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Biochemical and Biophysical Research Communications 354 (2007) 190–196
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RRM2 induces NF-κB-dependent MMP-9 activation and enhances cellular invasiveness

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Received 20 December 2006
Available online 29 December 2006

Abstract

Ribonucleotide reductase is a dimeric enzyme that catalyzes conversion of ribonucleotide 5′-diphosphates to their 2′-deoxynucleotide forms, a rate-limiting step in the production of 2′-deoxyribonucleoside 5′-triphosphates required for DNA synthesis. The ribonucleotide reductase M2 subunit (RRM2) is a determinant of malignant cellular behavior in a range of human cancers. We examined the effect of RRM2 overexpression on pancreatic adenocarcinoma cellular invasiveness and nuclear factor-κB (NF-κB) transcription factor activity. RRM2 overexpression increases pancreatic adenocarcinoma cellular invasiveness and MMP-9 expression in a NF-κB-dependent manner. RNA interference (RNAi)-mediated silencing of RRM2 expression attenuates cellular invasiveness and NF-κB activity. NF-κB is a key mediator of the invasive phenotypic changes induced by RRM2 overexpression.

Keywords: Pancreas; Pancreatic cancer; Pancreatic adenocarcinoma; Invasion; Invasiveness; NF-κB; MAPK; Ribonucleotide reductase; RRM2; MMP-9; Metalloproteinase

Pancreatic adenocarcinoma is characterized by a propensity for extensive locoregional invasion and early metastasis. Invasion into vital structures commonly precludes surgical resection, rendering the disease incurable. Determinants of cellular invasiveness represent a potential therapeutic target.

Ribonucleotide reductase (RR) is an enzyme of central importance in DNA synthesis [1]. RR catalyzes conversion of ribonucleotide 5′-diphosphates to their 2′-deoxynucleotide forms, a rate-limiting step in the production of 2′-deoxyribonucleoside 5′-triphosphates (dNTP) required for DNA synthesis [2–5]. The RR holoenzyme consists of M1 and M2 subunits, although a recently discovered p53-dependent subunit, p53R2 can also unite with the M1 subunit to form a functional enzyme of lower activity [6–8]. Holoenzymatic activity is modulated by levels of the M2 subunit (RRM2), expression of which varies during the cell cycle [9,10]. RRM2 itself is a dimer of two 44 kDa moieties, each containing a tyrosine free-radical and non-heme iron [11]. Cells overexpressing RRM2 exhibit enhanced cellular invasiveness [12], but the mechanisms through which RRM2 affects the invasive phenotype have not been closely studied. Here, we examine these mechanisms in the context of human pancreatic adenocarcinoma.

Materials and methods

Cell lines and culture. Capan2 and MIAPaCa2 pancreatic ductal adenocarcinoma cells were obtained from ATCC (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco-BRL, Gaithersburg, MD). Cells were maintained in a humidified (37 °C, 5% CO_2) incubator and passaged upon reaching 80% confluence.

Expression constructs and transfection. RRM2 cDNA inserts were produced by PCR (primer 1: 5′-CACCATGCTCTCCCTCCGTGT-3′; primer 2: 5′-TTAGAAGTCAGCATCCAAG-3′). The PCR products were inserted into the pcDNA3.1(+) expression vector by Proteintech Group Inc. (Chicago, IL). The I-κB (S32A/S36A) expression construct was obtained from Upstate (Upstate, NY). Transfection was performed

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doi:10.1016/j.bbrc.2006.12.177

using Lipofectamine 2000 Reagent (Invitrogen). Stable transfectants were selected on the basis of their resistance to G418 (0.5 mg/ml, Gibco).

