RRM1 is mediated by histone acetylation through gemcitabine resistance and contributes to invasiveness and ECM remodeling in pancreatic cancer

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Abstract. The invasiveness of pancreatic cancer and its resistance to anticancer drugs define its malignant potential, and are considered to affect the peritumoral microenvironment. Cancer cells with resistance to gemcitabine exposed to external signals induced by anticancer drugs may enhance their malignant transformation. Ribonucleotide reductase large subunit M1 (RRM1), an enzyme in the DNA synthesis pathway, is upregulated during gemcitabine resistance, and its expression is associated with worse prognosis for pancreatic cancer. However, the biological function of RRM1 is unclear. In the present study, it was demonstrated that histone acetylation is involved in the regulatory mechanism related to the acquisition of gemcitabine resistance and subsequent RRM1 upregulation. The current *in vitro* study indicated that RRM1 expression is critical for the migratory and invasive potential of pancreatic cancer cells. Furthermore, a comprehensive RNA sequencing analysis showed that activated RRM1 induced marked changes in the expression levels of extracellular matrix-related genes, including N-cadherin, tenascin-C and COL11A. RRM1 activation also promoted extracellular matrix remodeling and mesenchymal features, which enhanced the migratory invasiveness and malignant potential of pancreatic cancer cells. The present results demonstrated that RRM1 has a critical role in the biological gene program that regulates the extracellular matrix, which promotes the aggressive malignant phenotype of pancreatic cancer.

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Abbreviations: RRM1, ribonucleotide reductase large subunit M1; TME, tumor microenvironment; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; TNC, Tenascin-C

Key words: gemcitabine resistance, histone acetylation, pancreatic cancer, RRM1, ECM remodeling

Introduction

To date, pancreatic cancer is the third-highest cause of cancer-related deaths (~48,000) in the U.S (1). Its incidence is gradually increasing, and it is likely to become the second-highest cause of cancer-related deaths by 2030 (2). The treatment of pancreatic cancer includes radical methods such as surgical resection. However, the prognosis for such treatment remains poor because pancreatic cancer is usually advanced at the time of diagnosis. Furthermore, within two years of curative resection, 80% of patients develop a recurrence of pancreatic cancer (3).

Since 80% of patients have regional or metastatic cancer at diagnosis, chemotherapy or radiation therapy are the primary treatment options (1). Previous studies have investigated the use of combination chemotherapy regimens, including gemcitabine and nab-paclitaxel (GnP) or 5-fluorouracil, oxaliplatin, and irinotecan (FOLFIRINOX). These regimens have an important role in the treatment of advanced pancreatic cancer (4,5). Despite these advanced treatments, the prognosis of advanced pancreatic cancer remains poor. This may be related to drug resistance (6).

The mechanism of drug resistance is considered to include various pathological factors specific to pancreatic cancer, including a peritumor microenvironment containing dense fibrous stroma and an abnormal vascular network around the tumor (7,8). In pancreatic cancer, the tumor microenvironment consists of a rigid extracellular matrix (ECM) formed from collagen I, elastin and fibronectin (8,9). Pancreatic cancer causes specific changes in the ECM close to the tumor, resulting in a microenvironment that promotes tumor growth and multi-organ metastasis (10,11). Desmoplastic reaction, a large deposition of ECM, and pancreatic cancer cells have biochemical effects that increase the interstitial fluid pressure, which reduces tumor perfusion and inhibits the delivery of antitumor agents (12-15). This is exacerbated

by the lowered density of the tumor vasculature, reducing the effect of cytotoxic therapy against pancreatic cancer cells (13).

Ribonucleotide reductase large subunit M1 (RRM1), a rate-limiting enzyme in the DNA synthesis pathway, requires the conversion of ribonucleotides to dNTPs (16). RRM1 has an effect on the poor disease prognosis of several cancers, including pancreatic cancer (17-19). Furthermore, RRM1 has been associated with resistance to gemcitabine, an important drug in the treatment of pancreatic cancer (19-21). However, the way in which RRM1 is involved in the biology of pancreatic cancer, particularly related to cell motility, is poorly understood.

In the present study, the relationship between epithelial-mesenchymal transition (EMT), motility invasion capacity, histone acetylation and drug resistance in gemcitabine-resistant (GEM-R) cell lines was investigated. *In vitro* overexpression or siRNA experiments were also performed to examine the functions of RRM1, concentrating on the relationship between invasiveness and changes in the expression of factors such as N-cadherin. It was found that RRM1 contributed to the malignant phenotype of cancer cells, including increased motility and invasiveness, particularly during gemcitabine resistance. Therefore, RRM1 may be a therapeutic target for the treatment of pancreatic cancer.

Materials and methods

Materials. Anti-RRM1 (1:1,000; D12F12; cat. no. 8637), anti-acetyl histone H3 (Lys9) (1:1,000; C5B11; cat. no. 9649), anti-acetyl histone H3 (Lys27) (1:1,000; D5E4; cat. no. 8173), anti-histone H3 (1:2,000; D1H2; cat. no. 4499) and GAPDH (1:2,000; D16H11; cat. no. 5174) antibodies were obtained from Cell Signaling Technology, Inc. Anti-N-cadherin (1:1,000, cat. no. 610920) antibody was purchased from BD Biosciences. Tenascin-C (TNC) (1:200; E-9; cat. no. sc-25328) and fibronectin (1:200; P5F3; cat. no. sc-18827) antibodies were obtained from Santa Cruz Biotechnology, Inc. Anti-COL11A (1:200; cat. no. ab64883) antibody was purchased from Abcam. HAT inhibitor C646 (cat. no. SML0002) was obtained from MilliporeSigma.

Cell cultures. The human pancreatic cancer cell lines MIAPaCa2 and Panc1 were obtained from the American Type Culture Collection in August 2016. The cancer cell lines were authenticated by short tandem repeat analysis for DNA profiling, and all experiments were performed with mycoplasma-free cells. Cancer cells were maintained in high-glucose DMEM medium containing 10% FBS (FUJIFILM Wako Pure Chemical Corporation) and 1% penicillin/streptomycin in a humidified 5% CO_2 chamber at 37˚C.

Cell viability assay. Cell numbers were evaluated by WST-8 assay (Cell Counting Kit-8; Dojindo Molecular Technologies, Inc.), as previously described (19). Briefly, 5.0-7.5x10^3 cells per well were seeded onto 96-well plates and incubated overnight at 37˚C. After 72 h of gemcitabine treatment at each concentration from 1 nM to 10 μM, cell viability was determined according to the manufacturer's instructions. The absorbance of each well was measured at 450 nm, using an iMark™ microplate reader (Bio-Rad Laboratories, Inc.), and was determined to be within the linear range of the assay.

Establishment of a GEM-R cancer cell line. GEM-R pancreatic cancer cell subclones were generated using Panc1 cells in our laboratory, as previously described (22). Briefly, the Panc1 cells were serially cultured with exposure to incrementally increasing gemcitabine concentrations (10-100 nM) for two months.

