Heterogeneous nuclear ribonucleoprotein A2/B1 regulates the ERK and p53/HDM2 signaling pathways to promote the survival, proliferation and migration of non-small cell lung cancer cells

MIN KYU KIM[1*], MUN JU CHOI[1*], HYUN MIN LEE[1], HONG SEO CHOI[1], YOUNG-KWON PARK[2] and CHUN JEIH RYU[1]

[1]Department of Integrative Bioscience and Biotechnology, Institute of Anticancer Medicine Development, Sejong University, Seoul 05006; [2]Prevention and Management Center, Ulsan University Hospital, Ulsan 44033, Republic of Korea

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Abstract. Lung cancer is the most frequent cause of cancer-associated mortality worldwide. Upregulation of heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2/B1) has been reported in non-small cell lung cancer (NSCLC) cells, but its contribution to NSCLC remains poorly understood. hnRNPA2/B1 is involved in carcinogenesis by interacting with a number of proteins; however, little is known about its interaction with p53. The results of the present study revealed that hnRNPA2/B1 expression levels were upregulated in NSCLC cells under tumorsphere culture conditions and cisplatin treatment compared with those in cells under the adherent condition and dimethyl sulfoxide treatment, respectively, suggesting that hnRNPA2/B1 expression is induced under stress conditions. hnRNPA2/B1 knockdown decreased the number and size of NSCLC cell colonies in a clonogenic survival assay and led to a decreased migratory potential of NSCLC cells, suggesting that hnRNPA2/B1 may promote the survival, proliferation and migration of NSCLC cells. hnRNPA2/B1 knockdown induced G_0/G_1 phase arrest in NSCLC cells through cyclin E degradation and phosphorylation of cyclin-dependent kinase 2. In addition, hnRNPA2/B1 knockdown inhibited extracellular signal-regulated kinase (ERK)1/2 phosphorylation, suggesting that hnRNPA2/B1 may promote the G_1/S phase transition in NSCLC cells through ERK signaling. hnRNPA2/B1 knockdown resulted in increased expression levels of p21 and p27 in NSCLC cells, as well as p53 induction and phosphorylation. Additionally, hnRNPA2/B1 knockdown inhibited human double minute 2 protein (HDM2) stability and phosphorylation, whereas overexpression of hnRNPA2 induced the opposite effects. These results suggested that hnRNPA2/B1 may promote the survival, proliferation and migration of NSCLC cells through preventing the activation of p53, which is induced by ERK-mediated HDM2 activation. The results of the present study also indicated that the components of the hnRNPA2/B1/ERK/p53/HDM2 signaling pathway may be novel potential molecular targets for the treatment of patients with NSCLC.

Introduction

Lung cancer is the most frequent cause of cancer-associated mortality worldwide (1). Non-small cell lung cancer (NSCLC) represents ~85% of all lung cancer types (2). Multiple genetic alterations have been observed in patients with NSCLC, including mutations in the Kirsten rat sarcoma (KRAS; 25-30%), epidermal growth factor receptor (EGFR; 10-35%), fibroblast growth factor (20%) and anaplastic lymphoma kinase (5-7%) genes (3). The USA Food and Drug Administration has approved numerous drugs such as gefitinib and erlotinib against these mutated genes; however, the 5-year overall survival of patients with NSCLC remains low (~16%) (3,4). Therefore, there is an urgent need to identify novel oncogenes with the aim of improving the survival of patients with NSCLC.

Heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2/B1) has two isoforms, A2 and B1, which are derived from alternative splicing variants of the same gene (5). B1 has a 12-amino acid insertion at the N-terminus of A2 (5). hnRNPA2/B1 serves major roles in RNA processing, splicing, transport and stability through direct interaction with mRNAs (6). Previous studies have reported that hnRNPA2/B1 has multiple functions in numerous cellular

Correspondence to: Professor Chun Jeih Ryu, Department of Integrative Bioscience and Biotechnology, Institute of Anticancer Medicine Development, Sejong University, 209 Neungdong-ro, Gwangjin-gu, Seoul 05006, Republic of Korea
E-mail: cjryu@sejong.ac.kr

*Contributed equally

Abbreviations: HDM2, human double minute 2 protein; hnRNPA2/B1, heterogeneous nuclear ribonucleoprotein A2/B1; NSCLC, non-small cell lung carcinoma; hESCs, human embryonic stem cells

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processes, including proliferation (7), metabolism (8), migration (9), survival and apoptosis (10). hnRNPA2/B1 also regulates the stability of a number of downstream target genes such as CCR4-NOT transcription complex subunit (CNOT)13, CNOT4 and CNOT6 through a sequence-specific mRNA decay pathway (11). hnRNPA2B1 has also been reported to be a novel nuclear DNA sensor that initiates antiviral innate immunity (12). hnRNPA2/B1 is upregulated in various types of human cancer, including lung (13), colon (14), breast (15), pancreatic (16), gastric (17,18), brain (8,19) and liver (20) cancer. hnRNPA2/B1 promotes pancreatic cancer progression by providing a link between protein kinase B (AKT)/mammalian target of rapamycin and KRAS-dependent mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (ERK) signaling (21). hnRNPA2/B1 has been reported to contribute to the tumorigenic potential of breast cancer cells via signal transducer and activator of transcription 3 and ERK signaling (22), although this has been challenged by another study describing hnRNPA2/B1 as a negative regulator of breast cancer metastasis (23). Since hnRNPB1 has been considered to be an early detection marker for lung cancer (24), previous studies have demonstrated that high expression levels of hnRNPA2/B1 lead to NSCLC through the induction of cell proliferation and the epithelial-mesenchymal transition (EMT) (25,26). However, the precise signaling mechanism of the regulation of NSCLC growth and metastasis by hnRNPA2/B1 remains elusive.

Our previous study demonstrated that hnRNPA2/B1 maintained the self-renewal and pluripotency of human embryonic stem cells (hESCs) via the control of the G_1/S phase transition, and that hnRNPA2/B1 negatively regulated the EMT process in hESCs (27). The present study aimed to examine the effects of hnRNPA2/B1 on the survival, number and migration of NSCLC cells, and to provide a mechanistic insight into how hnRNPA2/B1 may regulate the survival, number and migration of NSCLC cells.