Cytotoxicity assays. Cell proliferation was quantified by 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Trevigen, Gaithersburg, MD) in accordance with the manufacturer's instructions and confirmed by cell counting. Results of the MTT assay have been shown to correlate well with [^{3}H]-thymidine incorporation in pancreatic adenocarcinoma cell lines [14]. Plates were read using a V_{max} microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm, referenced to 650 nm. Ten samples were used for each experimental condition and experiments were performed in triplicate. At identical time points, cell counting was performed. Cells were trypsinized to form a single cell suspension. Viable cells, determined by Trypan blue exclusion, were counted using a Neubauer hemocytometer (Hausser scientific, Horsham, PA). Cell counts were used to confirm MTT results.

Invasion assay. Invasion was quantified using a modified Matrigel Boyden chamber assay. The BD BioCoat Matrigel invasion chamber (BD Bioscience, Bedford, MA) was used according to the manufacturer's instructions. 2.5×10^4 cells in serum-free media were seeded onto Matrigel-coated filters. In the lower chambers, 5% FBS was added as a chemoattractant. After 18 h incubation, the filters were stained using the Diff-QuikTM kit (BD Biosciences), and cells that had invaded through the filter were counted under magnification (randomly selected high-power fields). The counting was performed in three random fields for each sample, and mean values from three independent experiments were used. The contribution of MMP-9 to cellular invasiveness was determined by performing the invasion assay in the presence of 10 μg/ml anti-MMP-9 neutralizing monoclonal antibody (Santa Cruz) or 10 μg/ml control (irrelevant) IgG.

MMP-9 activity assay. MMP-9 activities in whole cell lysates were assessed using the colorimetric Biotrak MMP-9 activity assay (Amersham) in accordance with the manufacturer's instructions. Optical densities were quantified using a V_{max} microplate spectrophotometer at a wavelength of 405 nm, referenced to 650 nm. Three samples were used for each experimental condition and experiments were performed in triplicate and mean values calculated.

Immunoblotting. Whole cell extracts were prepared using cell lysis buffer (20 mM Tris, pH 7.5, 0.1% Triton X, 0.5% deoxycholate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and cleared by centrifugation at 12,000*g*, 4 °C. Tissues were homogenized and lysed in the same lysis buffer. Total protein concentration was measured using the BCA assay kit (Sigma, St. Louis, MO) with bovine serum albumin as a standard. Cell lysates containing 30 μg total protein were analyzed by immunoblotting. Anti-RRM2 and anti-MMP-9 antibodies were obtained from Santa Cruz (Santa Cruz, CA). Anti-actin antibodies were obtained from LabVision (Freemont, CA). Chemoluminescent detection (Upstate, Lake Placid, NY) was performed in accordance with the manufacturer's instructions. Signal intensities were quantified using ImagePro Plus software version 4.0 and normalized to respective loading control as appropriate.

Ribonucleotide reductase assay. Cells (10^7) were collected, washed, and extracts were prepared on ice in the presence of RNAse A as previously described [15]. Protein concentration was measured by BCA assay and samples containing equal total protein were analyzed. Endogenous nucleotides were removed using a Sephadex G-25 spin column. Ribonucleotide reductase activity was quantified using a [^{14}C]CDP and DNA polymerase-based assay as described by Jong et al. [15]. Ribonucleotide reductase catalyzes conversion of CDP to [^{14}C]dCDP which is converted to [^{14}C]CTP by endogenous nucleotide diphosphate kinase and incorporated into nascent DNA by DNA polymerase. The duration of the ribonucleotide reductase reaction was 30 min and the duration of the coupling reaction was 50 min. Nascent DNA radioactivity was quantified using a liquid scintillation counter (Beckman Coulter Inc., Fullerton, CA). Negative controls included reactions with omission of polymerase and DNA primers. All experiments were performed three times with three determinations per cell line.

NF-κB luciferase assay. Cellular NF-κB activity was quantified using a commercially available NF-κB reporter construct (Stratagene, La Jolla, CA). Cells were transfected with the reporter construct in accordance with the manufacturer's instructions. Luciferase activity was assessed using a proprietary luciferase assay kit (BD BioSciences, San Jose, CA). Luminescence was quantified using a TD-20/20 luminometer (Turner BioSystems Inc., Sunnyvale, CA). Experiments were performed in triplicate with three determinations per condition.

