Original Research

Depletion of RNA-binding protein RBM8A (Y14) causes cell cycle deficiency and apoptosis in human cells

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Abstract

RBM8A (Y14) contains an RNA-binding motif and forms a tight heterodimer with Magoh. The heterodimer is known to be a member of the exon junction complex that forms on mRNA before export and it is required for mRNA metabolism processes such as splicing, mRNA export and nonsense-mediated mRNA decay. Recently, deficient cellular proliferation has been observed in RBM8A- or Magoh-depleted cells. These results prompted us to study the role of RBM8A in cell cycle progression of human tumour cells. The depletion of RBM8A in A549 cells resulted in poor cell survival and the accumulation of mitotic cells. After release from G1/S arrest induced by a double thymidine block, the RBM8A-silenced cells could not proceed to the next G1 phase beyond G2/M phase. Finally, the sub-G1 population increased and the apoptosis markers caspases 3/7 were activated. Silenced cells exhibited an increased frequency of multipolar or monopolar centrosomes, which may have caused the observed deficiency in cell cycle progression. Finally, silencing of either RBM8A or Magoh resulted in mutual downregulation of the other protein. These results illustrate that the RBM8A-Magoh mRNA binding complex is required for M phase progression and both proteins may be novel targets for anticancer therapy.

Keywords: RBM8A (Y14), Magoh, cell cycle, G2/M phase, apoptosis

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Introduction

The exon junction complex (EJC) forms on exon junctions in association with mRNA splicing and this complex consists of RBM8A (Y14), Magoh, eIF4A3, Btz, SRm160, Aly and other proteins.[1–3] RBM8A tightly forms a heterodimer with Magoh and it localizes onto mRNA through the RNA-binding motif of RBM8A. EJC is required for mRNA export and the nonsense-mediated mRNA decay (NMD) pathway and NMD-related factors assemble the decaying complex onto mRNA through EJC. After the first pioneer round of translation, EJC is removed from mRNA molecules and reused in the nuclei.[4–6] Recent research revealed that RBM8A can form a complex with STAT3, which regulates cytokine regulator pathways and its novel function in STAT3-mediated transcription has been implied.[7,8]

On the other hand, Le Hir *et al.*[1] demonstrated the association between RBM8A and Magoh and depletion of one of these proteins in *Drosophila* SL2 cells resulted in deficient cellular proliferation. Furthermore, Sudo *et al.*[9] performed loss-of-function screening of genes involved in growth in a human mesothelioma cell line. In addition to the *COPA* gene, they demonstrated the contribution of RBM8A to cell growth in a silencing experiment. Thus, RBM8A seems to be necessary for cell cycle progression and its depletion can alter the cell cycle and lead to cell death. On the other hand, there is a possibility that RBM8A depletion leads to apoptosis. RBM8A-depleted cells do not replicate probably because they are undergoing apoptosis and not because RBM8A depletion alters cell cycle progression. However, no mechanism has been proposed thus far for the growth defect in the RBM8A-depleted cells, although abnormal gene expression was estimated as the cause of growth deficiency.

Recently, the novel function of Magoh in neural stem cell division was reported in mice and the contribution of Magoh to mitotic progression was proposed by Silver *et al.*[10] Furthermore, Inaki *et al.*[11] revealed the contribution of Magoh to cyclin-dependent kinase (Cdk) regulation in a temperature-sensitive cell cycle mutant screening experiment. Because Cdk was required for mitosis progression, this finding strongly implies the participation of Magoh in cell cycle progression, independent of EJC formation.

Interestingly, RBM8A deficiency in humans had different phenotypic consequences than Magoh deficiency. The deletion of chromosome 1q21.1 has been frequently found in thrombocytopenia-absent radius (TAR) syndrome patients and *RBM8A* is mapped to this region. Recently, Albers *et al.*[12] reported that the TAR syndrome is caused by the compound inheritance of a 1q21.1 deletion and rare SNPs in *RBM8A*. TAR syndrome patients share no common phenotype with Magoh-deficient mice. Therefore, it is speculated that Magoh and RBM8A have independent functions in addition to their shared functions.

In the present study, we studied the role of RBM8A in cell division in silencing experiments using a human tumour cell line and our results revealed the role of RBM8A in cell cycle progression, particularly for mitotic progression. In our study, depletion of RBM8A resulted in coordinate decreasing of Magoh protein. Therefore, it is possible that RBM8A participates in cell cycle progression through Magoh, with which it associates in centrosome regulation.

Materials and methods

Cell culture

A549 and HeLa cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics. To arrest the HeLa cells at the G1/S boundary phase, double thymidine treatment was used. In brief, cells were incubated in 2.5 mM thymidine-containing medium (Sigma-Aldrich, St Louis, MO, USA) for 24 h in two separate periods that were typically separated by 12 h.