Materials and methods

Cell culture. The human NSCLC cell lines NCI-H358, A549, NCI-H1703 and NCI-H460 were purchased from the Korean Cell Line Bank and cultured according to the supplier's protocol. The 293T cell line was obtained from the American Type Culture Collection and cultured according to the supplier's protocol. Cell line authentication was performed by short tandem repeat DNA profiling (http://cellbank.snu.ac.kr). NCI-H358, A549, NCI-H1703 and H460 cells were cultured in RPMI-1640 medium (Welgene, Inc.), and 293T cells were cultured in DMEM (Welgene, Inc.). All cells were supplemented with 10% fetal bovine serum (VWR International, LLC) and an antibiotic-antimycotic (Welgene, Inc.). All cell lines were cultured at 37˚C in a humidified atmosphere containing 5% CO_2 and were monitored for mycoplasma contamination monthly using a Mycoplasma PCR Detection Kit (Intron Biotechnology, Inc.) according to the manufacturer's instructions. For anticancer drug treatment, A549 cells were treated with dimethyl sulfoxide or 2 μM cisplatin (Sigma-Aldrich; Merck KGaA) in RPMI-1640 medium for 28 days. The medium was changed every 2 days. Well-isolated, large-sized and propidium iodide (PI)-negative populations were regarded as cisplatin-resistant cells in flow cytometric analysis. All dead cells were washed out and remaining cells were subjected to western blot analysis. Tumorsphere culture was performed using A549 cells as previously described (28).

Flow cytometry. Flow cytometric analysis was performed as described previously (29). To detect apoptosis, cancer cells were analyzed with PI and Annexin V using an Annexin V-FITC/PI Apoptosis kit (BD Biosciences) according to the manufacturer's protocol. For cell cycle analysis, A549 cells transfected with siCon or siA2B1 were analyzed with bromodeoxyuridine (BrdU) as previously described (27). Briefly, A549 cells ($1x10^6$) were treated with 30 mM BrdU (Thermo Fisher Scientific, Inc.) for 4 h at 48 h post-transfection. Detached cells were fixed in 70% ethanol overnight at 4˚C. The cells were subsequently suspended in denaturation buffer (2 M HCl/0.5% Triton X-100) for 30 min and 0.1 M sodium borate for 2 min following washing. Then, the cells were incubated with an Alexa 488-conjugated anti-BrdU antibody (1:200 dilution; cat. no. 364105; BioLegend, Inc.) for 30 min at room temperature and incubated in an RNAase A (10 μg/ml) and PI (20 μg/ml) solution for 30 min at room temperature prior to flow cytometric analysis.

Small interfering RNA (siRNA) transfection. siRNA oligonucleotides targeting hnRNPA2/B1 (siA2B1) were synthesized by Bioneer Corporation and used with AccuTarget™ Negative Control siRNA (Bioneer Corporation). The sequences of siRNAs targeting human hnRNPA2/B1 were as follows: siA2B1 #1 sense, 5'-CGGUGGAAAUUUCGGACCTT-3' and antisense, 5'-UGGUCCGAAUUUCCACCGTT-3'; #2 sense, 5'-CUGAAG UUGUUUAGGUUCUTT-3' and antisense, 5'-AGAACCUAA ACAACUUCAGTT-3'; and #3 sense, 5'-UGAGACAGUUUC UUAGCUUTT-3' and antisense, 5'-AAGCTAAGAAACUGU CUCATT-3'. A549 cells ($1.5x10^5$) were transfected with 100 nM control siRNA (siCon) or each siA2B1 using Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The cells were incubated at 37˚C for 48 h before harvesting for analysis. For H460 cells, cells ($1.5x10^5$) were transfected the second time at 36 h following the first transfection and incubated for additional 36 h prior to subsequent analysis.

Overexpression of hnRNPA2 in 293T cells. The human hnRNPA2 gene was cloned into the pCMV-Myc-DDK vector (OriGene Technologies, Inc.) with Myc and DDK tags at the C-terminus. 293T ($1x10^6$) cells were transiently transfected with pCMV-DDK or pCMV-hnRNPA2-Myc-DDK plasmids (2 μg/well) in 6-well plates using polyethylenimine and cultured at 37˚C for 48 h following transfection . Transfected cells were suspended in a lysis buffer [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 2 μg/ml aprotinin, 100 μg/ml phenylmethylsulphonyl fluoride, 5 μg/ml leupeptin, 1 mM NaF and 1 mM Na_3VO_4]. The resulting cell lysates were centrifuged at 14,240 x g for 40 min at 4˚C, collected and used for western blotting.

Clonogenic survival and migration assays. siCon- or siA2B1-transfected A549 cells were harvested at 2 days post-siRNA transfection. The number of viable cells was

determined by the trypan blue exclusion assay. The cells ($1x10^4$) were plated in a 6-well plate with complete medium. Following 7-day incubation, the cells were stained with crystal violet (Sigma-Aldrich; Merck KGaA) at room temperature for 1 h, and the colonies were counted. Briefly, images of the plates were captured by digital camera, and the number of colonies were counted using CellCounter software (http://nghiaho.com/?page_id=1011). A cluster of ≥100 cells was counted as a colony. For the migration assays, A549 cells were suspended in serum-free medium ($2x10^4$ cells/ml) and placed in the upper chamber of Transwell inserts (Corning, Inc.), whereas RPMI1640 containing 10% fetal bovine serum was placed in the lower chamber. Following incubation at 37˚C for 24 h, the cells on the upper surface of the membrane were removed using cotton swab. The cells on the lower surface of the membrane were fixed with 4% paraformaldehyde at room temperature for 1 h. Crystal violet staining was performed at room temperature for 1 h, and cells were counted under a light microscope at x100 magnification in four sections per sample and three samples per group.

Sphere formation assay. siCon- or siA2B1-transfected A549 cells were harvested at 24 h post-siRNA transfection. Cells ($1x10^4$) were resuspended in TeSR™-E8™ medium (Stemcell Technologies, Inc.) and seeded in low-attachment 6-well plates (Corning, Inc.). The spheres were collected and counted under a light microscope at x200 magnification after 5 days of culture.

