Human RNA-binding motif protein 3 inhibits RNA translation *in vitro*.

Rihito Morita[*,**], Satomu Morita[***], Toshihisa Sumi[**], Katsuji Nishi[***], Yoshiko Minami[*] and Masahito Hitosugi[***]

[*] *Department of Biochemistry, Faculty of Science, Okayama University of Science, 1-1 Ridai-cho, Kita-ku, Okayama, 700-0005, Japan.*

[**] *Department of Biological and Chemical Systems Engineering, Kumamoto College, National Institute of Technology, 2627 Hirayamashinmachi, Yatsushiro, Kumamoto, 866-8501, Japan.*

[***] *Department of Legal Medicine, Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu City, Shiga, 520-2192, Japan.*

(Received October 29, 2021; accepted December 9, 2021)

Hypoxic cells express characteristic proteins that are termed hypoxia markers. The markers, RNA-binding motif protein 3 (RBM3) and hypoxia inducible factor-1 alpha (HIF-1α), promote global protein expression and are considered to protect cells and tissues from hypoxia. To reveal the detailed molecular function(s) of RBM3, we investigated the direct influence of purified recombinant RBM3 on translation *in vitro*. Unexpectedly, RBM3 inhibited translation in our *in vitro* cell-free translation system. This novel inhibitory activity of RBM3 contrasts that of HIF-1α. The difference of prognosis in some kinds of cancers is related to their expressed proteins, with RBM3 and HIF-1α leading to good and poor prognoses, respectively. The molecular function of RBM3 might be key to improving prognosis in cancers.

Keywords: RBM3, hypoxia markers, translation, prognosis, cancer.

1. Introduction

Some proteins, termed hypoxia markers, are expressed in hypoxic cells. We have previously evaluated hypoxia markers in the myocardium using immunohistochemical staining. In particular, we identified RNA-binding motif protein 3 (RBM3) as a useful marker of focal ischemic heart disease [1]. We observed RBM3-positive cells in the areas of old myocardial infarction, which were mostly fibrotic. Thus, we considered that RBM3 is a hypoxia marker that appears during a comparatively late phase. In forensic sciences, the measurement of hypoxic time and ischemia is very important in determining the agonal phase. Therefore, we have been investigating a method for measuring agonal time until death using RBM3.

There have been few reports of the molecular function(s) of RBM3. It has been reported that overexpression of RBM3 promotes global protein expression *in vivo* using N2a cells, B104 cells and prion-infected hippocampal slices from mice [2-4]. It has also been reported that RBM3 interacts directly with ribosomal proteins [2]. However, it is unclear whether or not the interaction between RBM3 and the translation machinery is related to the promotion of protein expression. In clinical reports, while associations have been made between increased expression of RBM3 and prolonged survival in some cancers [5-8], increased expression of hypoxia inducible factor-1 alpha (HIF-1α) is associated with a poor prognosis [9]. In general, the function of some of the hypoxia markers is to protect cells and tissues from hypoxia and consequently from death, which is exemplified by HIF-1α. As a consequence of this protection, tumor cells in hypoxic regions could adapt to conditions with a low oxygen tension.

Therefore, we hypothesize that RBM3 exhibits some functions that are distinct from those of other hypoxia markers. It has been reported that RBM3 increases the expression of proteins, as well as other hypoxia markers [4], but the actual function of RBM3 remains unclear. At the first step of our investigation, we attempted to assess the influence of RBM3 on translation. We initially overexpressed and purified

RBM3, and then we measured the fluorescence signal from GFP *in vitro* using a cell-free translation system with the addition of RBM3.

2. Materials and Methods

Materials --- Restriction enzymes and Human Cell-Free Protein Expression System (#3281) were purchased from TakaraBio (Kusatsu, Japan). DNA polymerase was from TOYOBO (Osaka, Japan). Yeast extract and polypeptone were from Difco (Detroit, MI). *Escherichia coli* strains DH5α and BL21(DE3) together with plasmids pET-28a were from Novagen (Madison, WI). Ni-NTA Agarose was from Qiagen (Hilden, Germany). TOYOPEARL-Phenyl 650M was from Tosoh (Tokyo, Japan). Synthesized DNA oligomers were from Sigma-Aldrich Japan (Tokyo, Japan). RBM3 cDNA was from ABGENT (Shanghai, China). The pGLO plasmid containing Green Fluorescent Protein (GFP) gene was from Bio-Rad (Hercules, CA). All other reagents used in this study were of the highest available commercial grade.