Silencing of gene expression using siRNA

One day before siRNA transfection, cells were seeded in a culture plate or dish. The RBM8A gene was silenced using Stealth Select RNAi^{TM} siRNAs (HSS115052: shown in '#1', HSS115053: shown in '#2', HSS115054: shown in '#3') and Magoh silencing was also performed in the same manner (HSS142861: shown in '#1', HSS142862: shown in '#2', HSS142863: shown in '#3'). siRNAs were obtained from Invitrogen, Life Technologies (Carlsbad, CA, USA) and Lipofectamine RNAiMAX (Invitrogen) was used to transfect siRNA. Two double-stranded molecules of the Stealth RNAi negative control kit, LO (L) MI (M) and HI (H), were used as negative controls. Because *RBM8A* partially overlaps with *GNRHR2*,[13,14] the target sequence of siRNA did not include the overlapping region.

Flow cytometric analysis

After gene silencing, cells were collected by trypsinization and fixed with 30% ethanol in phosphate-buffered saline (PBS). To collect floating M phase cells, the culture medium was combined with the trypsinized cell suspension. After fixation, cells were treated with 1 mg/mL RNase (Sigma-Aldrich) and stained with 1 mg/mL propidium iodide (Sigma-Aldrich). After staining, a FACSCalibur flow cytometer and Cell Quest Pro software (Becton Dickinson & Co., Franklin Lakes, NJ, USA) were used for data analysis.

Western blotting (WB)

To analyse the expression of RBM8A and Magoh, Western blotting was performed.[15] Mouse monoclonal anti-RBM8A antibody was purchased from Sigma (Y1253) and anti-Magoh antibody was purchased from Abcam plc (ab38768, Cambridge, UK). Apoptosis-related caspase and poly(ADP-ribose) polymerase (PARP) activation was analysed by Western blotting. Anticaspase and anti-PARP antibodies were picked up from Apoptosis sampler kit (#4445, Cell Signaling Technology, Inc., Danvers, MA, USA). Reference β-actin (ACTB) was detected by mouse monoclonal anti-β-actin antibody from Sigma (A5441). Finally, the signal from a horseradish peroxidase-conjugated second antibody (polyclonal goat antirabbit or antimouse antibody, P0447 or P0448, from Dako Denmark Inc., Glostrup, Denmark) was detected using ImmunoStar LD (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and LAS4000 (Fujifilm Corp., Tokyo, Japan).

Real-time PCR analysis

Total RNA was extracted from the cells by using QIAGEN RNeasy mini kit (Qiagen GmbH, Hilden, Germany). The quality of RNA was checked on the basis of the band intensity of 18 S and 28 S ribosomes determined using 1.2% agarose gel. Complement DNA (cDNA) was synthesized using Superscript III reverse transcriptase (Invitrogen, Life Technologies) and the cDNA was used as the template in real-time PCR; TaqMan Gene Expression assays were performed using the 7900HT Fast Real-time PCR system (Applied Biosystems, Life Technologies). The primer sets Hs04234932_g1 for RBM8A, Hs00830672_s1 for Magoh and Hs01060665_g1 for β-actin were purchased from Applied Biosystems, Life Technologies.

Measurement of the mitotic index

Cells were collected by trypsinization and fixed with Carnoy solution. Specimens were stained with Giemsa solution (Sigma-Aldrich). The frequency of mitotic cells per 800 cells was counted under a microscope using a blinded method.

Immunostaining

Our immunostaining protocol was described in a previous report.[16] Concretely, the cells were washed twice with excess PBS and fixed for 10 min in cold methanol (Wako) or 10% paraformaldehyde solution (TAAB Laboratories Equipment Ltd., Berks, UK) at room temperature. Cells were treated with 0.2% Triton X for 10 min following three washes with PBS. Blocking was done using PBS-diluted 1% bovine serum albumin (Wako) for 30 min at room temperature. After these procedures, cells were incubated with 200 x diluted rabbit anti-γ-Tubulin (T3559, Sigma) or 200 x diluted antipericentrin antibody (PRB-432 C, Covance Inc., Princeton, NJ, USA) for 2 h at room temperature. Primary antibody binding was detected using 800 x diluted Alexa

Fluor 594-conjugated secondary antibody solution (A11007, Molecular Probes, Life Technologies) for 1 h at room temperature and nuclei were detected by DAPI staining. Prolong Gold antifade reagent (Invitrogen) was used to avoid fading. Images were obtained and processed using an Axiovert 200 M camera (Carl Zeiss Co. Ltd., Jena, Germany). The number of centrosomes was counted in more than 200 cells.

Statistics analysis

For multiple testing, data were analysed by Dunnett's post hoc test for multiple comparisons to the control groups. Differences were considered statistically significant if $P < 0.05$.