Reverse transcription-quantitative PCR (RT-qPCR). The relative expression values of EMT-associated markers were analyzed by RT-qPCR using the $2^{-\Delta\Delta Cq}$ method as previously described (30,31). Total RNAs were extracted from H358, A549 and H1703 cells using the RNAiso Plus (Takara Bio, Inc.) according to the manufacturer's protocol. cDNAs were generated from total RNAs using 500 ng total RNAs and the PrimeScript RT Master Mix (Takara Bio, Inc.) at 37˚C for 15 min and 94˚C for 5 sec. qPCR was performed using Applied StepOne™ Real-Time PCR System with SYBR®-Green (Thermo Fisher Scientific, Inc.). The standard reaction contained 12.5 μl 2X PCR buffer (PowerUP™-SYBR™-Green Master Mix; Thermo Fisher Scientific, Inc.), 2 μl cDNA template, and 10 pM of forward and reverse primers in a total volume of 25 μl. The thermocycling conditions were 30 cycles of 1 min at 95˚C, 45 sec at 45-56˚C and 1 min at 72˚C. The target gene-specific primers for human transcripts encoding E-cadherin, hnRNPA2/B1, Snail, Twist, zinc finger E-box-binding homeobox 1 (ZEB1), human double minute 2 protein (HDM2) and GAPDH are presented in Table S1. Each sample was analyzed in triplicate.

Western blotting. A549 cells were lysed in lysis buffer at 4˚C for 30 min. The protein concentration of each sample was determined by bicinchoninic acid protein assay kit. Equal amounts of protein (60 μg) from each sample were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes, blocked with Tris-buffered saline with 0.1% Tween-20 (TBST) with 5% skimmed milk for 1 h at room temperature or TBST with 5% bovine serum albumin (MP Biomedicals, Inc.) for phosphoproteins. The membranes were then incubated with specific primary antibodies diluted to 1:1,000 in 5% skimmed milk overnight at 4˚C, followed by incubation with a secondary antibody for 1 h at room temperature. β-actin (cat. no. sc-84322, Santa Cruz Biotechnology, Inc.) or GAPDH (cat. no. CSB-PA00025A0Rb; Cusabio Technology, LLC) antibodies were used as the loading controls. HDM2 (cat. no. sc-965), cyclin-dependent kinase (CDK)2 (cat. no. sc-6248), p21 (cat. no. sc-6246), glycogen synthase kinase (GSK)-3β (cat. no. sc-7291), ERK1/2 (cat. no. sc-514302), EGFR (cat. no. sc-1005), vimentin (cat. no. sc-5565), Snail (cat. no. sc-28199), ZEB1 (cat. no. sc-25388), E-cadherin (cat. no. sc-7870), phosphorylated (p)-CDK2/cell division cycle (Cdc) 2 (T14/Y15; cat. no. sc-163), cyclin E (cat. no. sc-481), Cdc25A (cat. no. sc-97), p27 (cat. no. sc-528), AKT1 (cat. no. sc-1618), β-catenin (cat. no. sc-7199) and p-AKT1/2/3 (S473; cat. no. sc-7985) primary antibodies were purchased from Santa Cruz Biotechnology, Inc. FLAG antibody (cat. no. M185-3L) was purchased from MBL International Co. Cyclin D1 (cat. no. 2978S), p-CDK2 (T160; cat. no. 2561S), p53 (cat. no. 2524S), p-p53 (S15; cat. no. 9284S), p-p53 (S20; cat. no. 9287S), p-HDM2 (S166; cat. no. 3521S), p-GSK-3β (S9; cat. no. 9322S) and p-ERK1/2 (T202/Y204; cat. no. 9101S) primary antibodies were purchased from Cell Signaling Technology, Inc. C-X-C chemokine receptor type 4 (CXCR4) primary antibody (cat. no. CSB-PA006254GA01HU) was purchased from Cusabio Technology, LLC. Major vault protein (MVP) antibody (cat. no. OAAB16556) was purchased from Aviva Systems Biology Corp. Goat anti-mouse IgG-HRP (1:5,000; cat. no. AP-124P) was purchased from MilliporeSigma. Goat-anti-rabbit IgG-HRP (1:10,000; cat. no. ab6721) was purchased from Abcam. Immunostained protein bands were detected with an enhanced chemiluminescence kit (Advansta, Inc.). Quantifications of Western Blots were performed using ImageJ software (Version 1.8.0; National Institutes of Health).

Statistical analysis. Data are presented as the mean ± SD (n=3). The unpaired Student's t-test was used to analyze the differences between two populations. To compare the expression of five genes in three cell lines, the non-parametric Kruskal-Wallis test was used, followed by post hoc testing using the Mann-Whitney U test with the Bonferroni correction. Analyses were performed with Stata/SE version 13.1 (StataCorp, LLC.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

hnRNPA2/B1 drives the EMT, drug resistance and cancer stemness in NSCLC cells. Previous studies have suggested that hnRNPA2/B1 regulates the stemness and EMT process of hESCs and NSCLC cells (26,27). Among the three NSCLC cell lines examined in the present study (NCI-H358, A549 and NCI-H1703), EMT-associated markers were expressed at intermediate levels in A549 cells (Fig. S1). The levels of hnRNPA2/B1 were also detected at an intermediate level in A549 cells (Fig. S1), suggesting that the role of hnRNPA2/B1 may be representative in A549 cells. hnRNPA2/B1 expression was also induced in the sphere cultures of A549 cells (Fig. 1A),