Overexpression of RRM1 by stable transfection. A human RRM1 expression plasmid (pCMV6-RRM1; NM_001033) was purchased from Origene Technologies, Inc. A total of 1 μg RRM1 of expression vectors or corresponding empty vectors (pCMV6-Entry) were transfected into Panc1 cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions onto 12-well plates. After 72 h of transfection, the cells were incubated in culture medium containing 800 μg/ml G418. After culturing in the selection medium containing G418 for two weeks, stably transfected cells were selected and the expression of RRM1 protein was confirmed by western blotting.

Western blotting. Western blotting was performed as previously described (23). Protein bands were visualized and their intensities quantified using ImageQuant LAS 4000 mini (GE Healthcare). Western blot analyses were conducted at least three times with similar results, and representative blots are presented. A densitometric analysis was performed using ImageQuant TL (GE Healthcare) to calculate the intensity of each protein band. Figures for western blotting are cropped and displayed according to the appropriate molecular weight.

Gene silencing by small interfering (si)RNA. Loss-of-function analysis was performed using siRNAs specific for RRM1 (siRRM1#1: cat. no. HSS109388, Invitrogen; Thermo Fisher Scientific, Inc.; sense, 5'-CCCAGUUACUGAAUAAGC AGAUCUU-3' and antisense, 5'-AAGAUCUGCUUAUUC AGUAACUGGG-3') or scrambled negative control (siNC: #12935300; Stealth RNAi™ Negative Control Med GC Duplex #2; Invitrogen; Thermo Fisher Scientific, Inc.). An alternative sequence of siRNA targeting RRM1 (siRRM1#2; cat. no. HSS184469; Invitrogen; Thermo Fisher Scientific, Inc.; sense, 5'-CAGAAGCUUUGUUAUGGACUCAAUA-3' and antisense, 5'-UAUUGAGUCCAUAACAAAGCUUCU G-3') was also used. Each siRNA (20 nM) was transfected into pancreatic cancer cells using Lipofectamine RNA iMAX (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Cancer cells transfected with each siRNA were cultured at 37˚C for 72 h. The knockdown of RRM1 was confirmed by western blotting.

Reverse transcription-quantitative PCR (RT-qPCR). Extracted RNA using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was reverse transcribed into first-strand cDNA using SuperScript VILO cDNA Synthesis kits (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The expression of mRNA was determined using the following TaqMan Gene Expression Assays (Applied Biosystems; Thermo Fisher Scientific, Inc.):

CDH2, Hs00983056_m1; COL11A, Hs01097664_m1; and TNC, Hs01115665_m1. Gene expression values are presented as ratios between genes of interest and an internal reference gene (Hs99999901_s1 for eukaryotic 18S). These were then normalized against the value for the control (relative expression level) and analyzed by the comparative $2^{-\Delta\Delta Cq}$ method (24). Each assay was performed in duplicate for each sample.

Clonogenic assay. Long-term cell survival was evaluated by clonogenic assays, as previously described (25). Cells were seeded at $1.0x10^3$ per well into six-well plates, in triplicate. After overnight incubation, adherent cells were treated with gemcitabine for 24 h, at which point the culture medium was changed to fresh medium without gemcitabine. Cells were incubated for at least one week, and colonies were stained with a 0.3% crystal violet solution for 10 min at room temperature. A cluster of 50 or more stained cells was counted as one colony.

Transwell migration and invasion assays. Transwell migration and invasion assays were performed in 24-well modified Boyden chambers precoated already with (invasion) or without (migration) Matrigel (Transwell chamber; BD Biosciences) with 8-μm pore size membrane of Transwell chambers. Pancreatic cancer cells (Panc1, $5x10^4$ cells; and MIAPaCa2, $7.5x10^4$ cells per well) in serum-free medium were seeded onto the trans-membrane in the upper chamber, with 10% FBS in the lower chamber as a chemoattractant. After a 24-h incubation, cells that had migrated through the membrane were fixed and stained with a Diff-Quik Stain Set (Siemens AG) for 10 min at room temperature and counted under magnification (x100) in five randomly selected high-power fields. Each assay was performed in triplicate.

Scratch wound-healing assay. Cancer cells were cultured in six-well plates until confluent. Confluent cell layers were carefully scratched using a sterile 200-μl tip, washed twice with fresh medium, and cultured for 24 h. Images of the scratched cell layers were acquired with a phase contrast microscope linked to a charge coupled device camera, and the wound area was evaluated.

Cell adhesion assay. Cell adhesion assays were performed using the CytoSelect 48-Well Cell Adhesion Assay (ECM array; CBA-070, CellBiolabs) according to the manufacturer's protocol. Cancer cells ($1.0x10^5$ cells) were plated in triplicate on a precoated plate (Fibronectin, Collagen I, Collagen IV and Laminin I). After 60 min of incubation at 37˚C, adherent cells were fixed and stained by incubating for 10 min at room temperature. The stained solution was then removed and quantified colorimetrically at 560 nm, using a plate reader.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assaying was performed using a ChIP-IT Express Kit (Active Motif, Inc.) according to the manufacturer's protocol, as previously described (26). Cells were fixed with 1% formaldehyde/PBS for 10 min. DNA was sonicated to 500-1,000 bp for all experiments. Immunoprecipitated DNA enrichment was normalized to the input. The antibodies used were anti-H3K9ac (1:50; cat. no. 9649), anti-H3K27ac (1:50; cat. no. 8173; both from Cell Signaling Technology, Inc.), and anti-histone H3 (1 μg; cat. no. 07-690; MilliporeSigma). Normal rabbit IgG (1:50; cat. no. 2729; Cell Signaling Technology, Inc.) was used as a negative control for each assay. The primer set for qPCR was as follows: RPM1 sense, 5'-GCC TCTGCTCTGAAGAAAGTG-3' and antisense, GACAGA GTGCGAAGGGTTAGG-3'.

Immunofluorescence staining. Cells were cultured on four-chamber CultureSlides (Becton, Dickinson and Company). The cells were then fixed in 4% formaldehyde in PBS for 15 min, blocked with blocking buffer (PBS with 5% BSA and 0.2% Triton X-100) for 1 h, and incubated overnight with the primary antibody at 4˚C (anti-N-cadherin; 1:500). Alexa Fluor 488-conjugated goat anti-mouse IgG antibodies (1:1,000; cat. no. 4408; Cell Signaling Technology, Inc.) were used as secondary antibodies. The cells were viewed under a fluorescence microscope (BZ-700; Keyence Corporation).

RNA sequencing. Total RNA was quantified and qualified using a Qubit RNA Assay (Thermo Fisher Scientific, Inc.) and TapeStation RNA ScreenTape (Agilent Technologies, Inc.). cDNA synthesis followed by transcriptome libraries were performed using a NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England BioLabs, Inc.), where dUTP was incorporated during second strand cDNA synthesis, instead of dTTP, which blocks PCR amplification against the second strand templates, enabling strand-specific library preparation. The resulting transcriptome sequencing libraries were quantified by Qubit DNA Assay (Thermo Fisher Scientific, Inc.) and their fragment size distributions were confirmed by TapeStation D1000 ScreenTape (Agilent). The transcriptome libraries established were loaded onto a next-generation sequencing platform, HiSeq 4000 or equivalent (Illumina, Inc.). Sequencing was performed according to the manufacturer's instructions. Image analysis and base calling were performed using software on the HiSeq instrument. GOSeq (27) and TopGO software (https://bioconductor.org/packages/release/bioc/html/topGO.html) were utilized in the Gene Ontology (GO) enrichment analysis for differentially expressed genes. All processes were conducted at Azenta Life Sciences (formerly GENEWIZ).