Construction of RBM3 Expression Plasmids and Overexpression --- Sequence data for the *Homo sapiens* RBM3 was obtained from NCBI reference sequence NM_006743.4. Using this information, two primers (forward and reverse) for amplification of *rbm3* genes were designed and synthesized as follows; 5'-GGAATTCCATATGTCCTCTGAAGAAGGAAAGCTC-3' (forward) and 5'-CGGGATCCCTATCAGTTGTCATAATTGTCTCTGTAATTTCC-3' (reverse). The forward and reverse primers contained *Nde*I and *Bam*HI recognition sites (underlined), respectively. Using these primers, the RBM3 gene fragment was amplified from RBM3 cDNA by PCR. The amplified fragment was ligated into the pET-28a using the *Nde*I and *Bam*HI sites, creating pET28a/*rbm3*. Sequence analysis revealed that the construct was error-free. *E. coli* BL21(DE3) cells transformed by pET-28a/*rbm3* plasmid were grown at 37°C in 500 ml of LB medium containing 50 μg/ml ampicillin until the density of the culture reached 1×10^8 cells/ml. The cells were then incubated for a further 16 h in the presence of 40 μg/ml IPTG at 16°C and then harvested by centrifugation and stored at -20°C.

Purification --- All purification steps described below were carried out at 4°C. The purification method for RBM3 was as follows. Frozen cells (5 g) expressing RBM3 were suspended in 50 ml of buffer I [20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 10 mM Imidazole (pH 8.0)] and disrupted by ultrasonication on ice. The cell lysate was clarified by centrifugation at ~30,000 *g* for 20 min and the supernatant was collected. After 10 ml of Ni-NTA Agarose resin including Ni^{2+} was equilibrated with buffer I, it was resuspended with the supernatant. The resin was loaded into a column and washed with buffer I. It was subsequently washed with buffer II [20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 30 mM imidazole (pH 8.0)] and the proteins were eluted with 30 ml of buffer III [20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 300 mM imidazole (pH 8.0)]. Fractions containing RBM3 were pooled and ammonium sulfate was added to a final concentration of 1 M. This protein solution including ammonium sulfate was applied to a TOYOPEARL-Phenyl column (bed volume 10 ml) previously equilibrated with buffer IV (20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 M Ammonium Sulfate). Proteins were eluted with a linear gradient of ammonium sulfate from 1.0 to 0 M in a total volume of 200 ml of buffer IV. The collected solution containing RBM3 was dialyzed against buffer V [20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5% glycerol] at 4°C.

Construction of GFP Expression Plasmids --- Two primers (forward and reverse) for amplification of *gfp* genes were designed and synthesized as follows; 5'-CGGAATTCATGGCTAGCAAAGGAGAAGAAC-3' (forward) and 5'-GAAGATCTTTATTTGTAGAGCTCATCCATGCC-3' (reverse). Forward and reverse primers contained *Eco*RI and *Bgl*II recognition sites (underlined), respectively. Using these primers, the GFP gene fragment was amplified from pGLO plasmid by PCR. The amplified fragment was ligated into the pT7-IRES using *Eco*RI and *Bgl*II sites, creating pT7-IRES/*gfp*.