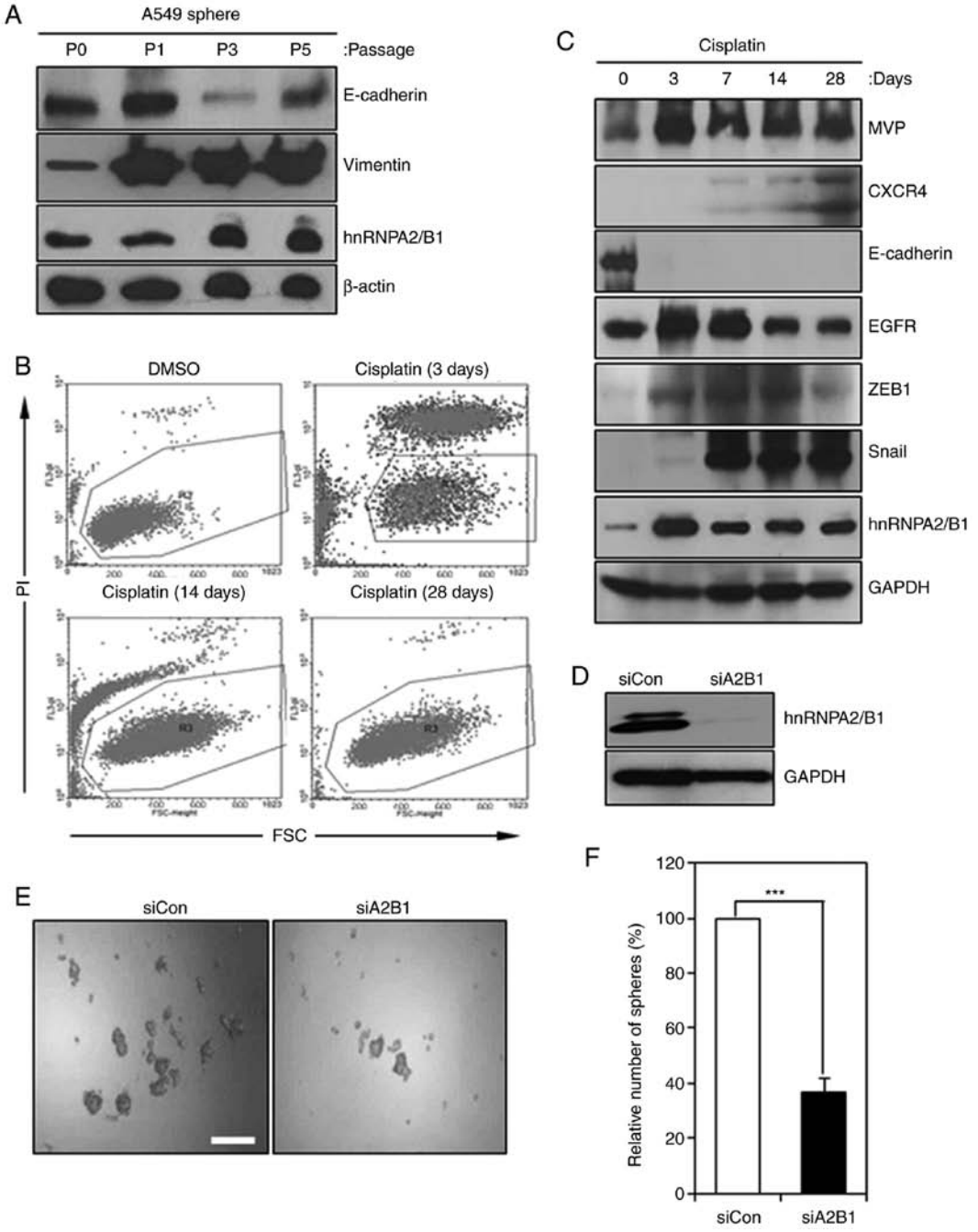

Figure 1. hnRNPA2/B1 regulates the epithelial-mesenchymal transition, drug resistance and cancer stemness in non-small cell lung cancer cells. (A) hnRNPA2/B1 expression was induced in sphere cells of A549. Cancer cells were cultured in sphere medium and subjected to western blot analysis to detect the expression levels of vimentin, E-cadherin and hnRNPA2/B1. β-actin was used as an internal control. (B and C) hnRNPA2/B1 expression was induced in cisplatin-treated A549 cells. A549 cells were treated with cisplatin for 28 days. (B) PI-negative cells (gated) were harvested and subjected to (C) western blot analysis to detect the expression levels of MVP, CXCR4, E-cadherin, EGFR, ZEB1, Snail and hnRNPA2/B1. GAPDH was used as an internal control. (D) Knockdown of hnRNPA2/B1 in A549 cells. Cells were transfected with siCon or siA2B1. Knockdown efficiency was determined by western blot analysis. (E) The sphere formation assays of control siCon- or siA2B1-transfected A549 cells. siCon- or siA2B1-transfected A549 cells were harvested after 24 h of siRNA transfection and were subjected to the sphere forming assays for 5 days. Scale bar, 200 μm. (F) Statistical analysis of the data in E. ***P<0.005. siCon, control small interfering RNA; hnRNPA2/B1, heterogeneous nuclear ribonucleoprotein A2/B1; siA2B1, small interfering RNA targeting hnRNPA2B1; PI, propidium iodide; MVP, major vault protein; CXC4, C-X-C chemokine receptor type 4; ZEB1, zinc finger E-box-binding homeobox 1; FSC, forward scatter.

suggesting that it may be induced during detachment stress. To determine whether hnRNPA2/B1 expression may be associated with NSCLC survival under stress conditions, the expression levels of hnRNPA2/B1 were examined for 28 days in A549 cells under cisplatin treatment as previously described (32). Cisplatin treatment markedly induced cell death (Fig. 1B). When the proteins from the surviving cells were analyzed by western blotting, the levels of mesenchymal markers Snail and ZEB1 were upregulated, whereas the levels of the epithelial marker E-cadherin were downregulated compared with those on day 0 (Fig. 1C). The levels of drug resistance-associated markers MVP, CXCR4 and EGFR were also upregulated 3 days after cisplatin treatment compared with those on day 0 (Fig. 1C). Under cisplatin treatment, hnRNPA2/B1 expression

