Stress response protein RBM3 attenuates the stem-like properties of prostate cancer cells by interfering with CD44 variant splicing

Yu Zeng¹,⁴, Dana Wodzenski¹, Dong Gao¹, Takumi Shiraishi¹, Naoki Terada¹, Youqiang Li¹, Donald J. Vander Griend³, Jun Luo¹, Chuize Kong⁴, Robert H. Getzenberg¹, Prakash Kulkarni¹,²

¹The James Buchanan Brady Urological Institute, Department of Urology, ²Oncology, The Johns Hopkins University School of Medicine, Baltimore, MD; ³The Section of Urology, Department of Surgery, the University of Chicago, Chicago, IL; ⁴Institute of Urology, Department of Urology, The First Hospital of China Medical University, Shenyang, China.

Corresponding Author: Yu Zeng, MD, PhD, Department of Urology, The First Hospital of China Medical University, 155 North Nanjing Street, Heping District, Shenyang, 110001, China. Phone: 86-24-22713586; Fax: 86-24-22703576; E-mail: zengyud@hotmail.com; and Prakash Kulkarni, PhD, Department of Urology, Johns Hopkins University School of Medicine, 600N, Wolfe Street, Baltimore, MD 21087. Phone: 410-502-4962; Fax: 410-502-9336; E-mail: pkulkar4@jhmi.edu.

Running Title: RBM3 and prostate cancer

Key Words: RBM3; prostate cancer; cancer stem cell; CD44; alternative splicing variant

Disclosure of Potential Conflicts of Interest: No potential conflicts of interest were disclosed.

Words of text: 3704
Words of abstract: 222
Number of figures: 7
**Precis:** This study focuses on a member of an little studied family of stress regulators in cancer, the cold shock proteins, offering new perspectives on how stress alters RNA splicing for a modulator of stem cell-like character in malignant disease.
Abstract

Stress response pathways play an important role in cancer. The cold-inducible RNA-binding protein RBM3 is upregulated in several types of cancer including prostate cancer (PCa), but its pathogenic contributions are undetermined. RBM3 is expressed at low basal levels in human fetal prostate or in CD133+ prostate epithelial cells (PrEC), compared to the adult prostate or to CD133- PrEC, and RBM3 is downregulated in cells cultured in soft agar or exposed to stress. Notably, RBM3 overexpression in prostate cancer cells attenuated their stem cell-like properties in vitro as well as their tumorigenic potential in vivo. Interestingly, either overexpressing RBM3 or culturing cells at 32°C suppressed RNA splicing of the CD44 variant v8-v10 and increased expression of the standard CD44 (CD44s) isoform. Conversely, silencing RBM3 or culturing cells in soft agar (under conditions that enrich for stem cell-like cells) increased the ratio of CD44v8-v10 to CD44s mRNA. Mechanistic investigations showed that elevating CD44v8-v10 interfered with MMP9-mediated cleavage of CD44s and suppressed expression of cyclin D1, whereas siRNA-mediated silencing CD44v8-v10 impaired the ability of prostate cancer cells to form colonies in soft agar. Together these findings suggested that RBM3 contributed to stem cell-like character in prostate cancer by inhibiting CD44v8-v10 splicing. Our work uncovers a hitherto unappreciated role of RBM3 in linking stress-regulated RNA splicing to tumorigenesis, with potential prognostic and therapeutic implications in prostate cancer.
Introduction

It is now widely accepted that most tumors harbor cancer stem cells (CSCs), which are crucial for their evolutionary capability. Consistent with such a function, CSCs display a greatly enhanced tumor-initiating capability and -sustaining capacity, a self-renewal potential, and the ability to spawn other subpopulations (1). Furthermore, the CSCs have been proposed to play a critical role in the initiation of secondary tumors in metastatic sites and in the recurrence of tumors after therapy (2).