Nuclear NF-κB enzyme-linked immunoassays. Levels of NF-κB consensus sequence DNA binding in nuclear extracts were detected using the BD Mercury Transfactor™ kit (BD Biosciences). This non-radioactive technique has 10-fold higher sensitivity than electrophoretic mobility shift assays [16]. NF-κB consensus sequence DNA (5′-GGGGACTTTCCC-3′) [17,18] immobilized on a 96-well plate is used to capture NF-κB from pancreatic adenocarcinoma nuclear extracts containing 20 μg of total protein. The DNA-bound transcription factor was detected and quantified by ELISA using NF-κB-specific antibodies and TMB-based detection, in accordance with the manufacturer's instructions. An oligonucleotide containing a mutated consensus sequence motif (5′-GGCGACTTTCCC-3′) was used as an additional negative control. Plates were read on a V_{max} microplate spectrophotometer at 650 nm. Experiments were reproduced three times with three samples per condition.

Retroviral RRM2 siRNA vector construction. Complementary oligonucleotide primers containing *Xho*I and *Xba*I overhangs and the required siRNA sequences joined by a linker sequence (5′-gactcctg-3′) were obtained from Sigma. The RRM2 gene (Accession No. BC001886) target sequence was 5′-AATGCTGTTCGGATAGAACAG-3′. A single-base mismatch control target sequence, lacking any significant gene sequence homology, was 5′-AATGCTTTTCGGATAGAACAG-3′. The oligonucleotides were annealed and inserted into the linearized pSuppressorRetro (psi) vector in accordance with the manufacturer's instructions (Imgenex, San Diego, CA). Vectors were expanded in competent *Escherichia coli* 293 packaging cells (ATCC) were transfected with retroviral vectors, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and selected for stable retrovirus production. Pancreatic adenocarcinoma cells were exposed to retrovirus-containing supernatant for 16 h in the presence of polybrene (Sigma, St. Louis, MO). Stable selection of transfected clones was achieved using G418 (Gibco). Clones were maintained in 200 μM G418.

Statistical analysis. Differences between groups were analyzed using Student's *t* test, multifactorial ANOVA of initial measurements and Mann–Whitney *U* test, for non-parametric data, as appropriate, using Statistica 5.5 software (StatSoft Inc., Tulsa, OK). In cases in which averages were normalized to controls, the standard deviations of each nominator and denominator were taken into account in calculating the final standard deviation. $P < 0.05$ was considered statistically significant.

Results

RRM2 overexpression enhances cellular invasiveness and upregulates MMP-9 expression and activity

Pancreatic adenocarcinoma cells differentially express RRM2 [19]. Capan2 was selected for RRM2 overexpression studies, as this cell line inherently expresses a low level of RRM2. Two transfectant clones of Capan2 exhibiting stable RRM2 overexpression were established: Capan2-R2.1 and Capan2-R2.2. Overexpression of RRM2 protein was confirmed in each clone by Western blot analysis (Fig. 1a). Capan2-R2.1 and Capan2-R2.2 expressed levels of RRM2 7.2- and 8.2-fold higher than that of empty vector transfectants (Capan2-Neo), respectively. RRM2 expression did not significantly differ between Capan2 and Capan2-Neo (empty vector) transfectants.

Cellular ribonucleotide reductase activities were compared using a polymerase-based assay [15]. Transfected RRM2 was functional as the cellular ribonucleotide reduc-