Translation Inhibition Assay --- The amount of translated protein was quantitated fluorescently using an FP-8300 (JASCO Corporation). Green fluorescent protein (GFP) was translated using a Human Cell-Free Protein Expression System. The

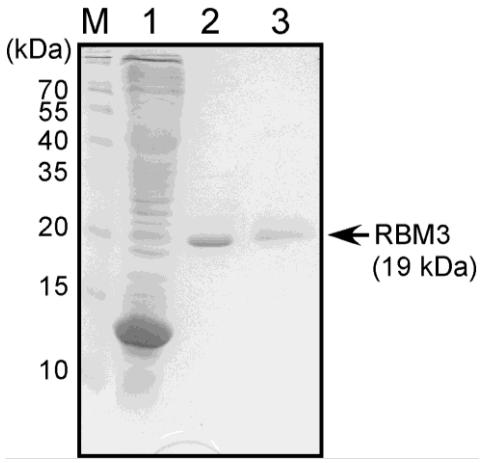

Fig. 1. **SDS-PAGE analysis of purified RBM3.** Lane M, protein markers. Lane 1, a flow through fraction from a Ni-NTA column, the first step of the purification of RBM3. Lane 2, the eluted fraction from the Ni-NTA column. The band for RBM3 was detected at ~20 kDa. Lane 3, the eluted fraction from hydrophobic interaction chromatography, the second step of the purification. A single band of purified RBM3 was detected.

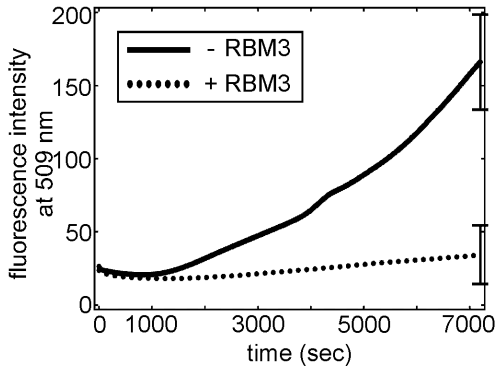

Fig. 2. **Time-dependent changes of the expressed GFP.** Expressed GFP from a cell-free expression system was detected by a spectrofluorometer in the presence (dotted line) or absence of 0.75 μM RBM3 (solid line). The fluorescence spectrum of the mixture was measured using an excitation wavelength of 395 nm. Dotted line, the protein expression system mixture with RBM3 (0.75 μM). Solid line, normal (without RBM3) condition.

GFP excitation and emission wavelengths were 395 nm and 509 nm, respectively. The method of GFP expression was as described in the manufacturer's instructions, except for the addition of RBM3 solution in the first step. The final reaction mixture was 55 μl, including 5 μl of RBM3 solution (0-8.23 μM) and 2.5 μl of pT7-IRES/*gfp* solution (268 ng/μl). All experiments were carried out at 32°C.

3. Results

Purification of RBM3 --- Recombinant RBM3 protein containing a histidine-tag at N-terminus was expressed in *E. coli*. We successfully purified the proteins from the *E. coli* cells with Ni-NTA and TOYOPEARL-Phenyl columns. The purity of the proteins was checked by SDS-PAGE (Fig. 1). A single protein band was detected at ~20 kDa in lane 3, the molecular mass of the His-tagged RBM3 was predicted to be 19.3 kDa. This purified protein was detected by Western blot using both anti-human RBM3 protein and anti-His-tag antibodies.

RBM3 inhibits Protein Synthesis --- It has been reported that mRNA encoding RBM3 is induced in mammalian cells following exposure to mild hypothermia, and that protein synthesis is generally inhibited in such cells. It has also been reported that RBM3 interacts directly with ribosomes. To investigate whether RBM3 affects translation directly, we measured the increase in fluorescence from translated GFP *in vitro* with a cell-free translation system in the presence of RBM3. In this experiment, GFP expression was clearly inhibited by RBM3. RBM3 inhibited the GFP expression level by about 92.4% after 7200 seconds.