Despite the central role of the CSCs however, their origin remains poorly understood and it has been argued that indeed, cancer cells may switch from a non-stem cancer cell (NSCCs) to a CSC-like cell phenotype. For example, such phenotypic switching by cancer cells has recently been demonstrated in melanoma where, CSCs defined by the presence of CD133+ or JARID1B+ are not only able to re-generate the progeny with marker heterogeneity but could also arise from cells that did not express CD133 or JARID1B (3, 4). Thus, the unstable traits of CSCs may reflect the flexibility that favors tumor adaption to facilitate tumor progression. These data strongly suggest that epigenetic rather than genetic changes may drive phenotypic switching in cancer cells. Emerging evidence suggests that microenvironmental factors may be crucial for generating and maintaining the CSC subpopulation within tumors (5-7). For instance, the proinflammatory cytokine IL6, which is a multifunctional chemokine in the tumor microenvironment, triggers the conversion of NSCCs into CSCs in breast and prostate cancer models (5). Similarly, hypoxia (6) and acidic stress (7) existing in the tumor microenvironment have been demonstrated to be involved in maintaining and promoting the cancer stem cell-like phenotype in human glioma.
Temperature is another important element of the physical microenvironment that is capable of regulating cellular ‘stemness’ properties (8). For example, a precise and optimum temperature is essential for hatching of bird eggs which of course are stem cells. Temperature effects are modulated at the cellular level by stress response pathways that include heat shock and cold shock proteins (9, 10). In contrast to heat shock proteins (HSPs) which are induced by increased temperatures, cold shock proteins (CSPs) are induced by lowering the temperatures but down-regulated when temperatures are elevated (11, 12). RNA binding motif 3 (RBM3) is an evolutionary conserved CSP(13) that has been shown to regulate the translation machinery and facilitate protein synthesis during hypothermic stress, and in brain development where it functions as a RNA chaperone to maintain RNA stability (14-18). Besides its effect on protein synthesis, RBM3 also has demonstrated roles in enhancing cell proliferation (19), promoting erythroid differentiation (20, 21), and protecting cell death due to hypothermia or other stress conditions (22-24). In the present study, we demonstrate that RBM3 is related to a neural differentiation and that forced expression of RBM3 greatly attenuates the stem cell-like properties of the prostate cancer (PCa) cells by inhibiting alternative splicing of exon v8-v10 of CD44.

**Materials and Methods**

**Patients and samples**

Primary PCa samples (n = 79) and histologically normal prostate tissues (n = 20) were obtained from patients undergoing radical prostatectomy (RP), from 1993 to 2007, at the Johns Hopkins Hospital. PCa patients were followed up from 1 to 14 years (median is 2 years). Biochemical recurrence was defined as a postoperative elevation of serum PSA (0.2 ng/ml or greater) after RP (25). The endpoint of the follow-up in this study was the time to biochemical recurrence.
Metastatic PCa tissues (n = 21) were collected from soft tissue metastasis of patients who died from PCa, as part of the Johns Hopkins Autopsy Study of lethal PCa. The clinical characteristics of the patients were described elsewhere (25). This study complies with the Declaration of Helsinki and was approved by the institutional ethics committee. Written informed consent was obtained from all patients and the samples used were done so unidentified. Alternatively, RNA samples isolated from different human adult normal tissues and cDNA samples from human fetal prostate and fetal testis were purchased from BioChain, where they obtained tissues from healthy donors. Among them, the fetal prostate tissue and the fetal testis tissue were obtained from a 36 week old male donor.

**Flow cytometry and cell sorting**

PrEC cells were stained with mouse anti-CD133-PE (AC141, Cell Signaling) and sorted upon CD133 expression on the cell surface using Fluorescence-activated cell sorting (FACS) system (BD FACSaria) as previously described (26).

**Soft agar clonogenic assay**

Two-layer soft agar clonogenic assays were conducted in 6-well plates. Cells were plated at 5000 cells per well. Colonies with more than 50 cells were counted after 4-6 weeks. Each line were plated in duplicate every time and the experiment were repeated at least three times.

**Prostasphere formation assay**

The cells from PC3-RBM3 clones or PC3-GFP clones were grown as suspension cultures in 96-well plates pre-coated with 6 mg/ml Polyhydroxyethyl methacrylate (PolyHEMA, Sigma) at a density < 1 cell/well. The Prostaspheres containing more than 50 cells were counted after two weeks.
Tumor xenograft in nude mice

Forty 6-week-old athymic nude mice (Harlan) were randomly divided into four groups with 10 in each. $2 \times 10^5$ cells of the PC3-GFP clone #1, #2 and PC3-RBM3 clone #1, #6 were separately inoculated into the mice of each group. Tumor growth was inspected and measured twice a week and the relative tumor volume was calculated.