Results

Effect of RBM8A depletion on colony-forming ability

To study the physiological function of RBM8A, we transfected the A549 cells with two siRNAs that target different regions of the RBM8A mRNA sequence. In this study, Western blot analysis revealed that specific siRNA reproducibly reduced the RBM8A levels to less than 15% of those in control siRNA-transfected cells two days after transfection (Figure 1a). Simultaneously, cells were harvested from plates and 200 cells were inoculated in 6-cm-diameter dishes. After two weeks of incubation, the colonies formed were stained and counted. The number of obtained colonies is shown in Figure 1b. RBM8A-depleted cells displayed significantly reduced colony-forming ability. The depletion of RBM8A causes the arrest of cell growth or cell death and this observation appears to be consistent with published data.[1]

Effect of RBM8A depletion on the mitotic index

RBM8A-depleted cells exhibited a significant increment in the number of mitotic cells (Figure 1c). However, the number of chromosomes was not affected by RBM8A silencing and no chromosomal aberration has been observed by microscopic examination to this point (data not shown). Another M phase indicator, phosphorylated histone H3 positivity, was also assessed and this marker also revealed a similar increment in the number of mitotic cells (Supplementary Figure 1).[17]

RBM8-depleted cells have defects in M phase progression

Next, we analysed whether RBM8A-depleted cells proceed through M phase. One day after the transfection of siRNA, HeLa cells were blocked at the G1/S border by a double thymidine block. Eight hours after release from the double thymidine block, almost all cells entered G2/M phase and proceeded to the next G1 phase after 12 h. As shown in Figure 2, both control siRNA- and RBM8A siRNA-transfected cells arrested at the G1/S boundary phase and entered G2/M within 8 h of release from the thymidine block. After 12 h of incubation, more than 60% of control cells proceeded to the next G1 phase and less than 30% remained in G2/M phase. However, only 40% of RBM8A-silenced cells proceeded to the next G1 phase and more than 30% of cells remained in G2/M phase (Figure 2). These results indicated that substantial fraction of RBM8A-depleted cells did not proceed through G2/M phase promptly and RBM8A depletion may result in defects in M phase progression.

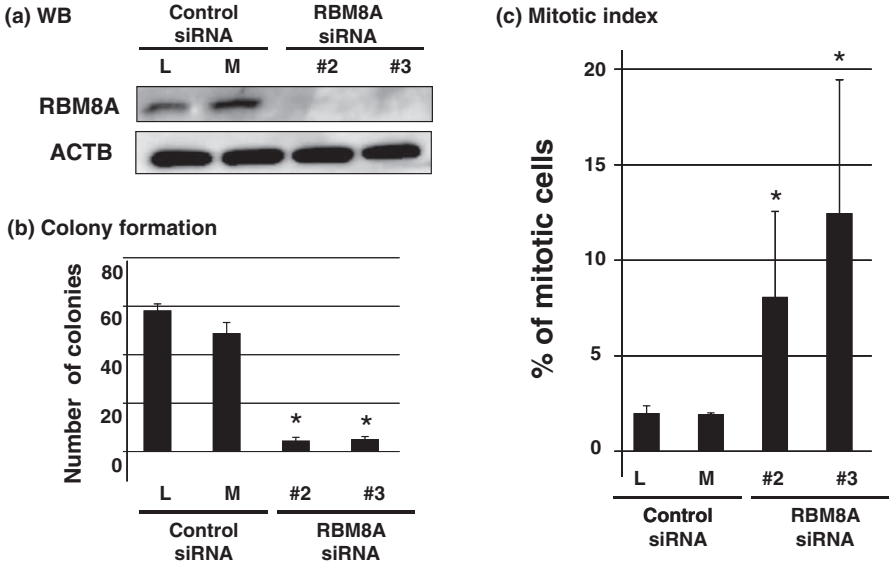


Figure 1 siRNA-mediated depletion of RBM8A results in poor colony-forming ability. The A549 cells were transfected with control stealth siRNA (L and M) or anti-RBM8A stealth siRNA (#2 and #3). After one day, cells were harvested and inoculated into dishes. Simultaneously, total protein was extracted and RBM8A expression was assessed by Western blotting. (a) A representative image. β-actin (ACTB) was used as a loading control. (b) The number of colonies. (c) After silencing, karyotype analysis was performed and the number of chromosomes was counted (c). L and M show control siRNA data and #2 and #3 show RBM8A-specific siRNA data. The bars indicate SD. The presented data are representative of three independent experiments. Asterisk shows $P < 0.05$