Statistical analysis. The drawing of figures, fitting of curves, IC_{50} calculations and statistical analyses were performed with GraphPad Prism 7 software (GraphPad Software, Inc.). Unless otherwise specified, independent experiments were conducted in triplicate and the values presented are their means, compared using the Student's t-test for single comparison or a one-way ANOVA with a Tukey's post hoc test and a two-way ANOVA with Sidak's post hoc test for multiple comparisons, as appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell motility and histone H3 acetylation are increased in GEM-R Panc1 cells. During the acquisition of drug resistance, cancer cells refractory to drugs are considered to be involved in a variety of malignant processes, including the acquiring of stem cell properties, increased motility, and the induction

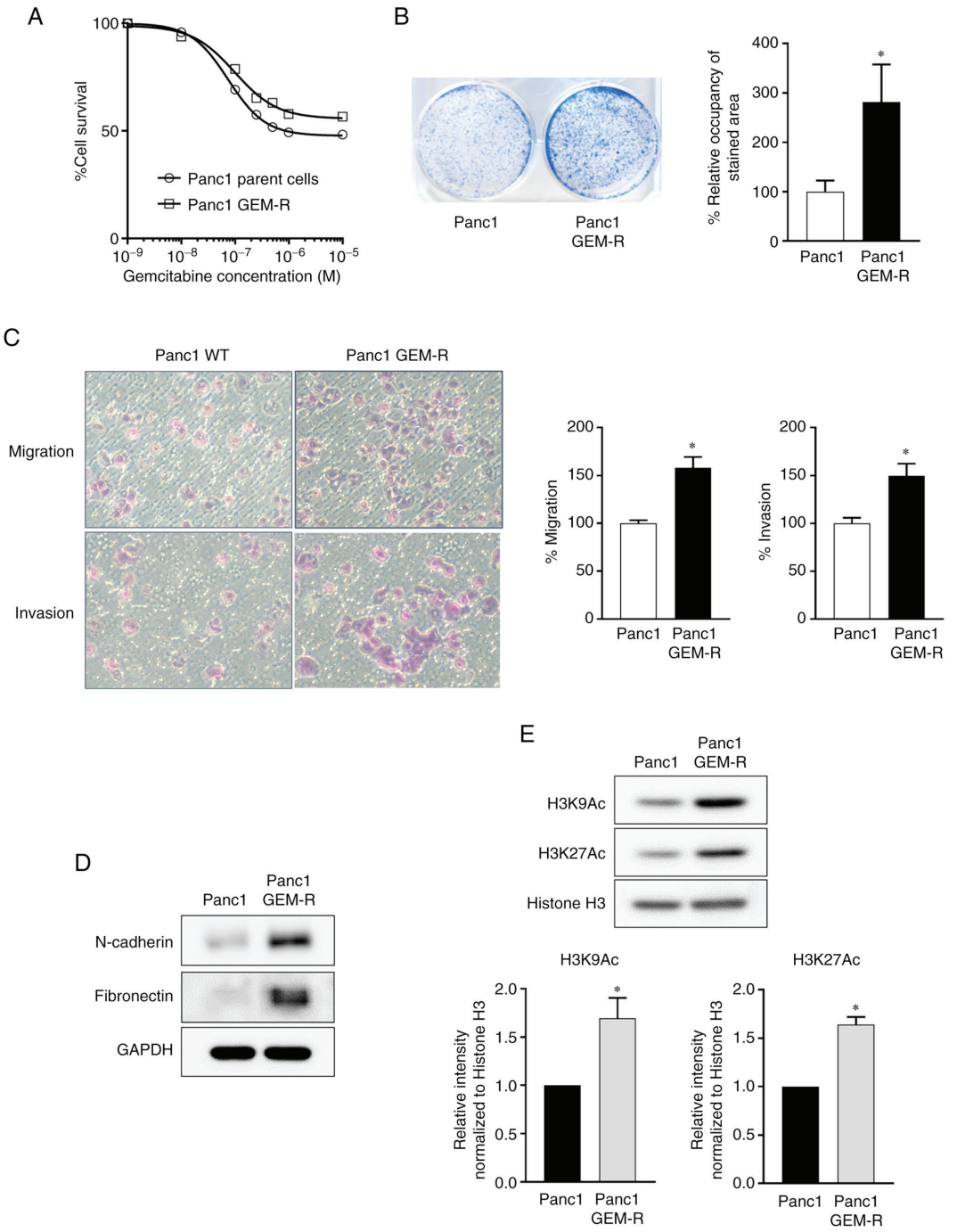

Figure 1. Cellular migration and invasion, and histone H3 acetylation are increased in gemcitabine-resistant Panc1 cells. (A and B) Comparison of cell viability between Panc1 parental cells and GEM-R cells after multiple dose gemcitabine treatment by (A) WST-8 assay (cancer cells were treated with each concentration of gemcitabine for 72 h) and (B) clonogenic assay (long duration of cell survival after gemcitabine treatment). GEM-R Panc1 cells showed increased cell viability upon gemcitabine treatment. Experiments were performed in duplicate. Error bars represent the mean ± SD. *P<0.05 vs. parental controls. (C) Effects of gemcitabine resistance on cellular migration and invasion. GEM-R cells increased the cellular migration of Panc1 cancer cells (1.6-fold increase, *P<0.05 by t-test). Similarly, gemcitabine resistance increased the cellular invasion of the Panc1 cell line (1.5-fold increase for Panc1, *P<0.05 by t-test). Error bars represent the mean ± SD. Each assay was performed in duplicate. (D) Effects of gemcitabine resistance on mesenchymal markers by western blotting. GEM-R Panc1 cells increased N-cadherin and fibronectin protein expression levels. (E) Effects of gemcitabine resistance on histone H3 acetylation by western blotting. GEM-R Panc1 cells significantly activated histone H3 acetylation (H3K9Ac: 1.7-fold increase; H3K27Ac: 1.6-fold increase; *P<0.05 by t-test). Error bars represent the mean ± SD. Each assay was performed in triplicate. GEM-R, gemcitabine-resistant.

of EMT. GEM-R Panc1 cells were generated and their effects on cancer motility, including migration and invasion, and their relevance to EMT and drug resistance were evaluated.

The IC_{50} of the parent cells was 75 nM at 72 h, whereas it was 100 nM for GEM-R Panc1 cells, 1.3 times higher than the parent cells (Fig. 1A). A colony formation assay for gemcitabine treatment revealed a 2.8-fold increase in the length of cell survival (Fig. 1B), demonstrating that Panc1 cells had acquired drug resistance to gemcitabine.