Next, to investigate the inhibition quantitatively, we carried out inhibition assays with several concentrations of RBM3. These experiments showed that RBM3 reduced the amount of protein synthesized in a concentration-dependent manner (Fig. 3). The results were fitted to an equation for non-competitive inhibition, and the apparent inhibition constant was calculated as 62 ± 43 nM. The shape of this curve, especially in case of low RBM3 concentrations, suggests that RBM3 has no activity to promote translation solely *in vitro*, even though there is the possibility that the inhibition resulted from binding to mRNA. Therefore, we

c o n c l u d e d t h a t

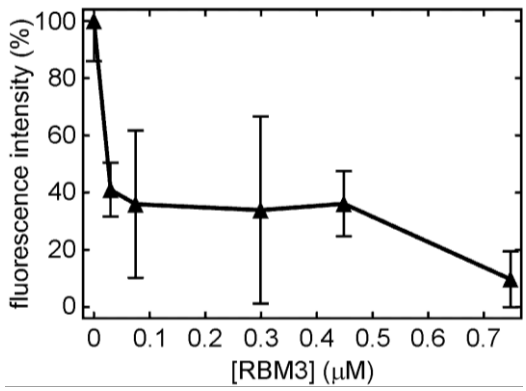

Fig. 3. **Protein expression inhibition by purified RBM3.** Protein expression inhibition assays with several concentrations of RBM3. Fluorescence of samples without RBM3 (0 μM) was taken as 100%. The fluorescence spectrum of the mixture was measured using an excitation wavelength of 395 nm. The reaction time was 1.5 hours.

RBM3 inhibits translation caused by direct interaction with translation machinery including mRNA.

4. Discussion

The RBM3 gene is induced by cold shock and low oxygen tension. While the induction of RBM3 is suspected to protect the cell, the molecular mechanism remains to be determined. In this study, we investigated whether RBM3 promotes or inhibits translation using a recombinant purified RBM3 protein. Our results from the *in vitro* cell-free translation system showed that RBM3 inhibits translation. It is considered that this inhibitory effect on translation is due to the ribosomal binding activity or RNA binding activity of RBM3 [2]. This finding is similar to previous reports of mammalian cells in hypothermia and hypoxia [10, 11], but was different from *in vivo* investigations of N2a cells, B104 cells and prion-infected hippocampal slices from mice [2-4].

The recent studies of the effects of overexpression of RBM3 suggest that RBM3 promotes global protein expression *in vivo*, which is in contrast to our findings [2-4]. However, RBM3 is also involved in microRNA processing [12]. These findings suggest that the increase in global translation is due to the effect on miRNA processing and that inhibition of translation has little effect *in vivo*. Therefore, we hypothesized that the inhibition of translation by RBM3 represents a novel function in the cell.

In clinical medicine, there have been several reports of associations between high expression of RBM3 and prolonged survival in some cancers, including prostate cancer [5], urothelial bladder cancer [6], epithelial ovarian cancer [7], malignant melanoma [8], etc. These associations are in contrast with the other hypoxia markers; specifically, HIF-1α is known as a poor prognostic marker of cancers. In general, tumor cells in hypoxic regions begin to adapt to low oxygen tension conditions through the activation of several survival pathways. Activation of HIF-1α transcription factor is the most well-recognized pathway adopted by hypoxic cells in this harsh microenvironment. By contrast, in this study we revealed that RBM3 interacts directly with translation machinery to inhibit translation. This function might be essential for improving the prognosis.

According to previous reports, RBM3 led to the induction of some proteins that could prolong survival through promotion of translation *in vivo*. RBM3 may exhibit the same function(s) as other hypoxia markers. For example, in the stem cell of prostate cancer, overexpression of RBM3 suppressed RNA splicing of the CD44 variant v8-v10 and increased expression of the standard CD44 (CD44s) isoform [13].

We revealed that direct interaction between RBM3 and translation machinery inhibited translation *in vitro*. This function is completely opposite to that of HIF-1α. However, global protein expression was increased by RBM3 *in vivo*, as well as by HIF-1α, which can be explained as a consequence of adjustment for processing by RBM3 as noted above. These facts suggest that RBM3 might act as a stabilizer in hypoxic cells. This new molecular function of RBM3 might be the key to improving prognosis in some cancers.

5. Acknowledgement

This work was supported in part by Grant-in-Aid for Scientific Research 26860464 (to S. Morita) from the Ministry of Education, Science, Sports and Culture of Japan. We thank Professor Kenji Ohshima for his help with the fluorimetric assay, and Dr. Jun-ya Motoki for providing us with the GFP gene.