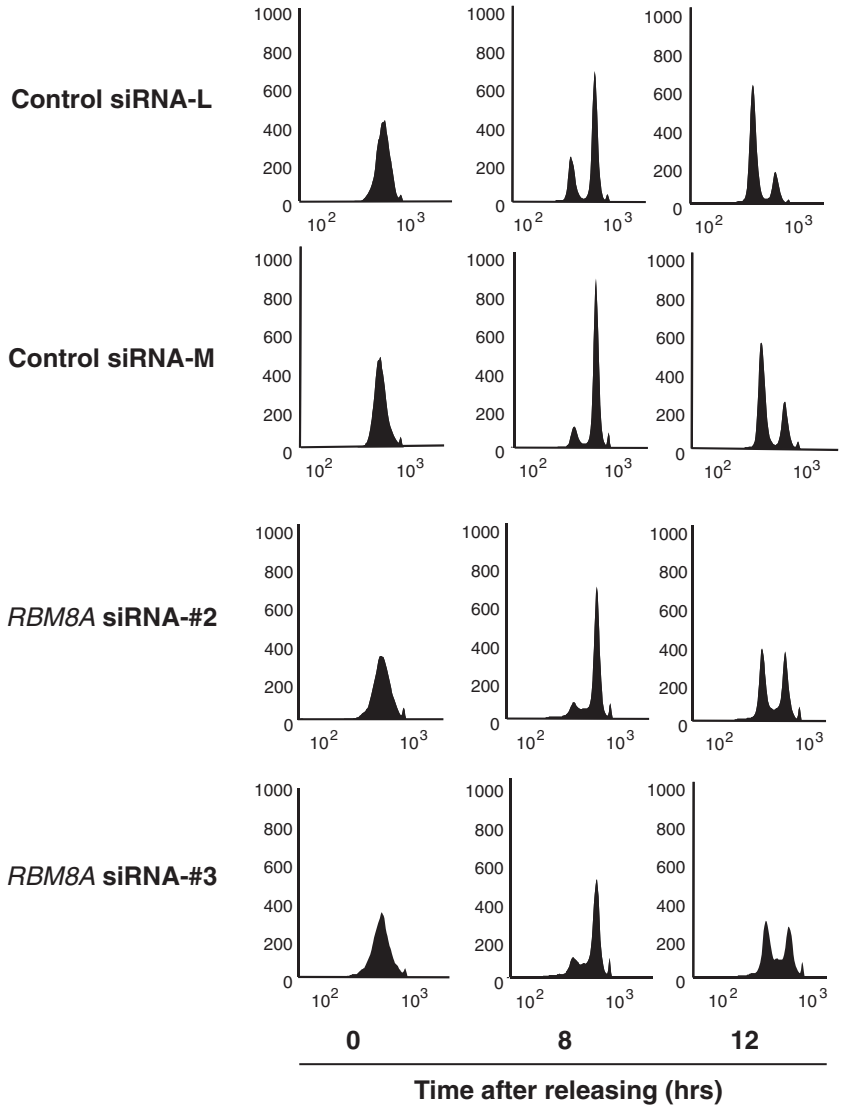


Figure 2 RBM8A cells have a defect in M phase progression. Transfected HeLa cells were blocked at the G1/S boundary phase by the double thymidine method and 8 or 12 h after release, cells were fixed and stained. A representative flow cytometry result is shown. The majority of control siRNA-transfected cells proceeded to the next G1 phase through G2/M but only limited numbers of RBM8A-silenced cells (#2 and #3 siRNA were used) proceeded to the next G1 phase

The sub-G1 fraction increased in RBM8A-depleted cells

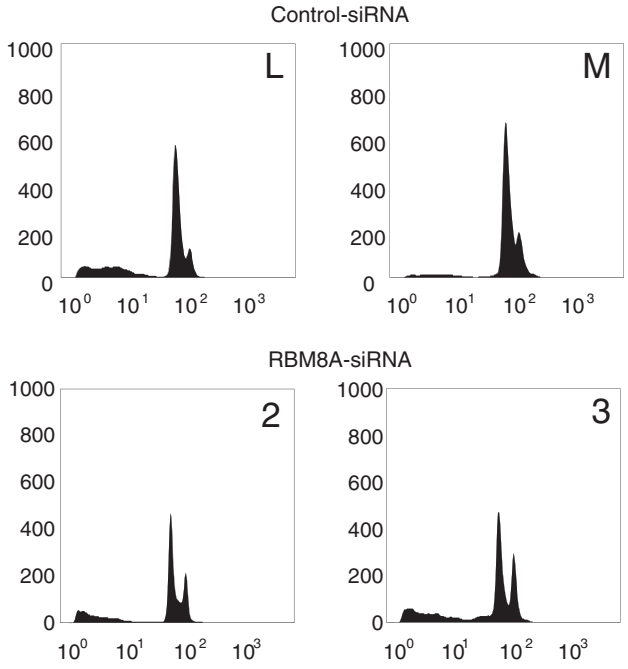
To reveal the effect of RBM8A silencing on cell cycle progression, flow cytometric analysis was performed. This analysis also revealed the accumulation of cells in M phase two days after transfection (Figure 3). Our results demonstrated the increment of the G2/M and apoptotic sub-G1 phases. In accordance with the results shown in Figure 1, we concluded that the arrest in M phase followed by apoptosis may be the cause of the low colony-forming ability of RBM8A-depleted cells.