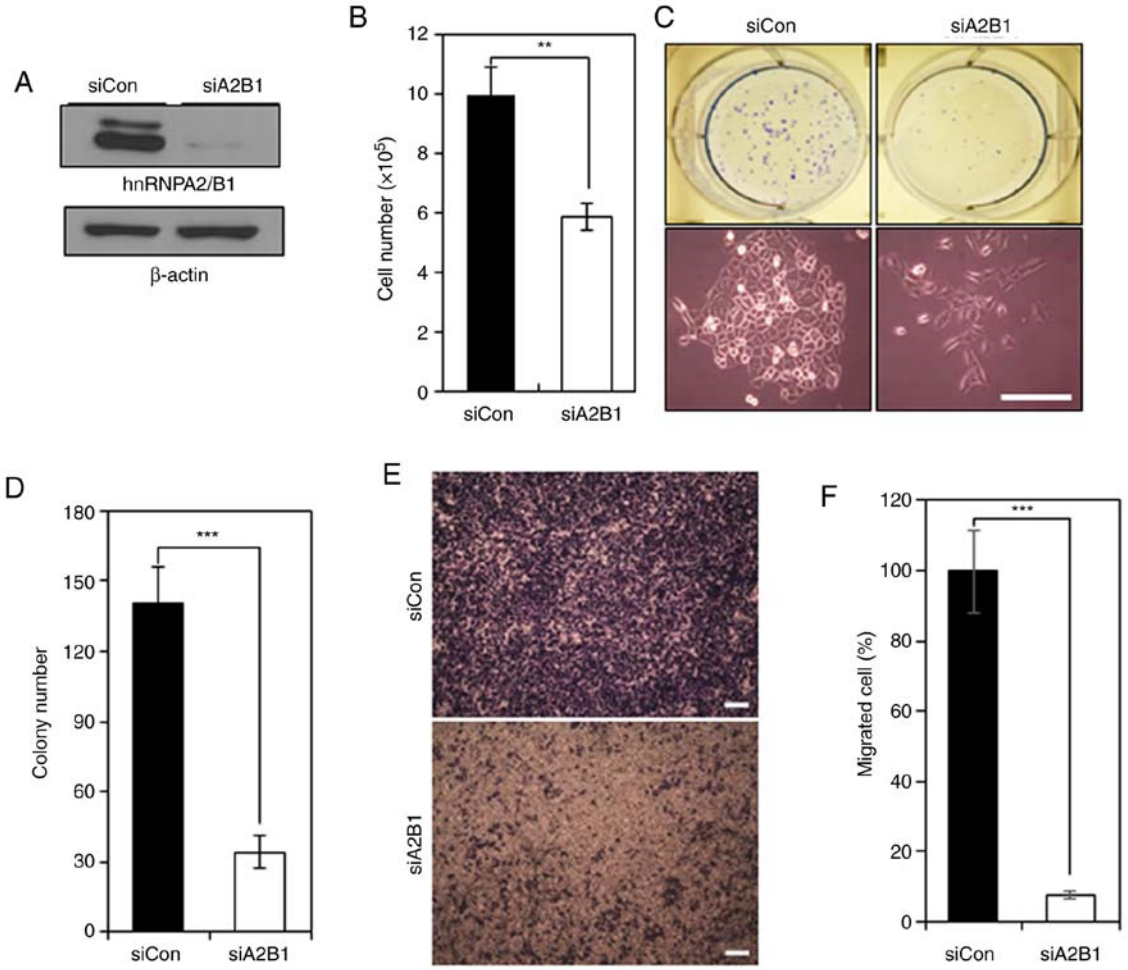

Figure 2. hnRNPA2/B1 is involved in the survival, proliferation and migration of non-small cell lung cancer cells. (A) Knockdown of hnRNPA2/B1 in A549 cells. Cells were transfected with siCon or siA2B1, and knockdown efficiency was determined by western blot analysis. (B) Viability of siCon- or siA2B1-transfected A549 cells was determined by the trypan blue exclusion assay. (C and D) Clonogenic survival assays of siCon- or siA2B1-transfected A549 cells. Transfected cells were cultured for 7 days, and colonies were stained by crystal violet and counted. Scale bar, 100 μm. (E and F) Transwell migration assays of siCon- or siA2B1-transfected A549 cells. Scale bar, 100 μm. **P<0.01 and ***P<0.005. siCon, control small interfering RNA; hnRNPA2/B1, heterogeneous nuclear ribonucleoprotein A2/B1; siA2B1, small interfering RNA targeting hnRNPA2B1.

levels were also upregulated in the surviving cells (Fig. 1C). These results suggest that hnRNPA2/B1 expression may be induced in a stressful environment.

Cancer stemness is associated with EMT and anticancer drug resistance, and the sphere forming assay has been previously used to enrich potential cancer stem cells *in vitro* (28,33). To further analyze whether hnRNPA2/B1 may regulate the stemness of A549 cells, the effects of hnRNPA2/B1 knockdown by RNA interference on the sphere formation ability of A549 cells were evaluated. The sphere forming capacity of hnRNPA2/B1-knockdown A549 cells was decreased by ~63% compared with that of the control A549 cells (Fig. 1D-F), suggesting that hnRNPA2/B1 may regulate the stemness of A549 cells. Taken together, these results suggested that hnRNPA2/B1 may drive the EMT, drug resistance and cancer stemness in NSCLC cells.

hnRNPA2/B1 is required for the survival, proliferation and migration of NSCLC cells. To determine the role of hnRNPA2/B1 in NSCLC cells, hnRNPA2/B1 was knocked down in A549 cells by RNA interference. hnRNPA2/B1 expression was detected at low levels by western blot analysis in A549 cells following hnRNPA2/B1 knockdown (Fig. 2A). hnRNPA2/B1 knockdown decreased the number of A549 cells by ~41% compared with that in the control group (Fig. 2B). hnRNPA2/B1 knockdown decreased the number of A549 colonies by ~80% in the clonogenic assay compared with that in the control group (Fig. 2C and D). However, the number of Annexin V-positive cells was not altered in A549 cells following hnRNPA2/B1 knockdown (Fig. S2), suggesting that hnRNPA2/B1 may promote the clonogenic survival of NSCLC cells irrespective of apoptosis. The migratory potential of A549 cells was decreased by ~92% following hnRNPA2/B1 knockdown compared with that in the control group (Fig. 2E and F). In addition, hnRNPA2/B1 knockdown decreased the expression levels of the mesenchymal marker vimentin and increased the levels of the epithelial marker E-cadherin compared with those in the control group (Fig. S3). Thus, hnRNPA2/B1 may positively regulate the survival, proliferation and migration of NSCLC cells.