References

1. Morita, S., Furukawa, S., Nishi, K. Immunohistochemical evaluation of hypoxia markers in the myocardium. *Aust J Forensic Sci*. 2015 May;47(2):138-146.
2. Dresios, J., Aschrafi, A., Owens, GC., Vanderklish, PW., Edelman, GM., Mauro, VP. Cold stress-induced protein Rbm3 binds 60S ribosomal subunits, alters microRNA levels, and enhances global protein synthesis. *Proc. Natl. Acad. Sci. USA.* 2005 Feb;102(6):1865–1870.
3. Smart, F., Aschrafi, A., Atkins, A., Owens, GC., Pilotte, J., Cunningham, BA., Vanderklish, PW. Two isoforms of the cold-inducible mRNA-binding protein RBM3 localize to dendrites and promote translation. *J. Neurochem.* 2007 Jun;101(5):1367–1379.
4. Peretti, D., Bastide, A., Radford, H., Verity, N., Molloy, C., Martin, MG., Moreno, JA., Steinert, JR., Smith, T., Dinsdale, D., Willis, AE. Mallucci, GR. RBM3 mediates structural plasticity and protective effects of cooling in neurodegeneration. *Nature*. 2015 Feb;518(7538):236-239.
5. Grupp, K., Wilking, J., Prien, K., Hube-Maqq, C., Sirma, H., Simon, R., Steurer, S., Budaus, L., Haese, A., Izbicki, J., Sauter, G., Minner, S., Schlomm, T., Tsourlakis, MC. High RNA-binding motif protein 3 expression is an independent prognostic marker in operated prostate cancer and tightly linked to ERG activation and PTEN deletions. *Eur J Cancer*. 2014 Mar;50(4):852-861.
6. Boman, K., Segersten, U., Ahlgren, G., Eberhard, J., Uhlen, M., Jirstrom, K., Malmstrom, PU. Decreased expression of RNA-binding motif protein 3 correlates with tumour progression and poor prognosis in urothelial bladder cancer. *BMC Urol*. 2013 Apr;13:17.
7. Ehlen, O., Nodin, B., Rexhepaj, E., Brandstedt, J., Uhlen, M., Alvarado-Kristensson, M., Ponten, F., Brennan, DJ., Jirstrom, K. RBM3-regulated gene promote DNA integrity and affect clinical outcome in epithelial ovarian cancer. *Transl Oncol*. 2011 Aug;4(4):212-221.
8. Nodin, B., Fridberg, M., Jonsson, L., Bergman, J., Uhlen, M., Jirstrom, K. High MCM3 expression is an independent biomarker of poor prognosis and correlates with reduced RBM3 expression in a prospective cohort of malignant melanoma. *Diagn Pathol*. 2012 Jul;7:82.
9. Masoud, GN., Li, W. HIF-1α pathway: role, regulation and intervention for cancer therapy. *Acta Pharm Sin B*. 2015 Sep;5(5):378-389.
10. Burdon, RH. Temperature and animal cell protein synthesis. *Symp Soc Exp Biol*. 1987;41:113-133.
11. Wassmann, H., Greiner, C., Hulsmann, S., Moskopp, D., Speckmann, EJ., Meyer, J., Van Aken, H. Hypothermia as cerebroprotective measure. Experimental hypoxic exposure of brain slices and clinical application in critically reduced cerebreal perfusion pressure. *Neurol. Res*. 1998;20:S61–S65.
12. Pilotte, J., Dupont-Versteegden, EE., Vanderklish, PW. Widespread Regulation of miRNA Biogenesis at the Dicer Step by the Cold-Inducible RNA-Binding Protein, RBM3. *PLoS One*. 2011 Dec;6(12):e28446.
13. Zeng, Y., Wodzenski, D., Gao, D., Shiraishi, T., Terada, N., Li, Y., Griend, DJ., Luo, J., Kong, C., Getzenberg, RH., Kulkarni, P. Stress response protein RBM3 attenuates the stem-like properties of prostate cancer cells by interfering with CD44 variant splicing. *Cancer Res*. 2013 Jul.73(13): 4123-4133.