Panc1 GEM-R cells exhibited significantly enhanced motility in migration and invasion assays, compared with control cells (migration: 1.6-fold; invasion: 1.5-fold; P<0.05 in

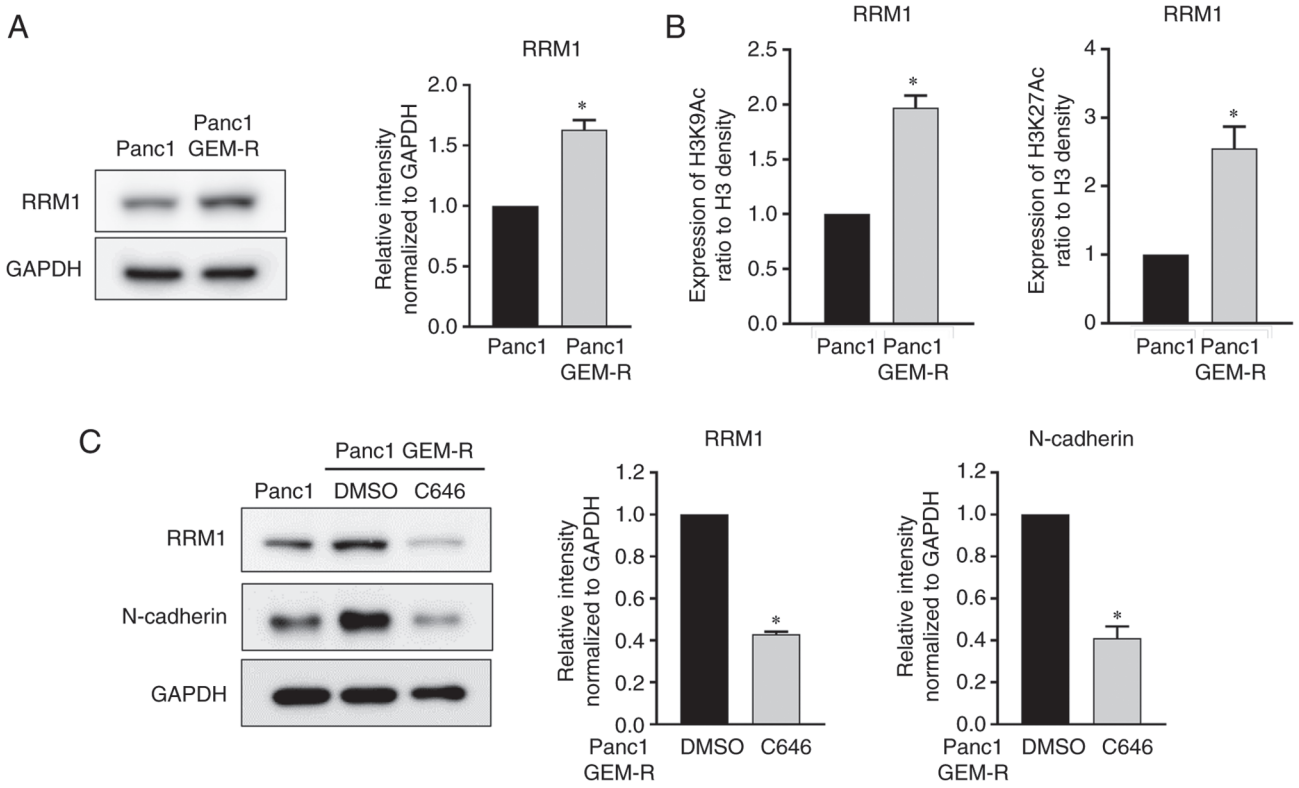

Figure 2. Activated RRM1 expression is regulated by histone acetylation in GEM-R Panc1 cells. (A) The effect of gemcitabine resistance on RRM1 expression. RRM1 activation was confirmed in Panc1 cells with gemcitabine resistance by western blotting (1.6-fold increase by densitometric analysis). Error bars represent the mean ± SD. *P<0.05 vs. parental controls. Each assay was performed in duplicate. (B) Quantitative ChIP analysis of RRM1 in GEM-R Panc1 cells compared with parental controls. H3K9 and H3K27 acetylation levels at the promoter region of RRM1 were significantly increased by gemcitabine resistance (H3K9Ac: 2.0-fold increase; H3K27Ac: 2.6-fold increase; *P<0.05 vs. controls by t-test). Error bars represent the mean ± SD. Each assay was performed in duplicate. (C) Effects of C646 treatment on RRM1 and N-cadherin expression levels. C646, a histone acetyltransferase inhibitor, was incubated with the cells for 72 h. RRM1 and N-cadherin expression levels were downregulated by the inhibition of histone acetylation (RRM1: 0.4-fold decrease; N-cadherin: 0.4-fold increase; *P<0.05 vs. controls by t-test). Error bars represent the mean ± SD. Each assay was performed in duplicate. RRM1, ribonucleotide reductase large subunit M1; GEM-R, gemcitabine-resistant.

both cases) (Fig. 1C). The expression levels of N-cadherin and fibronectin, which are mesenchymal markers in EMT, were upregulated (Fig. 1D). Notably, histone H3 acetylation was enhanced in Panc1 GEM-R cells (H3K9Ac:1.7-fold; H3K27Ac: 1.6-fold; P<0.05 in both cases) (Fig. 1E). Thus, Panc1 cells that acquired gemcitabine resistance showed enhanced drug resistance, increased migration and invasion, EMT and histone H3 acetylation.

Increased RRM1 expression in GEM-R Panc1 cells is regulated by histone acetylation. The RRM1 gene is upregulated when gemcitabine resistance is acquired, and may be associated with a worse prognosis in pancreatic cancer, as previously reported by the authors (19,20). Indeed, the protein level of RRM1 was increased 1.6-fold in the Panc1 GEM-R cell line (Fig. 2A). Next, the relationship between upregulated histone H3 acetylation in cells with gemcitabine resistance and the increased expression of RRM1 was examined. Chip-PCR experiments showed that the RRM1 gene transcription level was significantly enhanced by H3K9 and H3K27 acetylation, indicating that RRM1 transcriptional activity was partly regulated by histone H3 acetylation (Fig. 2B).

The increased expression levels of RRM1 and N-cadherin as well as histone H3 acetylation were observed in the GEM-R cells. Notably, C646, a histone acetylation inhibitor, inhibited histone H3 acetylation at concentrations sufficient to inhibit the acetylation of histone H3 (Fig. S1) and concurrently decreased the expression of RRM1 to 43%, compared with controls. Furthermore, histone acetylation inhibition reduced N-cadherin protein levels to 41%, compared with controls (Fig. 2C).

The aforementioned findings indicated that histone H3 acetylation was upregulated after the acquisition of gemcitabine resistance, leading to the increased expression of RRM1. N-cadherin was also involved in histone acetylation.

RRM1 expression is associated with the migration and invasion of pancreatic cancer cells. Next, the effect of increased RRM1 expression on motility, including migration and invasion, was investigated. A Panc1 cell line overexpressing RRM1 was generated, which was 3.8 times more active than the parent cell line, as revealed by western blotting (Fig. 3A). The increased RRM1 expression significantly enhanced the migration ability of Panc1 cells in a wound-healing assay, compared with normal counterparts (Fig. 3B), and their migration and invasion abilities were significantly increased, 2.2 and 2.3-fold respectively, in a chamber assay (Fig. 3C).