hnRNPA2/B1 knockdown induces G_0/G_1 phase arrest in NSCLC cells. Previous studies have reported that hnRNPA2/B1 promotes G_1/S transition in carcinoma cells and

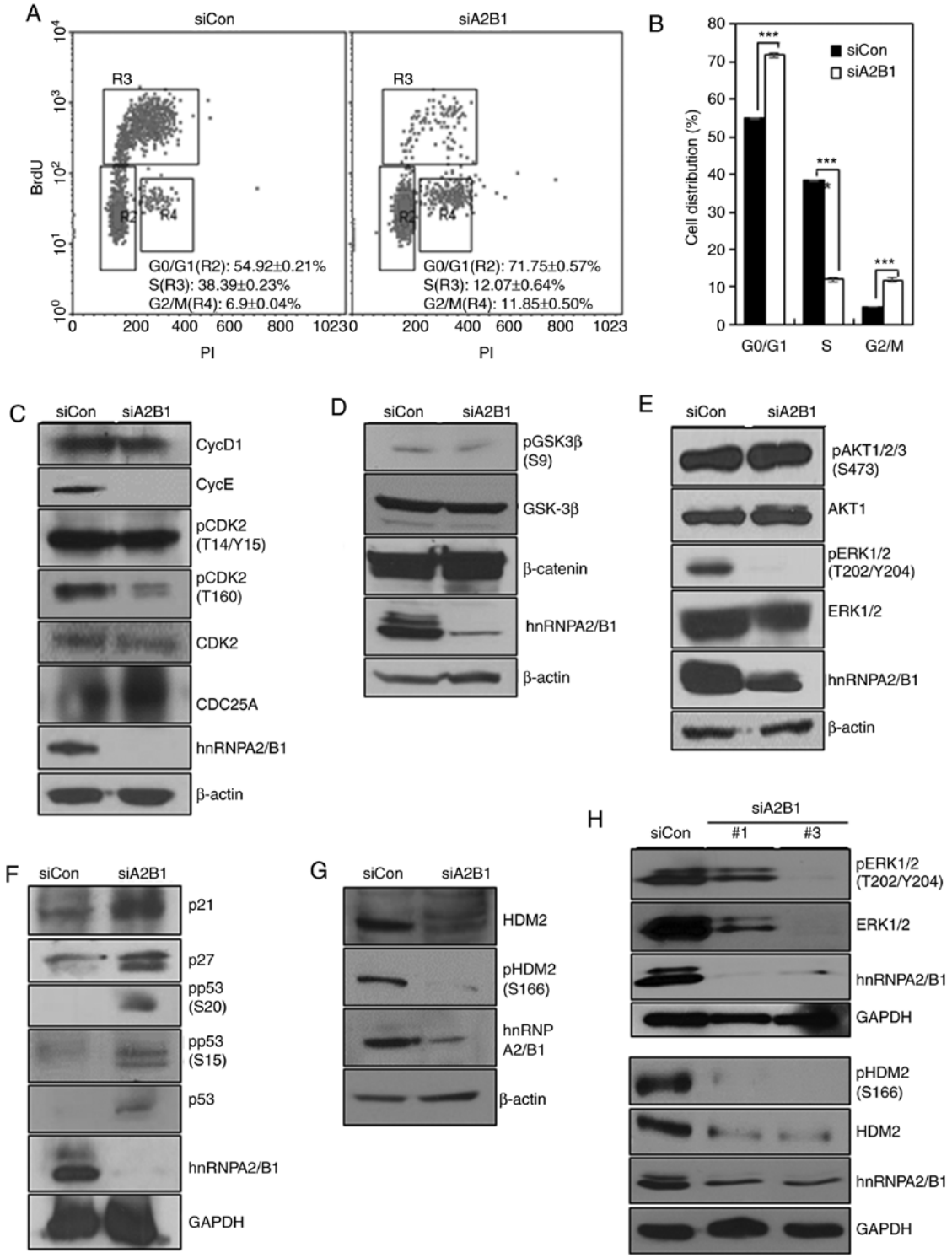

Figure 3. hnRNPA2/B1 promotes the G_1/S phase transition through ERK signaling, suppresses expression of p21 and p27, and prevents p53 activation via HDM2. (A) Cell cycle analysis of BrdU incorporation of siCon- or siA2B1-transfected A549 cells. (B) Statistical analysis of the data presented in A. ***P<0.005. (C-H) Western blot analysis of the protein expression levels of (C) cyclins, CDK2, p-CDK2 and Cdc25A, (D) GSK-3β, p-GSK-3β and β-catenin, (E) Akt1, p-Akt1/2/3, ERK1/2 and p-ERK1/2, (F) p21, p27, p53 and p-p53, (G) HDM2 and p-HDM2, and (H) ERK1/2, p-ERK1/2 (upper panels), HDM2 and p-HDM2 (lower panels) in siCon- or siA2B1-transfected A549 cells. Two different siRNAs (#1 and #3) against hnRNPA2/B1 were used in H. siCon, control small interfering RNA; hnRNPA2/B1, heterogeneous nuclear ribonucleoprotein A2/B1; siA2B1, small interfering RNA targeting hnRNPA2B1; BrdU, bromodeoxyuridine; PI, propidium iodide; p-, phosphorylated; Cdc25A, cell division cycle 25A; GSK-3β, glycogen synthase kinase 3β.

hESCs (7,27,34). The results of the present study demonstrated that hnRNPA2/B1 knockdown induced a substantial accumulation of A549 cells in the G_0/G_1 phase compared with that of the control cells; the proportion of hnRNPA2/B1-knockdown A549 cells in the G_0/G_1 phase was increased by ~17%, whereas the proportion of hnRNPA2/B1-knockdown A549 cells in the S phase was decreased by ~26% (Fig. 3A and B). Thus, hnRNPA2/B1 knockdown may inhibit A549 cell proliferation by blocking the cell cycle progression from the G_1 to the S phase.