RBM8A depletion results in caspase activation

As shown in Figure 3, in RBM8A-depleted cells, the fraction of the sub-G1 phase increased by two- to three-fold. To confirm the induction of apoptosis, we performed Western blotting to detect the apoptosis-related enzymes caspases 3/7.[18–20] The cleaved forms of caspases 3/7 were detected only in RBM8A-depleted cells by using three independent siRNA for RBM8A and their activation was confirmed (Figure 4). Furthermore, PARP, which is a downstream target of caspases 3/7, was also cleaved and activated. Thus, we concluded that RBM8A-depleted cells accumulate in M phase and ultimately undergo apoptotic cell death.

RBM8A depletion results in aberrant centrosome maturation

To analyse why RBM8A-silenced cells cannot proceed to G1 phase after mitosis, we examined the number of centrosomes after siRNA transfection. After siRNA transfection, centrosomes were immunostained with anti-γ-tubulin antibody. As shown in Figure 5a, chromosome formation proceeded with two centrosomes in control siRNA-transfected cells. However, the RBM8A-depleted cells exhibited aberrant numbers of centrosomes. As shown in Figure 5c and e,



%	subG1	G1	S	G2/M
L	7.4	41.3	25.4	20.5
M	5.0	44.6	25.4	21.5
2	13.0	32.7	17.0	28.7
3	13.1	32.5	19.5	27.8

Figure 3 RBM8A depletion resulted in an increase in the sub-G1 fraction. Two days after siRNA transfection, the A549 cells were harvested and fixed. To detect sub-G1 cells, cells were stained with propidium iodide. A representative flow cytometric analysis result is shown. The lower table shows the proportion of each fraction

mono- and multipolar centrosomes were detected. We randomly selected the mitotic cells and counted the number of centrosomes. Results were presented in graphical form in Figure 6. After anti-γ-tubulin staining in the RBM8A-depleted cells, the percentage of cells with multiple (more than two) and single centrosomes increased. A similar result was obtained using pericentrin staining.

Depletion of RBM8A resulted in the downregulation of its binding partner Magoh

The RBM8A-deficient patients with TAR syndrome display no brain abnormalities even though Magoh-deficient mice display microcephaly.[10] This finding in mice is caused by deficient cell division in the brain but it is not clear whether RBM8A contributes to this defect. To elucidate the effect of RBM8A depletion on Magoh expression, we silenced RBM8A or Magoh and analysed the expression of each protein by Western blotting. As shown in Figure 7, silencing RBM8A or Magoh resulted in the downregulation of both the target gene and the binding partner. Three siRNAs against Magoh had different level of knockdown and a proportional effect on RBM8A protein expression was observed. We performed real-time PCR to determine whether the mRNA expression levels influenced this downregulation. The results of our analysis are shown in Figure 8. The levels of RBM8A mRNA reduced in RBM8A-knockdown cells but not in Magoh-knockdown cells. The levels of Magoh mRNA reduced in Magoh-knockdown cells but not in RBM8A-knockdown cells. Therefore, we conclude that the reduction in the levels of each protein is interdependent and that both the proteins are required to be present for the stable expression of each other in the cells.

Discussion

Our results revealed that the deficiency of RBM8A in human cells resulted in the failure of cell cycle progression, particularly progression through mitosis. This defect was caused by abnormal centrosome maturation and RBM8A-deficient cells ultimately underwent apoptosis via caspases activation. Our results showed that RBM8A is required for cell cycle progression and its depletion can cause the cell death from cell cycle deficiency.

As shown in Figure 1, RBM8A-depleted cells accumulate in G2/M phase in A549 cells. In addition, almost all RBM8A-depleted cells could not proceed from G2/M to next G1 phase and only limited part of siRNA-transfected cells could proceed to next G1, as shown in Figure 2. Therefore, RBM8A depletion can cause the G2/M arrest