By contrast, the functional suppression of endogenous RRM1 levels in MIAPaCa2 and Panc1 cells by siRNA suppressed the motility of cancer cells. A total of two different RRM1 siRNAs were used and it was found that the downregulation of RRM1

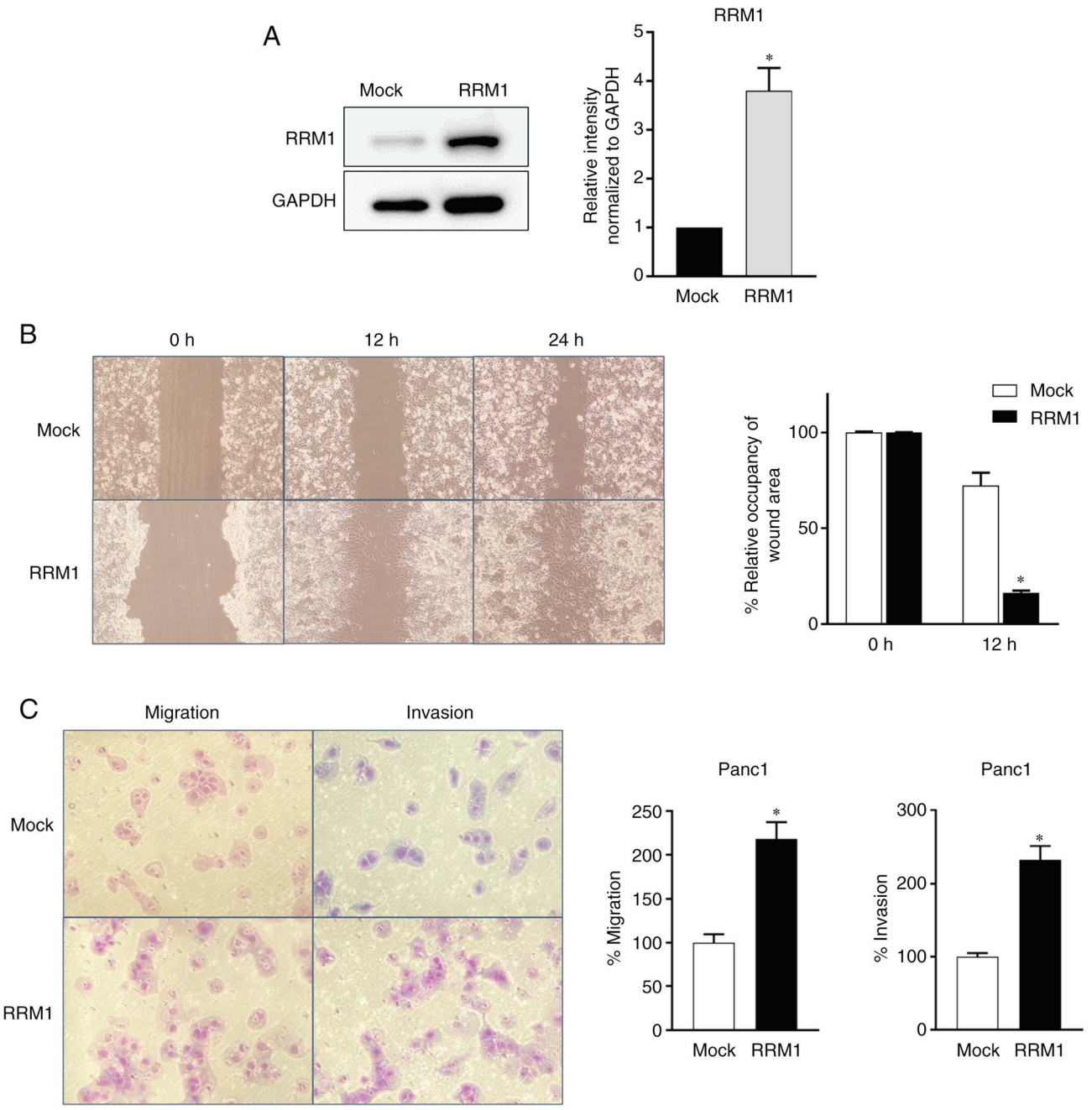

Figure 3. RRM1 overexpression promotes cell migration and invasion. (A) Overexpression of RRM1. Cells were transfected with pCMV6-Mock (empty vector) or pCMV6-RRM1. The overexpression of RRM1 in stably transfected Panc1 cells was confirmed by western blotting (3.8-fold increase, *P<0.05 vs. controls by t-test). Error bars represent the mean ± SD. Each assay was performed in duplicate. (B) Effects of RRM1 overexpression on cellular wound-healing activity. Established transfected cells were evaluated by a scratch wound-healing assay. The forced overexpression of RRM1 increased the cellular migration of Panc1 cells compared with the empty vector transfected controls. Each assay was performed in duplicate. Occupancy of the wound area was also evaluated. The ability of migration was significantly increased in RRM1 overexpressed Panc1 cells. Error bars represent the mean ± SD. *P<0.05 by two-way ANOVA. (C) Effects of RRM1 overexpression on cellular migration and invasion. Established transfected cells were evaluated by migration and invasion assays. The forced overexpression of RRM1 increased the cellular migration and invasion of Panc1 pancreatic cancer cells (by 218 and 232%, respectively; *P<0.05 by t-test) compared with empty vector transfected controls. Error bars represent the mean ± SD. Each assay was performed in triplicate. RRM1, ribonucleotide reductase large subunit M1.

expression significantly reduced the migration and invasion of MIAPaCa2 and Panc1 cells, as assessed by wound-healing assays (Fig. 4A and B). Therefore, RRM1 may promote the malignant transformation of cancer cells by enhancing their motility. Furthermore, the suppression of RRM1 reduced the levels of N-cadherin (Fig. S2) and the migratory and invasive capacity of cancer cell lines (Fig. 4C and D). Therefore, the RRM1/N-cadherin axis may be associated with the acquisition of malignant traits by pancreatic cancer cells.

RRM1 overexpression increases the expression of ECM-related genes. Next, the effects of RRM1 overexpression were compared with empty vector transfected in Panc1 cells, using RNA sequencing. A total of 302 statistically significant