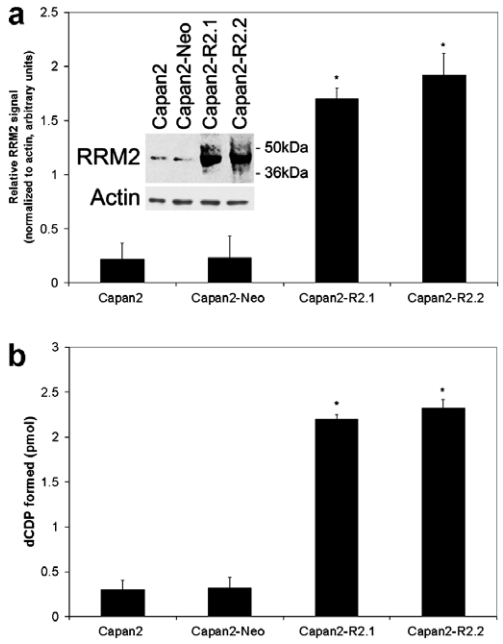

Fig. 1. (a) Stable overexpression of RRM2 was confirmed in Capan2-R2.1 and Capan2-R2.2 transfectants by Western blotting. There was no significant difference in RRM2 expression in Capan2 cells and Capan2-Neo transfectants. Blot is representative of triplicate blots. Densitometric values are means (±SD). $^{*}P < 0.05$ versus Capan2-Neo transfectants. (b) Ribonucleotide reductase activities were quantified using a polymerase-based assay [15]. Capan2-R2.1 and Capan2-R2.2 transfectants exhibited significantly greater levels of ribonucleotide reductase activity than Capan2-Neo transfectants. Assays were performed in triplicate with three samples per transfectant. Values are means (±SD). $^{*}P < 0.05$ versus Capan2-Neo transfectants.

tase activities of Capan2-R2.1 and Capan2-R2.2 transfectants were 6.9- and 7.3-fold greater than that of Capan2-Neo transfectants (Fig. 1b). Doubling times of the Capan2-R2.1 and Capan2-R2.2 transfectants did not significantly differ from those of control cells (data not shown).

Cellular invasiveness was quantified using Matrigel-coated Boyden chambers. Both Capan2-R2.1 and Capan2-R2.2 transfectants demonstrated greater cellular invasiveness than Capan2-Neo transfectants (Fig. 2a). We have previously shown that altered RRM2 expression affects MMP-9 activity [20] and we confirmed significantly higher levels of MMP-9 expression and activity in Capan2-R2.1 and Capan2-R2.2 (Fig. 2b and c).

The contribution of MMP-9 to the increased cellular invasiveness induced by RRM2 overexpression was determined by performing the invasion assay in the presence of anti-MMP-9 neutralizing antibody. MMP-9 immunoneutralization reduced the cellular invasiveness of Capan2-R2.1 and Capan2-R2.2 by approximately 80% (Fig. 2a).

Enhanced cellular invasiveness induced by RRM2 overexpression is NF-κB-dependent

The nuclear factor kappa B transcription factor (NF-κB) is an important determinant of pancreatic adenocarcinoma malignant cellular behavior [21–24], and RRM2 overexpression is associated with differential binding activity of NF-κB in human cancer cells [25]. We compared NF-κB activities of Capan2-R2.1 and Capan2-R2.2 with control transfectants, using a luciferase reporter assay, and assessed NF-κB nuclear translocation using DNA binding ELISA. Both Capan2-R2.1 and Capan2-R2.2 exhibited significantly greater total and nuclear NF-κB activities than that of control transfectants (Fig. 3a and b).

Next, we sought to determine whether NF-κB activity was necessary for the effects on cellular invasiveness and MMP-9 expression induced by RRM2 overexpression to occur. NF-κB activity was inhibited by transfection of an expression construct encoding the inhibitor of nuclear factor kappa B (I-κB). I-κB transfection markedly attenuated Capan2-R2.1 and Capan2-R2.2 transfectant cellular invasiveness and MMP-9 expression (Fig. 3c and d). These observations indicate that RRM2 overexpression increases cellular invasiveness, at least in part, via an NF-κB-dependent increase in MMP-9 expression and activity.