To examine the mechanism by which hnRNPA2/B1 promoted G_1/S transition in NSCLC cells, cyclin expression levels were analyzed in hnRNPA2/B1-knockdown A549 cells by western blotting. Cyclin D1 expression levels were slightly decreased, and cyclin E expression levels were markedly reduced in A549 cells following hnRNPA2/B1 knockdown compared with those in the control cells (Fig. 3C). Although CDK2 dephosphorylation at T14/Y15 or Cdc25A degradation were not altered, the levels of CDK2 phosphorylation at T160 were decreased in A549 cells following hnRNPA2/B1 knockdown compared with those in the control group (Fig. 3C). These results suggested that cyclin E degradation and decreased phosphorylation of CDK2 at T160 may be associated with the G_0/G_1 phase arrest in NSCLC cells following hnRNPA2/B1 knockdown.

hnRNPA2/B1 promotes G_1/S transition through ERK signaling. To determine how hnRNPA2/B1 regulated G_1/S transition in NSCLC cells, the present study examined the GSK-3β/β-catenin, PI3K/AKT and MEK/ERK signaling pathways. The levels of inhibitory phosphorylation of GSK-3β at S9 or β-catenin expression were not altered in hnRNPA2/B1-knockdown A549 cells compared with those in the control group (Fig. 3D), suggesting that β-catenin signaling was not involved in the G_1/S transition of A549 cells following hnRNPA2/B1 knockdown. Although hnRNPA2/B1 knockdown decreases AKT signaling in pancreatic, cervical cancer cells and hESCs (21,27,35), it did not cause a significant change in AKT1/2/3 phosphorylation in A549 cells (Fig. 3E). However, hnRNPA2/B1 knockdown abolished ERK1/2 phosphorylation in A549 cells compared with that in the control group (Fig. 3E). Taken together, these results suggested that hnRNPA2/B1 may regulate G_1/S transition in NSCLC cells through the ERK signaling pathway.

hnRNPA2/B1 suppresses the expression of p21 and p27, and prevents p53 activity via increased stability and activation of HDM2. The transition from G_1 into S phase is also regulated by CDK inhibitors (36). In the present study, hnRNPA2/B1 knockdown increased the expression levels of p21 and p27 in A549 cells compared with those in the control group (Fig. 3F), indicating that hnRNPA2/B1 may promote the G_1/S transition in NSCLC cells through the inhibition of p21 and p27 expression. Our previous studies reported that hnRNPA2/B1 knockdown increased p53 expression and phosphorylation at S15, suggesting that hnRNPA2/B1 also regulated the G_1/S phase transition by controlling p53 activity (27). In the present study, increased expression levels of p53 was detected in A549 cells following hnRNPA2/B1 knockdown compared with those in the control group, and the levels of phosphorylation of p53 at S15 and S20 were also increased (Fig. 3F). N-terminal phosphorylation of p53 at S15 and S20 reduces the affinity of p53 for its negative regulator HDM2, and it recruits the transcriptional coactivators CBP/p300, leading to p53 stability and activation; AKT and ERK1/2 physically associate with HDM2, phosphorylate it at S166, and increase p53 degradation (37). hnRNPA2/B1 knockdown decreased the stability of HDM2 in A549 cells and reduced the levels of HDM2 phosphorylation at S166 compared with those in the control group (Fig. 3G). These results suggested that the increased activation and stability of p53 protein may be due to the decreased activation of HDM2 caused by reduced ERK signaling activity (Fig. 3E-G). Therefore, hnRNPA2/B1 knockdown may induce the G_0/G_1 phase arrest through the activation of p53, which is in turn induced by HDM2 inactivation.

To exclude the potential off-target effects of siRNAs, hnRNPA2/B1 was also knocked down using two additional siRNAs (Fig. S4), and the expression and phosphorylation levels of ERK1/2 were decreased in A549 cells with hnRNPA2/B1 knockdown using two siRNAs (Fig. 3H upper panels). The expression and phosphorylation levels of HDM2 were also decreased in A549 cells following hnRNPA2/B1 knockdown compared with those in the control group (Fig. 3H, lower panels), suggesting that the decreased activation of HDM2 was caused by reduced activity of ERK signaling in hnRNPA2/B1-knockdown A549 cells.

To further examine the knockdown effects of hnRNPA2/B1 on another NSCLC cell line, hnRNPA2/B1 was also depleted in NCI-H460 cells. The expression and phosphorylation levels of ERK and HDM2 tended to decrease in NCI-H460 cells following hnRNPA2/B1 knockdown compared with those in the control group, although the knockdown efficiency of hnRNPA2/B1 was low in these cells (Fig. S5). Next, the mRNA expression level of HDM2 in hnRNPA2/B1-knockdown A549 cells was analyzed by qPCR. However, the mRNA expression levels of HDM2 were not significantly changed in hnRNPA2/B1-knockdown cells compared with those in the control A549 cells (Fig. S6), suggesting that the effects of the hnRNPA2/B1-ERK-HDM2/p53 regulatory pathway were not mediated through the regulation of HDM2 mRNA transcription.

To further demonstrate whether hnRNPA2/B1 regulated the G_1/S phase transition through the suppression of p53 activity and the activation of HDM2, the effects of the overexpression of hnRNPA2 on p53 and HDM2 were examined in 293T cells. The overexpression of hnRNPA2 induced the dephosphorylation of p53 at S15 and the phosphorylation of HDM2 at S166, which was opposite to the effects of hnRNPA2/B1 knockdown (Fig. 4A). Taken together, these suggested that hnRNPA2/B1 may promote the G_1/S phase transition through the suppression of p53 activity, which in turn is induced by ERK-mediated HDM2 activation.