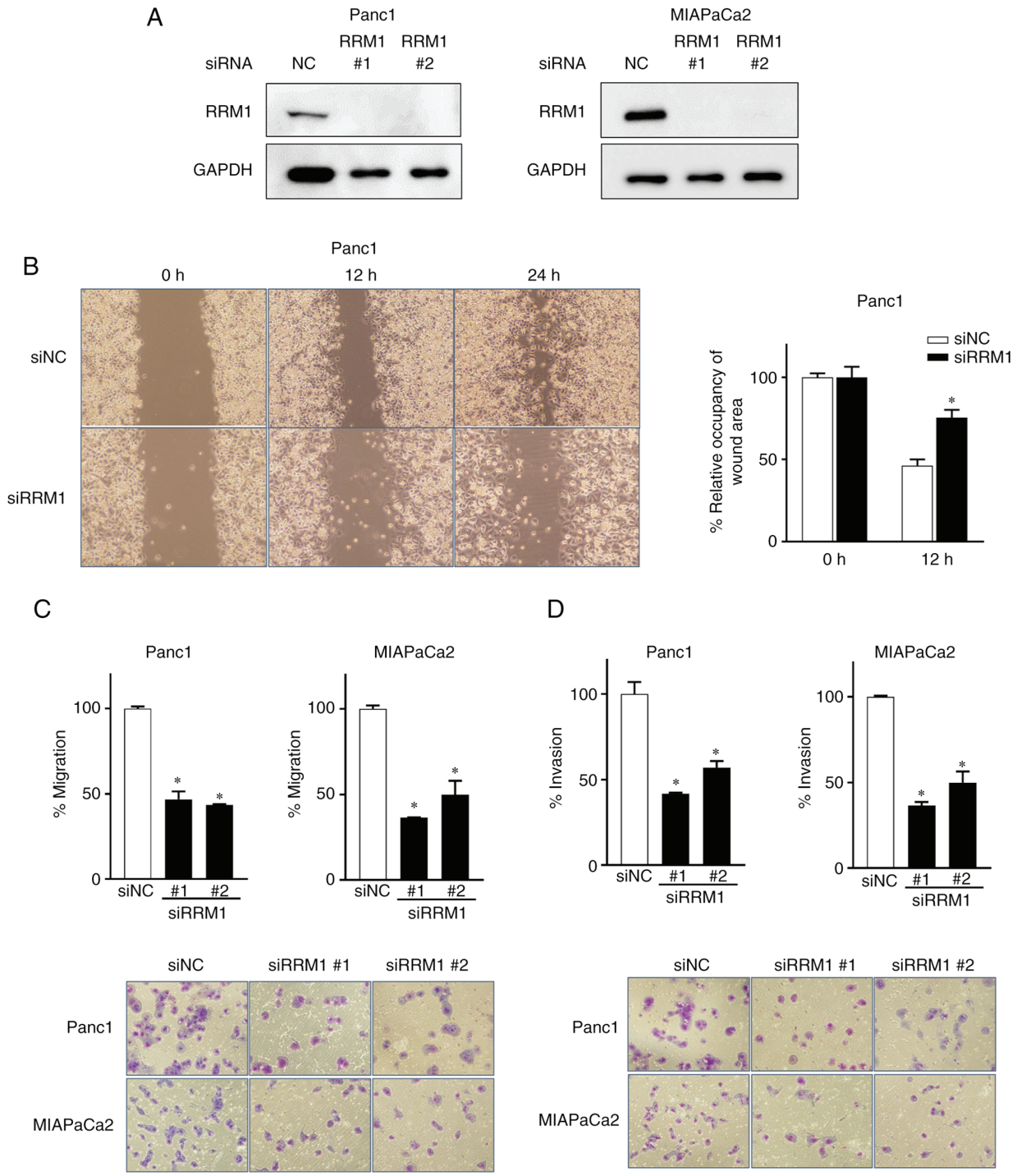

Figure 4. RRM1 gene silencing reduces cell migration and invasion. (A) Effects of RRM1 gene silencing on RRM1 expression. Cells were treated with RRM1-specific siRNAs or negative control-siRNA for 72 h. It was confirmed that RRM1 was suppressed by siRNA treatment in Panc1 and MIPaCa2 cells. (B) Effects of RRM1 gene silencing on cellular wound-healing activity. Cancer cells were treated with siRRM1 or siNC for 24 h prior to wound scratch assay for 24 h. RRM1 gene silencing decreased the cellular migration of siRRM1 treated Panc1 cells compared with siNC control cells. Each assay was performed in duplicate. Occupancy of the wound area was also evaluated. The ability of migration was significantly decreased in siRRM1 cells. Error bars represent the mean ± SD. *P<0.05 by two-way ANOVA. (C and D) Effects of RRM1 gene silencing on (C) cell motility and (D) invasion. Cells were treated with RRM1-specific siRNAs or negative control-siRNA for 24 h, then subjected to migration or invasion assays using Boyden chambers without or with Matrigel. Gene silencing of RRM1 reduced the cellular migration of two pancreatic cancer cell lines (46.9 and 43.7% decrease in Panc1 cells compared with siNC, 36.6 and 49.9% decrease in MIAPaCa2 cells, respectively; *P<0.05 by Tukey's test). Similarly, gene silencing of RRM1 reduced the cellular invasion of two pancreatic cancer cell lines (41.8 and 57.1% decrease in Panc1 cells, 36.6 and 49.8% in MIAPaCa2 cells, respectively; *P<0.05 by Tukey's post hoc test). Each assay was performed in duplicate. RRM1, ribonucleotide reductase large subunit M1; siRNA, small interfering RNA; NC, negative control.

altered genes were identified. A volcano plot and heat map showing the 302 genes significantly associated with RRM1 overexpression also revealed that more upregulated than downregulated genes (log2FC; 2.5) were observed (Fig. 5A). RRM1 overexpression resulted in 175 upregulated genes and 127 downregulated genes.