PCR array and micro-RNA PCR array

The RT² Profiler™ pathway-focused PCR array specific for human stem cell signaling and the micro-RNA (miRNA) PCR array specific for human cancers were purchased from SABiosciences. The complete gene lists for the PCR array and miRNA PCR array are available on supplier’s website (http://www.sabiosciences.com/). PCR data was analyzed using web-based software provided by the supplier. Fold change was determined by comparing the gene expression of PCR-RBM3 clones to PC3-GFP clones.

PCR screening for CD44 mRNA splicing variants and sequencing

CD44 splice products were screened using a PCR-based method that was described previously (27). The primer sequences used for screening are described by the previous study (27). PCR products at the expected sizes were carefully extracted, purified and directly sequenced on Applied Biosystems 3730xl DNA Analyzer (Life Technologies).

ELESA assay

Soluble ectodomain of human standard CD44 was measured using the human CD44 ELISA kit (abcam) following the manufacture’s protocol. Conditioned media from cell cultures were analyzed with a spectrophotometer at wavelength of 450 nm.

Statistical analysis
Comparisons between two groups are made using the Student’s t-test. Differences among more than two groups are analyzed by one way ANOVA test. Kaplan-Meier survival curves are used to evaluate the difference on tumor incidence among groups. The correlation coefficient (Pearson’s $r$) is used to assess the relationship between two data sets. Two-sided $P$ values of less than 0.05 are considered significant.

**Other materials and methods see Supplementary documents.**

**Results**

**RBM3 expression is down-regulated in prostatic stem cell-like cells**

While RBM3 mRNA is widely expressed in adult tissues (Supplementary Fig. S1A), it is significantly decreased in the fetal testis and fetal prostate (Fig. 1A). To determine whether the lower levels of RBM3 in fetal tissues are related to the poorly differentiated but a more stem-like state of the fetal cells, CD133$^+$ cells and CD133$^-$ cells from normal prostate epithelial cells (PrEC) were separated by flow cytometry. In these primary cells, the CD133$^+$ subpopulation has been demonstrated to have stem-like features (26). As expected, RBM3 mRNA expression is extremely low in CD133$^+$ cells in comparison to the CD133$^-$ subpopulation (Fig. 1B). In contrast, the expression of another CSP, namely cold inducible RNA binding protein (CIRBP), was found to be slightly increased rather than decreased in the same CD133$^+$ cells suggesting that the two CSPs may function differently (Supplementary Fig. S1B).

RBM3 mRNA was significantly increased in the primary PCa samples in comparison to the normal prostate tissues. However, its expression was relatively decreased in the metastatic PCa samples (Fig. 1C) suggesting a potential association between a decreased expression of RBM3 and cancer aggressiveness which renders cancer cells more capable of metastasis. However,
these findings do not preclude a role for RBM3 in the early events in PCa where it is significantly up-regulated. Further, immunohistochemical staining in the primary PCa tissues demonstrated that RBM3 was highly expressed in cancer lesions but not in the adjacent normal glands (Fig. 1D).

**RBM3 over-expression attenuates the stem cell-like feature of PC3 cells**

PC3 and DU145 cells were heated to 43°C or treated with Paclitaxel, and then allowed to recover (28). RBM3 expression was found to be significantly lower in the surviving cells than the parental cells (Fig. 2A). In addition, it was determined that the expression of RBM3 in PC3 cells and DU145 cells that formed colonies in soft agar were significantly decreased as compared to their respective counterparts that were routinely grown in 2-D cultures (Fig. 2B). Considered together, these results suggest that decrease of RBM3 expression is a more general response of cancer cells to stresses including thermal-, chemo- and re-colonization stress. At the same time, this result also suggests that a decreased RBM3 expression is compatible with an enhanced feature that favors cell survival in soft agar or after intensive cytotoxic stress that is commonly attributed to stem cell-like characteristics. In addition, PC3 cell clones over-expressing RBM3 (Supplementary Fig. S1C,D) showed a greatly attenuated capability to form colonies in soft agar (Fig. 2C,D) and to grow into prostaspheres from single cells when cultured without adhering to any physical surface compared to clones that were permanently transformed with the GFP control (PC3-GFP) (Fig. 2E). Furthermore, PC3-RBM3 clones showed a significant reduction in tumor formation as compared to the PC3-GFP clones when cells were inoculated in nude mice. Thus, these data strongly suggest that enhanced expression of RBM3 significantly inhibit the stem cell-like phenotype of PC3 cells.
RBM3 over-expression induces cell differentiation trend and alters CD44 expression pattern in PC3 cells