RRM2 knockdown decreases cellular NF-κB activity and nuclear localization and attenuates cellular invasiveness

Next, we examined the effect of stable suppression of RRM2 expression in MIAPaCa2 pancreatic adenocarcinoma cells, a highly invasive and tumorigenic cell line that, under standard culture conditions, inherently expresses relatively high levels of both RRM2 and MMP-9 [19,26]. Cells were stably transfected with one of two retroviral vectors that generated either RRM2-specific siRNA (psiRRM2) or control siRNA (psiControl). Empty retroviral vector (psiNeo) transfectants served as additional negative controls. Following selection of transfectants, based on their resistance to G418, stable suppression of RRM2 expression was confirmed by Western blot analysis (Fig. 4a). Consistent with our previous observations [20], NF-κB activity and nuclear localization were significantly decreased by stable suppression of RRM2 expression (Fig. 4b and c). Cellular invasiveness was significantly impaired in psiRRM2 transfectants, relative to both psiControl and psiNeo transfectants (Fig. 4d).

Discussion

Although RR is recognized to have importance in maintaining DNA integrity, the results of this study support the hypothesis that RRM2 has additional functions, including participation in oncologically important signaling events that influence the invasive phenotype. High RR activity is associated with tumor progression and resistance to cellular stressors such as chemotherapeutic agents and ionizing radiation [12,19,27–29]. Increased RR activity is reported to be present in highly metastatic tumor cells [30], and overexpression of RRM2 in human oral carcinoma cells has been shown to be associated with increases in their invasive potential [12]. Despite these observations, the

