility and metastasis by suppressing activation of transcription factor CREB and nWASP expression. *Oncotarget* 5, 7458–7470.

**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Heatmap of highly expressed lncRNAs in lung cancer tissues using TCGA database.

**Fig. S2.** qPCR was performed to investigate the lncRNA levels in H1299 cells after transfected with si-HMGB1 and pcDNA3.1-HMGB1.

**Fig. S3.** ChIP analysis of RSF1-IT2 promoter regions used anti-HMGB1 antibody.

**Fig. S4.** The sub-cellular localization of RSF1-IT2.