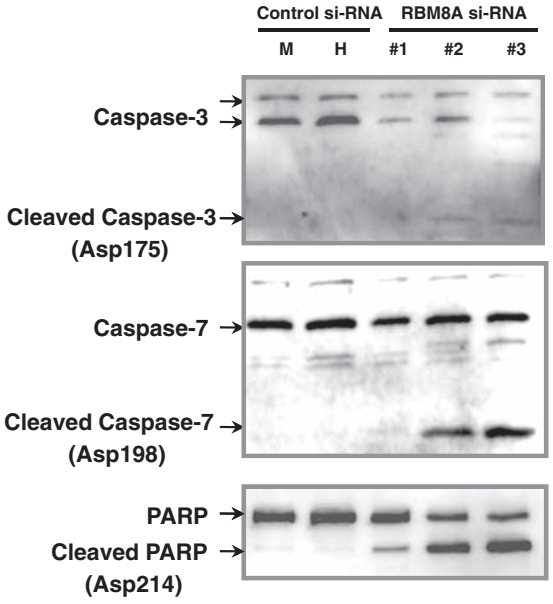


Figure 4 RBM8A depletion caused caspases 3/7 and PARP activation. A549 cells were inoculated and transfected with siRNAs. Two control siRNAs (M and H) and three RBM8A-specific siRNAs (1, 2 and 3) were used. Three days after the transfection, cell lysate was obtained and analysed for the activation of caspases 3/7 by inducing the expression of the cleaved form of the caspases; PARP in the cell lysate was determined by Western blotting

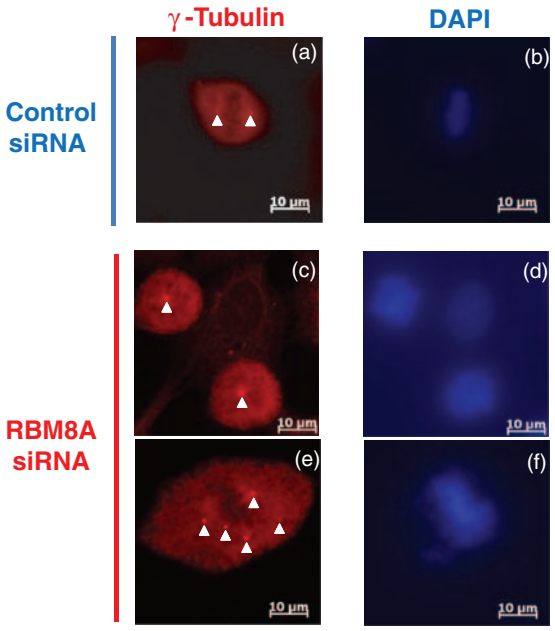


Figure 5 Abnormal centrosome numbers were observed after RBM8A depletion. The A549 cells were transfected with siRNA and stained with anti-γ-Tubulin antibody or DAPI. A representative result with control-M and anti-RBM8A siRNA-2 is shown. (a), (c) and (e) anti-γ-Tubulin staining (b), (d) and (f): DAPI staining. Triangle indicates the position of centrosome. (A color version of this figure is available in the online journal)

in human tumour cells and this indicated the requirement of RBM8A in cell cycle progression. Overall defect in cell replication might be the cause of deficiency of G2/M phase progression but our study could not reveal it. The role of RBM8A in mitotic phase progression is still unknown. Le Hir *et al.*[1] revealed the growth deficiency in RBM8A-depleted cells and his results imply that cell cycle arrest was not caused by the general inhibition of splicing. Dual coordinative functions of NMD-related factors in RNA and DNA processing have been proposed. However, it remains unclear whether the abnormal protein expression of RBM8A-depleted cells caused the M phase arrest and apoptosis observed in this study. Recent studies revealed the requirement of RBM8A for STAT3 activation.[7,8] STAT3 is a transcription factor that regulates a very wide range of cellular activities including cell cycle progression and STAT3 activation has also been reported in centrosome duplication.[21] On the other hand, Magoh is involved in the regulation of mouse Cdks.[11] Totally, it is possible that RBM8A-depleted human cells in our study also have similar deficiency and G2/M arrest via their alternation.

EJC serves as a platform for NMD factors and their various roles in cell cycle progression have been reported. For example, the stable depletion of Upf2 had little effect on the growth and survival of HeLa cells even though its expression is essential for mouse and zebrafish embryonic development.[22–24] Another NMD factor, Upf3b, associates with RBM8A and forms EJC on mRNA and this gene is mutated in individuals with syndromic and non-syndromic mental retardation.[25] However, Upf3b mutations are not lethal and this implies that Upf3b is not essential for fetal cell viability. Silencing of Upf1, another mediator of NMD, results in a deficiency in S phase progression and poor colony-forming ability in human cells.[26] The PI-3 kinase SMG1, which phosphorylates Upf1 on recognition of the premature termination codon on mRNA, also functions as a DNA damage sensor.[27–29] Collectively, NMD activity do not seem to be related to cell cycle progression because no common symptom is not observed among various NMD factor deficient cells. Therefore, loss of RBM8A may result in deficiency of NMD activity but defects in cell cycle progression or the DNA damage response in silenced cells are not due to the loss of NMD activity. Thus, it appears that specific NMD or EJC factors play a role in DNA metabolism in dependent on RNA processing and it is possible that RBM8A also processes the function in cell cycle progression in addition to RNA metabolism.