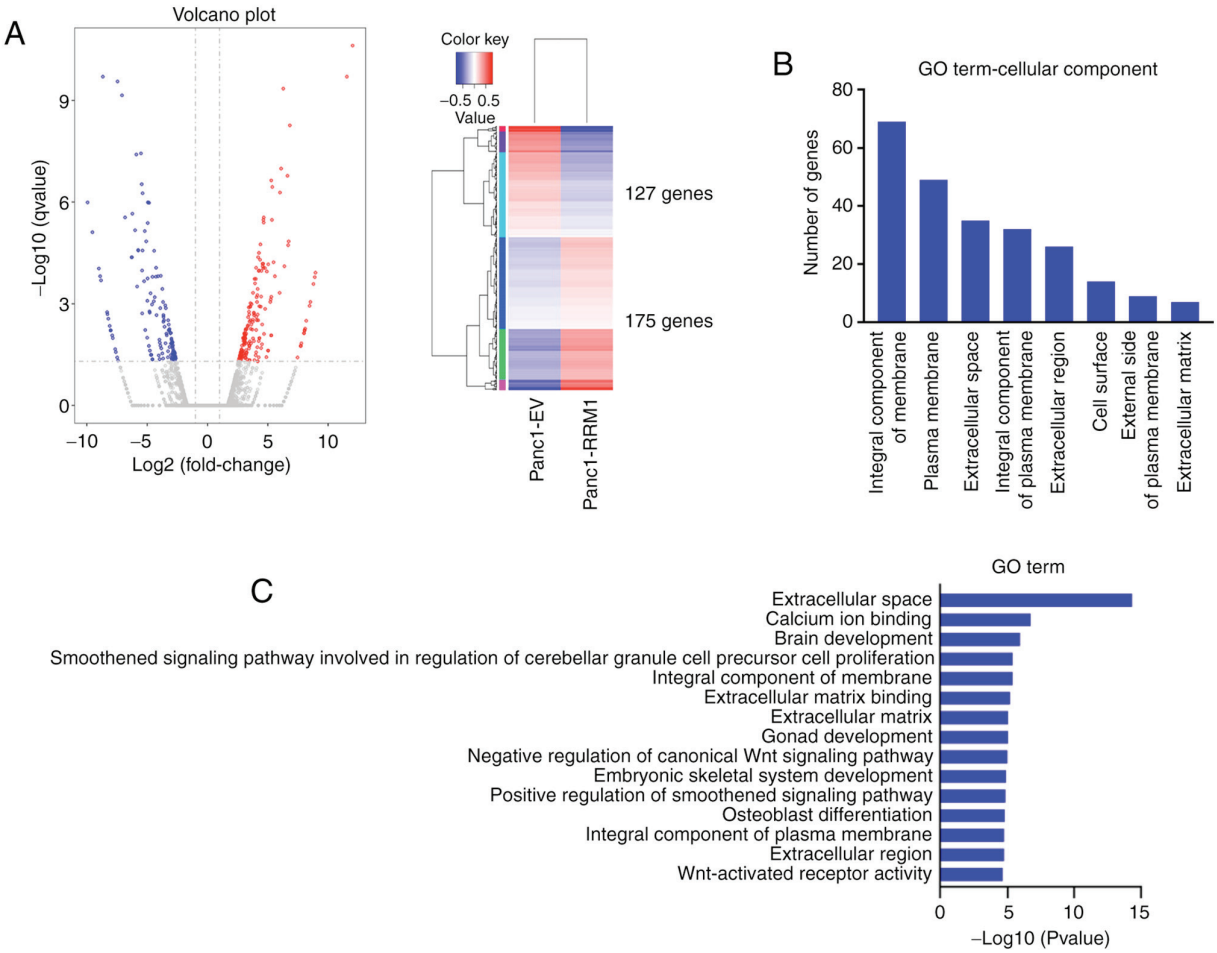

Figure 5. RNA sequencing analysis comparing RRM1 overexpression and Mock transfected in Panc1 cells. (A) Genes with an FDR <0.01 and log2 fold change of 2.5 were considered significant. Left panel: volcano plots showing log10 (P-value) vs. log2 (Fold Change). Colored points represent P<0.05, log2FC >2.5 (red), and P<0.05 and log2FC <-2.5 (blue). Right panel: a heat map of 302 significantly changed genes in Panc1-RRM1 cells compared with Panc1-Mock cells. The 175 significantly upregulated genes are colored red. The 127 significantly downregulated genes are colored blue. (B) Results of GO analysis of cellular components. The highest number of genes with a significantly altered expression were selected by terms related to the integral component of the membrane: 69 genes were selected. This was followed by 49 genes related to the plasma membrane and 35 genes related to the extracellular space. (C) Results of GO analysis significantly upregulated by RRM1 expression showed that changes in gene expression associated with the extracellular space were the most significant. In addition, gene expression changes related to the integral component of membrane or extracellular matrix were also significant, indicating that cytoskeletal-related gene expression changes were induced exclusively. RRM1, ribonucleotide reductase large subunit M1; GO, Gene Ontology.

Using GO analysis, with a focus on cellular components, the top differentially-expressed hallmark gene sets enriched in RRM1-overexpressing Panc1 cells were analyzed. RRM1 induced genes that encoded proteins that are an integral component of membranes, plasma membranes and the extracellular space (Fig. 5B). A group of 14 genes associated with the extracellular space had the most significant expression changes, compared with RRM1 expression. Other notable expression changes were related to the cytoskeleton, including the ECM and ECM binding (Fig. 5C). The expression of N-cadherin was also upregulated in this gene set.

The increased N-cadherin mRNA expression after RRM1 overexpression was confirmed by RT-qPCR. RRM1 overexpression also increased N-cadherin protein levels. N-cadherin expression changes were confirmed by western blotting and fluorescence staining. However, there was no change in the expression of fibronectin (Fig. 6A-C).

In addition to N-cadherin, the increased expression of RRM1 altered the expression levels of genes involved in the ECM, including TNC and COL11A (Fig. 6D and E). Finally, to evaluate the relevance of EMT, relative cell attachment activity was assessed by an adhesion assay (28). The increased expression of RRM1 decreased the adhesion of cells to substrates, including laminin and fibrinogen, and induced EMT, which may contribute to the increased motility and malignant transformation of cancer cells (Fig. 6F). It was previously reported by the authors that cytoplasmic RRM1 activation was associated with gemcitabine resistance (19). In the present study, a gene expression analysis approach was used to confirm that RRM1 activation was characterized by changes in the cytoplasmic skeleton associated with remodeling of the ECM.

Discussion

Gemcitabine is a key drug in the treatment of pancreatic cancer. However, the molecular mechanisms involved in the susceptibility of cells to gemcitabine are poorly understood although its pharmacological effects are clear (16). Detailed analyses of the global molecular mechanisms of gemcitabine resistance have shown that the acquisition of drug resistance