Discussion

hnRNPA2/B1 is associated with carcinogenesis through its interactions with various proteins; however, its interaction with p53 remains unclear. A549 cells exhibit wild-type p53, but harbor a mutation in the KRAS gene (38). In our previous study, hnRNPA2/B1 knockdown was demonstrates to induce p53 expression and activation in hESCs (27). In the present study, hnRNPA2/B1 knockdown also induced p53 expression and activation in A549 cells. Furthermore, hnRNPA2/B1 knockdown resulted in decreased stability and phosphorylation of HDM2 in A549 cells compared with that in the control group. These results were also supported by the opposite effects observed following hnRNPA2 overexpression. Thus, hnRNPA2/B1 may regulate the survival, proliferation and migration of NSCLC cells via the p53/HDM2 pathway. Previous studies have reported that hnRNPA2/B1 positively regulates AKT signaling in pancreatic and cervical cancer cells (21,35). However, the results of the present study demonstrated that hnRNPA2/B1 expression was not involved in AKT signaling in A549 cells. Another study also revealed that AKT

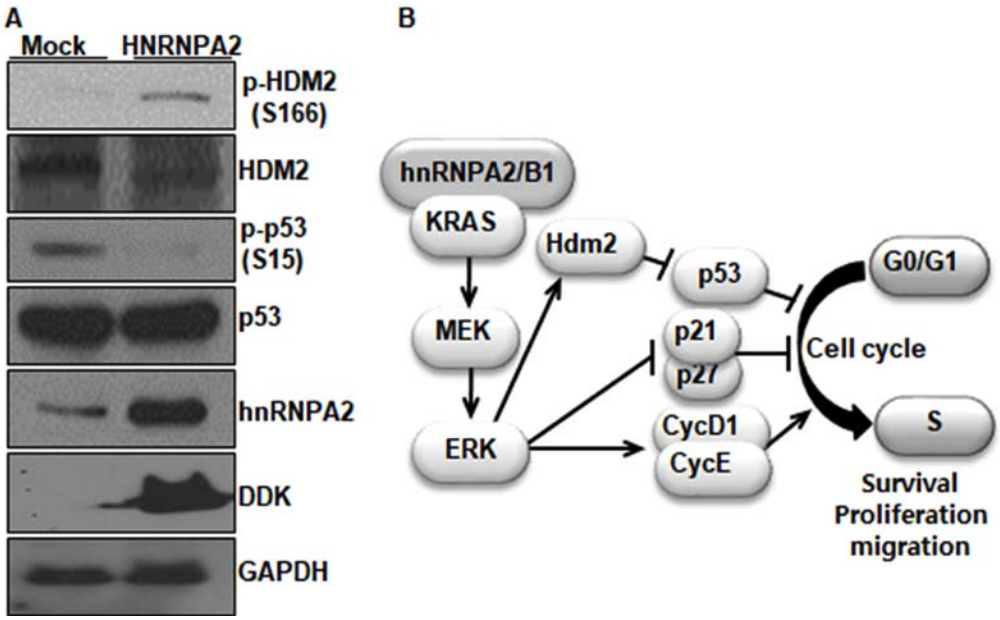

Figure 4. Overexpression of hnRNPA2/B1 increases clonogenic survival and suppresses p53 activity via HDM2 activation in 293T cells. (A) Western blot analysis of the expression levels of HDM2, p-HDM2, p53, p-p53, hnRNPA2 and DDK in 293T cells overexpressing DDK-tagged hnRNPA2. GAPDH was used as an internal control. (B) The proposed model for the role of hnRNPA2/B1 in NSCLC cells. hnRNPA2/B1 is required for the survival, proliferation and migration of NSCLC cells. hnRNPA2/B1 promotes the G_1/S phase transition via the ERK signaling pathway, which induces cyclin D1 and E expression and inhibits that of p21 and p27. hnRNPA2/B1 also prevents p53 activity via ERK-mediated HDM2 activation. hnRNPA2, heterogeneous nuclear ribonucleoprotein A2; p-, phosphorylated; hnRNPA2/B1, heterogeneous nuclear ribonucleoprotein A2/B1; NSCLC, non-small cell lung cancer; HDM2, human double minute 2 protein; DDK, DBF4-dependent kinase.

signaling is not associated with hnRNPA2/B1 expression in A549 cells (39). Therefore, it was concluded that the reduced phosphorylation of HDM2 at S166 was due to the decreased phosphorylation of ERK1/2 induced by hnRNPA2/B1 knockdown, suggesting that hnRNPA2/B1 may promote the survival, proliferation and migration of NSCLC cells by preventing p53 activation, which is induced by ERK-mediated HDM2 activation. This phenomenon was not mediated through the regulation of HDM2 mRNA levels, suggesting that hnRNPA2/B1 may regulate the activity of HDM2 at the posttranscriptional level. A proposed model for the role of hnRNPA2/B1 in NSCLC cells is presented in Fig. 4B. Further studies on different NSCLC cell lines should be performed to verify the proposed model. The present results also suggested that the hnRNPA2/B1/ERK/p53/HDM2 pathway may contain novel potential molecular targets for the treatment of patients with NSCLC who exhibit wild-type p53 and mutated KRAS.

Our previous study reported that hnRNPA2/B1 was required for maintaining the epithelial phenotypes of hESCs (27), suggesting that hnRNPA2/B1 may inhibit the EMT in hESCs. A recent study has also reported that hnRNPA2/B1 serves a role as a negative regulator in breast cancer metastasis (23). By contrast, in pancreatic cancer, hepatocellular carcinoma, prostate cancer and NSCLC, hnRNPA2/B1 promotes the EMT and metastatic phenotypes (26,40-42). In the present study, hnRNPA2/B1 knockdown resulted in decreased expression levels of vimentin, increased levels of E-cadherin and reduced migration in NSCLC cells compared with those in the control group, which was consistent with the results of the aforementioned previous studies (26,40-42). Thus, hnRNPA2/B1 exerts contradictory effects on cell proliferation, EMT, invasion and migration. hnRNPA2/B1 serves as a key regulator of a number of target genes and signaling pathways (6), such as the KRAS (21), PI3K/Akt (21) and STAT/ERK1/2 (22,40) signaling pathways. Therefore, the different effects of hnRNPA2/B1 may be due to combined effects of the hnRNPA2/B1 downstream genes and signaling pathways under different culture conditions and in different cell lines. The detailed mechanism by which hnRNPA2/B1 serves different roles in cell proliferation, EMT, migration and invasion remains elusive.

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Availability of data and materials

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

Authors' contributions

MKK and CJR conceived and designed the study. MJC contributed to the production of this manuscript following the peer review. HML and HSC acquired and analyzed the

data. CJR wrote the manuscript. MKK revised the manuscript. YKP contributed to the statistical analysis. MKK and CJR confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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