As shown in Figures 5 and 6, aberrant centrosomes were observed in the RBM8A-depleted cells. These cells might proceed with severe defect of genome construction and this can cause the cell death seen in Figures 3 and 4. On the other hand, there is another possibility that RBM8A depletion causes apoptosis directly and this cell death can cease the cell cycle progression. At least, the substantial part of the G1/S boundary-synchronized cells could proceed to G2/M phase, as shown in Figure 2, but our results using flow cytometer and Western blot analysis cannot differentiated the cell cycle stage that apoptosis induced. To reveal this point, further study is required.

Depletion of either RBM8A or Magoh resulted in the downregulation of both these proteins (Figure 7). Because RBM8A and Magoh expression is related to mRNA metabolism, we examined the mRNA levels for these proteins in

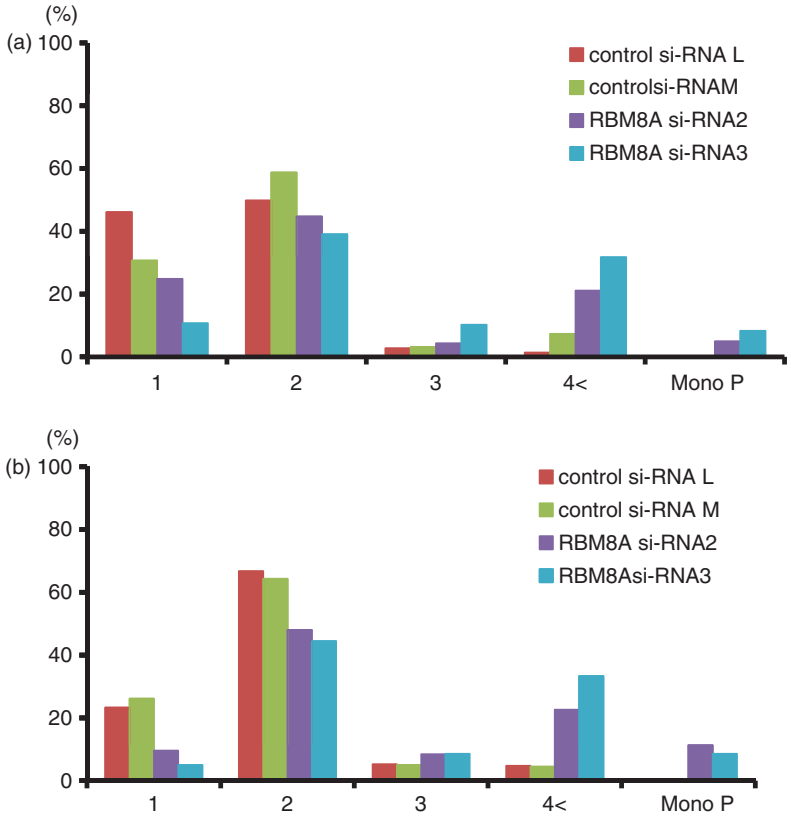


Figure 6 Distribution of centrosome numbers in RBM8A-depleted cells. A549 cells were transfected with siRNAs and cultured in a slide chamber. The cells were either stained with (a) anti-γ-tubulin antibody or with (b) antipericentrin antibody. The number of centrosomes in randomly selected 200 cells was counted. Metaphase was distinguished on the basis of chromosome formation. (A color version of this figure is available in the online journal)