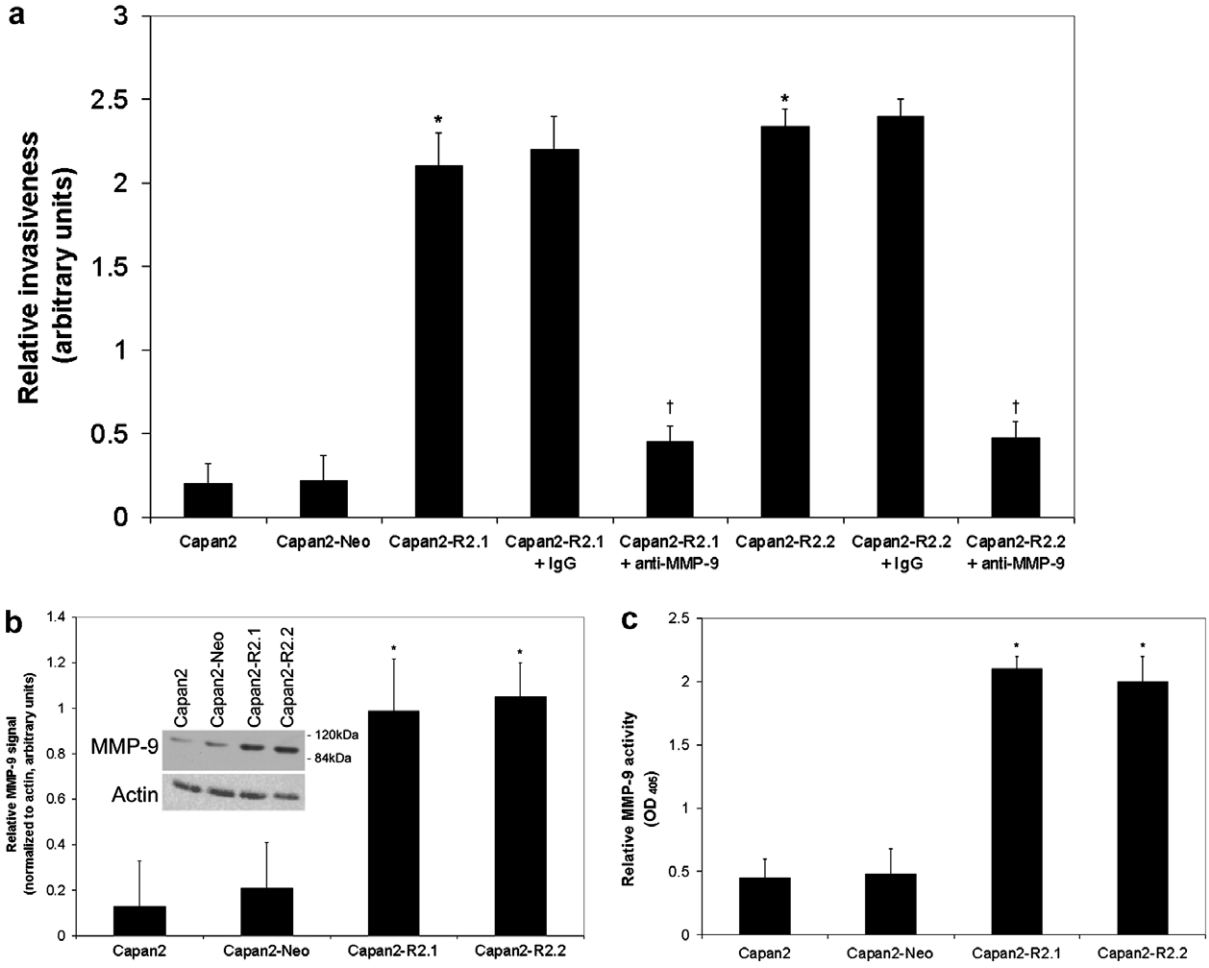

Fig. 2. (a) Cellular invasiveness, assayed by modified Matrigel Boyden chamber, was increased in Capan2-R2.1 and Capan2-R2.2 transfectants. There was no significant difference in invasiveness between Capan2 cells and Capan2-Neo transfectants. Anti-MMP-9 antibody suppressed cellular invasiveness by 80%, relative to IgG-treated controls. Mean values (±SD) from triplicate experiments derived from three samples per chamber are shown. $^{*}P < 0.05$ versus Capan2-Neo. $^{\dagger}P < 0.05$ versus control IgG. Cellular MMP-9 expression, quantified by Western blot (b), and cell lysate MMP-9 activity, quantified by colorimetric assay (c), were both significantly increased in Capan2-R2.1 and Capan2-R2.2 transfectants, relative to Capan2 cells and Capan2-Neo transfectants, which exhibited no significant difference in their respective levels of cellular MMP-9 expression and lysate MMP-9 activities. Means (±SD) values from triplicate experiments. $^{*}P < 0.05$ versus Capan2-Neo.

intracellular mechanisms through which RRM2 influences cellular invasiveness have received little attention.

This study demonstrates a role for NF-κB in mediating the oncogenic effects of RRM2 overexpression and strongly implicates NF-κB as a key mediator of the effects of RRM2 overexpression on the invasive phenotype. Transcription factors of the Rel/NF-κB family regulate diverse aspects of pancreatic adenocarcinoma tumor biology, including cellular invasiveness [21–24]. Zhou et al have shown that the KBGem clone, which overexpresses RRM2, exhibits differential NF-κB binding activity when compared to wild type KB cells [25]. The NF-κB transcription factor modulates expression of metalloproteinases in a range of human cancers [31–35]. We focused on MMP-9 as its promoter contains motifs homologous to the binding sites for the NF-κB protein and levels of NF-κB activity have been shown to modulate MMP-9 transcription [36,37]. We have also found MMP-9 activity to be decreased by RRM2 silencing [20]. These observations together with those reported here are consistent with a model in which NF-κB activation, resulting from RRM2 overexpression, enhances MMP-9 activity and promotes cellular invasiveness. However, we interpret these observations cautiously. While MMP-9 is an important mediator of pancreatic adenocarcinoma cellular invasiveness, other proteases and modulators of cellular motility not studied here play additional roles in determining tumor cell invasiveness. Despite this limitation, MMP-9 contributes significantly to the invasive phenotype of pancreatic adenocarcinoma cells [38]. RRM2 is reported to interact synergistically with a range of oncogenes such as H-ras [39], forming a point of convergence for a diversity of extracellular and signal-induced pathways [40]. The nature of the mechanisms through which RRM2 influences nuclear transcription factor activity requires further investigation.