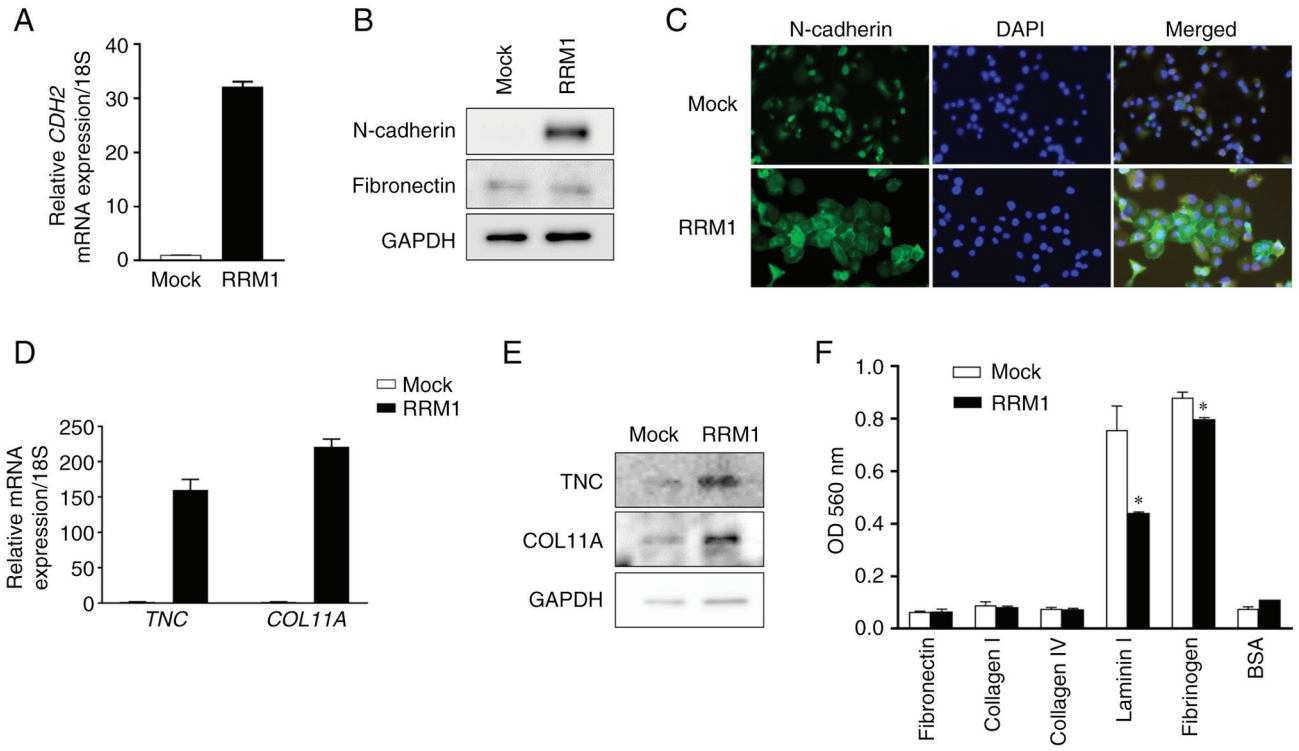

Figure 6. RRM1 overexpression increases the expression of extracellular matrix-related genes. (A) N-cadherin mRNA expression activated by RRM1 overexpression. Reverse transcription-quantitative PCR analysis showed that N-cadherin mRNA expression was increased more than 30-fold by RRM1 overexpression, compared with Mock transfected counterparts. Error bars represent the mean ± SD. Each assay was performed in duplicate. (B and C) N-cadherin protein expression was induced by RRM1 overexpression. RRM1 expression remarkably enhanced N-cadherin protein levels as revealed by (B) western blotting and (C) immunofluorescence staining analysis. (D and E) Upregulated expression levels of extracellular matrix-related genes was induced by RRM1 overexpression. RRM1 expression increased the (D) mRNA and (E) protein expression levels of TNC and COL11A. Error bars represent the mean ± SD. Each assay was performed in duplicate. (F) Cell attachment assay comparing RRM1 overexpression and Mock transfected counterparts. There were no significant differences between the two groups in adherent cells with fibronectin, collagen I and collagen IV. RRM1 overexpression significantly decreased adherent cells with laminin and fibronectin, suggesting that EMT was induced. Error bars represent the mean ± SD. *P<0.05 vs. Mock controls. Each assay was performed in duplicate. RRM1, ribonucleotide reductase large subunit M1.

by cancer cells is associated with a variety of phenotypic changes (29).

If cancer cells survive their exposure to anticancer drugs, various molecular programs are initiated during the acquisition of drug resistance, leading to the promotion of malignant properties by EMT (30,31).

The current study confirmed findings of previous studies that cells resistant to gemcitabine, a key drug in the treatment of pancreatic cancer, induced EMT and promoted the migration and invasion of cancer cells (31,32). When the GEM-R strain was created, it was judged that a 2-month treatment period was appropriate to generate GEM-R cells, based on previous studies (22,33). Our GEM-R Panc1 cells were less altered than those reported in previous studies. However, as revealed in the present study, the newly generated GEM-R cell line does demonstrate GEM-R traits, including increased expression of EMT markers such as N-cadherin and fibronectin and increased motor invasiveness, indicating that gemcitabine exposure does indeed induce malignant traits. One additional finding was that histone H3 acetylation was necessary for the acquisition of drug resistance to gemcitabine. It was previously reported by the authors that p300, a histone acetylation cofactor, bound to chromatin during the acute phase of gemcitabine exposure, and that its suppression enhanced the sensitivity of pancreatic cancer cells to gemcitabine (25). Meidhof *et al* (34) reported that HDAC inhibitors induced MET and enhanced gemcitabine sensitivity, which is consistent with our finding that increased histone acetylation is associated with resistance to gemcitabine.

RRM1 is an enzyme necessary for the conversion of ribonucleotides to deoxyribonucleosides, and may be involved in DNA synthesis and repair. A previous study reported that RRM1 gene expression was correlated with worse prognosis in various cancers and was associated with gemcitabine resistance (17-19). The survival rate of pancreatic cancer patients with high RRM1 expression treated with gemcitabine was significantly lower than that of patients with low RRM1 expression (18,19). A meta-analysis of pancreatic cancer prognosis in patients treated with gemcitabine indicated the prognostic relevance of RRM1 for pancreatic cancer (20). However, the functional importance of RRM1 is poorly understood. The current study investigated the association of RRM1, which is upregulated in GEM-R cells, with cancer cell migration and invasion. It was found that the stable expression of RRM1 increased motility-related traits such as invasion.

Wang *et al* (17) reported that RRM1 was present in the cytoplasm and nucleus of gastric cancer cells. During serum deprivation it was predominant in the cytoplasm, and upon serum repletion it translocated to the nucleus, confirming the functional changes of RRM1 under certain stress signals,

such as hypoxia. It was previously reported that RRM1 cytoplasmic expression was upregulated further after exposure to gemcitabine (19). In the current study, it was identified that the functional significance of the increased cytoplasmic expression of RRM1 is associated with global changes in cellular matrix remodeling, including the induction of EMTs including N-cadherin, TNC and COL11A.

The association of N-cadherin with motility has been reported in numerous carcinomas. The suppression of RRM2 in lung cancer cells reduced their migration and invasion, similar to the reduction of N-cadherin expression (35). RRM1 expression in cervical cancer was associated with EMT by upregulating N-cadherin, which altered the cytoplasmic expression of p53 (36). p53R2, a ribonuclease subunit-related protein, binds to RRM1. Its inhibition in cervical cancer cells induced EMT and increased the expression of N-cadherin through the Akt signaling pathway (37). The association of N-cadherin to prognosis was also reported in pancreatic cancer (38).

Global changes in the ECM, including N-cadherin and the cytoskeleton, may enhance the motility of cancer cells. COL11A is upregulated in pancreatic cancer and was correlated with numerous clinical parameters as well as increased motility and invasiveness via EMT (39). Similarly, TNC is upregulated in pancreatic cancer and contributes to enhanced motility and invasion (40,41). Furthermore, COL11A and TNC were associated with resistance to gemcitabine by modulating BAX/BCL-2 functions and activating ERK/NF-κB signaling, respectively (42,43). These ECM-related genes were previously reported to be associated with the prognosis of pancreatic cancer patients (44,45), indicating that RRM1 may be a master gene located upstream of these genes and involved in regulating their expression. Thus, the mechanisms involved and how they regulate the expression levels of ECM genes require further study.

When the HAT inhibitor C646 was used to inhibit increased histone acetylation in Panc1 GEM-resistant cells and migration and invasion assays were performed, the migratory ability of these cells was not affected. This result is considered a limitation to the present study. The main reason for this result is that C646 comprehensively suppresses histone acetylation, which has certain effects on the regulation of gene expression, and thus affects the expression of various genes other than the RRM1 gene, meaning that its effects on motor activity such as migration and invasion cannot be accurately assessed. In the current study, only the motor migration activity associated with the RRM1 gene was investigated.

As aforementioned, RRM1 expression has been shown to be associated with worse prognosis in pancreatic cancer (19). However, the biological significance of the RRM1 gene has not yet been clarified. In the present study, the biological significance of RRM1 expression associated with cell motility was reported. It was demonstrated that RRM1 transition is activated by histone H3 acetylation, and induces activation of biologically significant related genes such as N-cadherin, TNC, and COL11A, which involves ECM remodeling. Therefore, RRM1 is critical for the acquisition of a malignant phenotype, including increased invasiveness, in pancreatic cancer cells. It could not be demonstrated that the expression levels of ECM genes are regulated by histone acetylation. It is possible that ECM expression may be regulated by downstream factors of RRM1 signaling, such as through some transcription factors.

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Availability of data and materials

The data generated in the present study are available from the corresponding author on reasonable request and may be found in the DDBJ Sequenced Read Archive repository (https://www.ddbj.nig.ac.jp/dra/index-e.html) with accession number DRA015584.

Authors' contributions

HO conceived the study, performed the experiments and wrote and edited the manuscript. YM, TK and YA performed the experiments. SW, HY and KO were involved in data curation. DA, YI, HU and KA contributed to the conception of the study and analysis and interpretation of data. AK, ST, and MT supervised and directed the research and edited the manuscript. YM, TK and MT confirm 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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