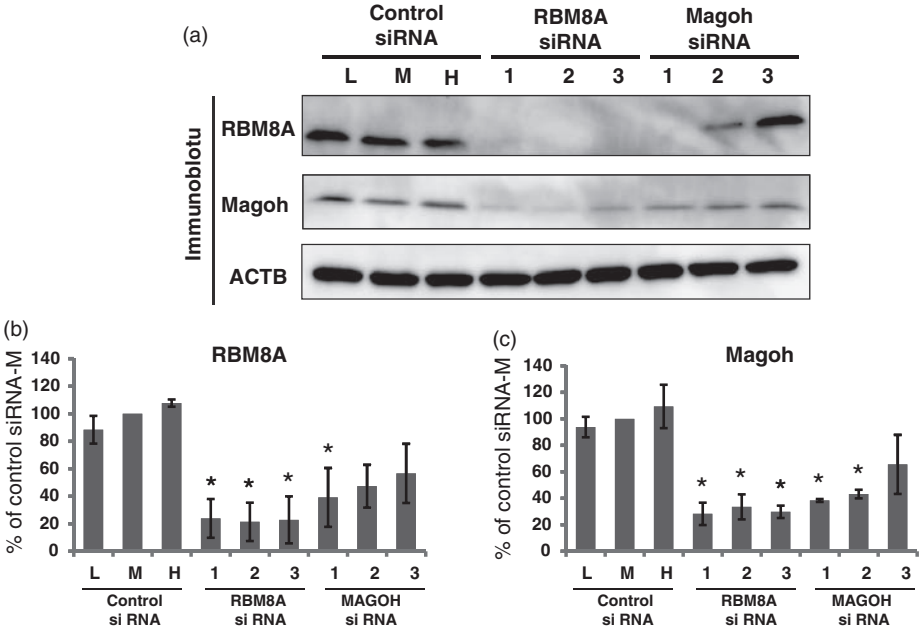


Figure 7 RBM8A and Magoh protein expression after silencing. The A549 cells were transfected with anti-RBM8A or anti-Magoh siRNA. Two days after transfection, cells were harvested and analysed by Western blotting. β-actin (ACTB) was used as a loading control. (a) Representative image of Western blotting. (b and c) The graph shows the average and standard error of the three independent experiments: (b) RBM8A expression calibrated with ACTB and (c) Magoh expression calibrated with ACTB. Asterisk indicates $P < 0.05$

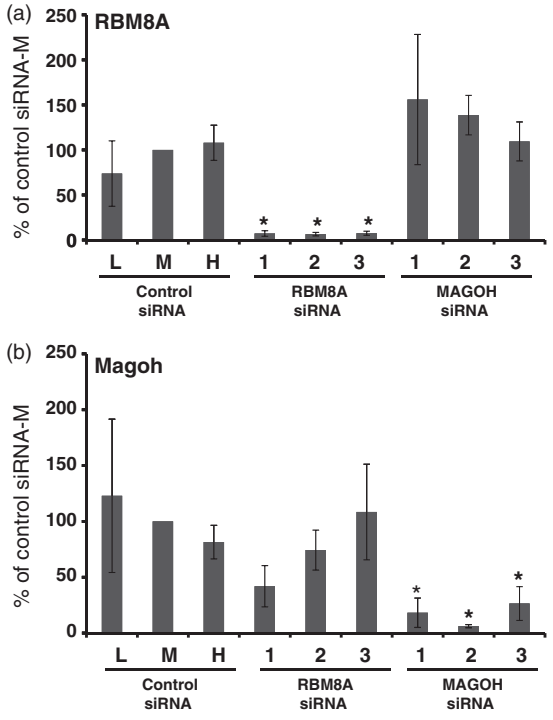


Figure 8 RBM8A and Magoh mRNA expression after silencing. The A549 cells were transfected with anti-RBM8A or anti-Magoh siRNA. Two days after the transfection, total RNA was extracted and analysed to determine the amount of (a) RBM8A mRNA and (b) Magoh mRNA by performing real-time PCR. β-actin (ACTB) was used as a reference and each value was calibrated. The graph shows the results of more than three independent experiments. Asterisk indicates $P < 0.05$

the knockdown cells and we observed siRNA-target specific mRNA depletion; no interdependent relation was observed (Figure 8). Therefore, we concluded that deficiency in the levels of either RBM8A or Magoh alters the expression of both RBM8A and Magoh. Depleted levels of either RBM8A or Magoh showed similar phenotypes in the cultured cells.[1] Therefore, we speculate that the complex formation of RBM8A and Magoh is important for stable protein expression in the cells. On the other hand, the results of this study show that deficiency of RBM8A or Magoh can lead to similar phenotype in the cultured cells. However, the characteristics of patients with TAR syndrome with deficient RBM8A have no resemblance with those observed in Magoh-deficient mice. In humans, defects in RBM8A expression probably alter the radius development and maturation in platelet thrombocytes and do not influence brain development.

In the present study, we found an M phase-related defect in RBM8A-depleted cells and we speculate that the unknown abnormal expression pattern induced by RBM8A silencing causes defects in M phase progression or RBM8A itself can regulate M cell cycle progression through Magoh and other pathways. The aberrant centrosome maturation was observed in RBM8A-depleted human cells. Similar abnormal mitotic apparatus maturation have been observed in cells treated with microtubule-stabilizer or microtubule-depolymerizer and the treatments resulted in apoptotic cell death of tumour cells. Then, some of them have been developed as anticancer drugs.[30,31] Because we observed apoptosis induction in various types of human tumour cell lines, RBM8A may be a candidate target for anticancer agents.

Author Contributions: All authors participated in the design and interpretation of the studies, analysis of the data and review of the manuscript. KI and NT conducted the experiments, MH performed flow cytometric analysis, YI, TS, TT and YN performed other experiments and YI, YN and NT wrote the manuscript.

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CONFLICT OF INTEREST

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