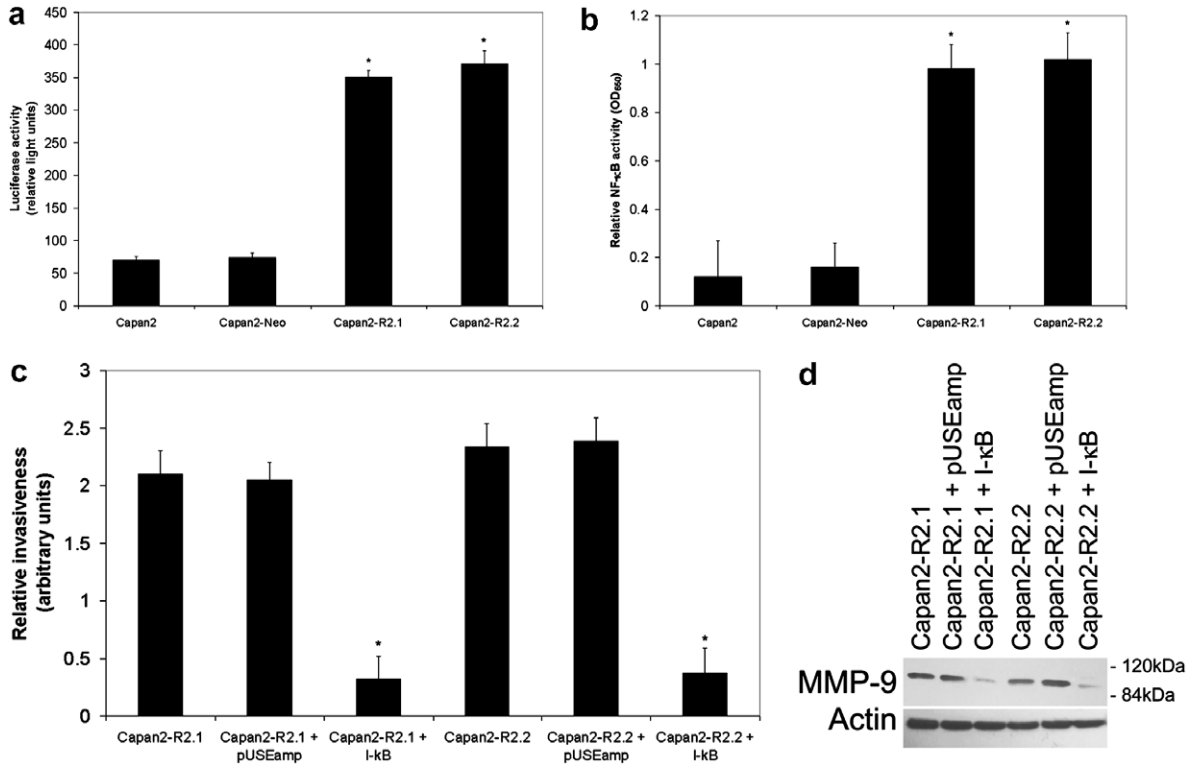

Fig. 3. Cellular NF-κB activity was quantified by luciferase reporter assay (a). Nuclear NF-κB translocation was quantified by NF-κB ELISA (b). Increased levels of NF-κB cellular activity and nuclear translocation were observed in Capan2-R2.1 and Capan2-R2.2 transfectants, relative to both Capan2 cells and Capan2-Neo transfectants. Experiments were reproduced three times with three samples per condition. Mean values (±SD) are shown. $^{*}P < 0.05$ versus Capan2-Neo. (c) Transfection of I-κB decreased the cellular invasiveness of Capan2-R2.1 and Capan2-R2.2 transfectants by 84% and 85%, respectively. Impaired invasiveness was associated with decreased MMP-9 expression (d). Values are means (±SD) from triplicate experiments. $^{*}P < 0.05$ versus Capan2-Neo.

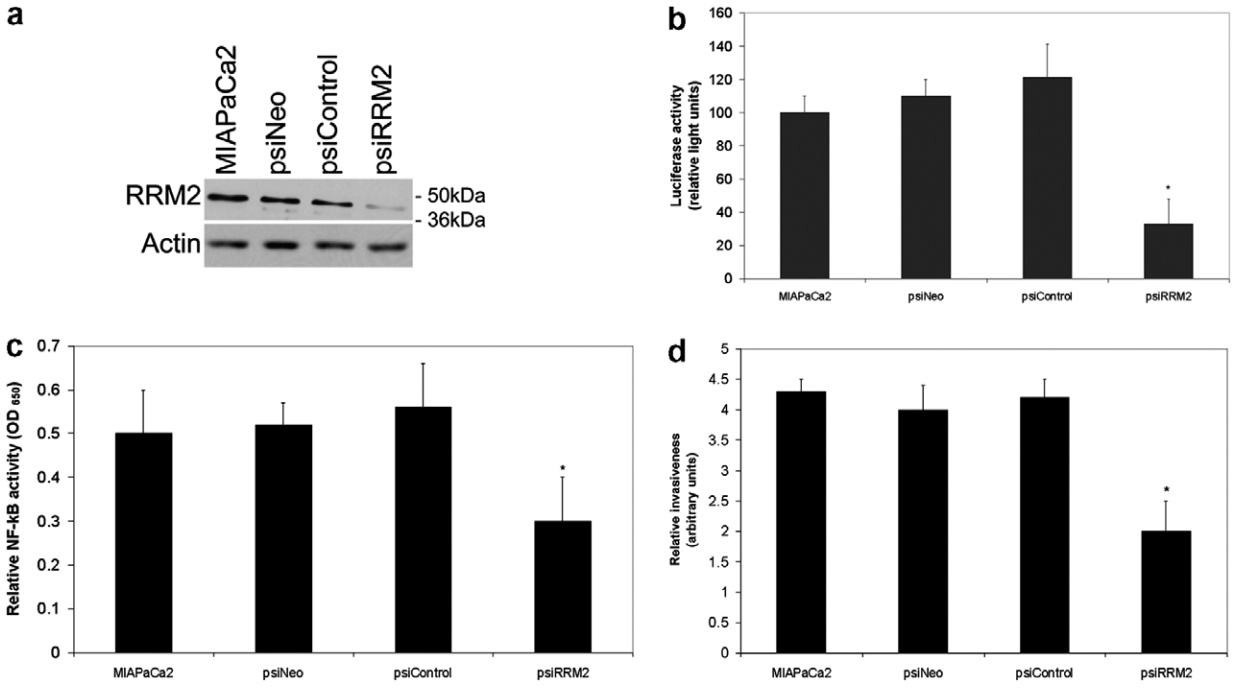

Fig. 4. (a) Stable knockdown of RRM2 expression by the RRM2-specific siRNA generating retroviral vector (psiRRM2), but not the control vector (psiControl), was confirmed by Western blot analysis. Empty vector transfectants (psiNeo) served as additional controls. Representative example of triplicate blots. NF-κB activities (b) and nuclear localization (c) of Capan2-R2.1 and Capan2-R2.2 transfectants were significantly decreased by RRM2 knockdown. (d) RRM2 knockdown attenuated cellular invasiveness. $^{*}P < 0.05$ versus psiControl.

In summary, increased invasiveness induced by RRM2 overexpression is associated with, and dependent upon, NF-κB activation. MMP-9 appears to be an important effector of the enhanced invasiveness that RRM2 overexpression induces. In addition to its role as a regulator of the dNTP pool and DNA synthesis, levels of RRM2 modulate oncologically important intracellular signaling events that lead to changes in cellular invasiveness. Targeting RRM2 and its downstream signaling intermediaries represents a rational approach for developing novel anticancer therapeutics.

Acknowledgments

The authors acknowledge the secretarial assistance of Jan Rounds. This study was supported by NIH Grant RO1 CA114103 and American Cancer Society Grant RSG-04421-01-CCE.

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