In order to determine the molecular mechanisms that may underline the effects of RBM3 over-expression, the expression of a panel of stem cell-related genes in the two clones was examined. Among the genes with altered expression, 60% (6/10) of the genes that were significantly increased \((P < 0.05)\) are related to cell differentiation, while more than 50% (5/9) of the decreased genes are associated with cell self-renewal (Fig. 3D and Supplemental Table S1). In addition to mRNA expression profiling, the expression of a panel of cell differentiation-related, in particular neural differentiation-related miRNAs, was found to be induced by RBM3 over-expression (Supplemental Fig. S2A). β3-tubulin protein, which is a neuron-differentiation marker, was observed to be increased in the PC3 clones over-expressing RBM3 and co-expressed with RBM3 in the nerve fibers observed in human prostate tissue (Supplemental Fig. S2B,C). Taken together, these results suggest that over-expression of RBM3 in PC3 cells tends to induce neural differentiation.

CD44 mRNA levels were found to be decreased upon RBM3 expression in the PCR array assay as described above and also confirmed by quantitative RT-PCR (Fig. 3E). In light of the intriguing relationship between CD44 expression and PCa stem cell-like properties (29), CD44 protein expression was examined in the RBM3 over-expressing PC3 cells. Surprisingly, standard CD44 protein that is about 85 kD in molecular weight was not decreased but increased in PC3-RBM3 clones as compared with PC3-GFP clones. However, an intense band above 180 kD was detected in the PC3-GFP clones that was extremely low in either of the two PC3-RBM3 clones (Fig. 3F). Further, CD44 tends to be clustered into foci at the edge of the cellular processes in PC3-RBM3 cells but not in PC3-GFP cells (Fig. 3G). This result suggests that, in addition to the...
expression level, the expression pattern of CD44 is also much altered upon RBM3 over-expression in PC3 cells.

**RBM3 inhibits splicing of the CD44 alternate exons v8-v10**

CD44 proteins range in molecular weight from 85-230 kD, presumably due to alternate splicing (29). Using the strategy established previously (Fig. 4A) (27, 30), a band at a size of around 650 bp was found to be decreased in the two PC3-RBM3 clones compared to the control PC3-GFP clones when using c13 primer to amplify all of potential variant exons (Fig. 4B). It was conjectured that the 650 bp-product corresponds to a CD44 variant with inclusion of exon v8-v10, while the 270 bp-product corresponds to the CD44 standard mRNA (Supplementary Table 2). This speculation was subsequently verified by direct DNA sequencing (Supplementary Fig. S3A), and by siRNAs specific to CD44 variant exons v8-v10 (Fig. 4C,D and Supplementary Fig. S3B,C). These results suggest that the differential expression of the high molecular CD44 protein between PC3-GFP and PC3-RBM3 clones results from alternative splicing of the CD44 variant exons v8-v10. Indeed, by using the variant-specific primers (Supplementary Fig. S3B), it was confirmed that the expression of CD44v8-v10 was decreased and the expression of CD44s was increased in the PC3-RBM3 cells compared to the PC3-GFP cells (Fig. 4E). On the other hand, decreasing the expression of RBM3 by specific siRNA in the PC3-RBM3 cells resulted in an increased expression of CD44v8-v10 but a decreased expression of CD44s, which significantly increased the ratio of CD44v8-v10 to CD44s (Fig. 4F, Supplementary Fig. S3D). In contrast, when the expression of RBM3 in PC3 cells was induced under 32°C, the ratio of CD44v8-v10 to CD44s was decreased accordingly (Fig. 4G). Together, these results suggest that RBM3 inhibit splicing of CD44 variant v8-v10 while consequently increase the expression of its standard isoform.
CD44v8-v10 enhances cancer stem cell-like features by interfering CD44 cleavage

When splicing of CD44 v8-v10 was silenced by exon-specific siRNAs, the colony formation of the parental PC3 cells as well as the PC3-GFP clones in soft agar was significantly decreased (Fig. 5A). At the same time, the expression of CD44v8-v10 was found to be increased while the expression of CD44s decreased in the cell colonies that formed in soft agar as compared to the cells grown in 2D culture condition (Fig. 5B). The same expression pattern of CD44v8-v10 and CD44s was observed in DU145 cells (Fig. 5C) as well, although they showed a significantly decreased ability to form colony in soft agar as compared to PC3 cells (data not shown). Indeed, when the general primers that amplify all the variants of CD44 was used in a conventional RT-PCR, it was found that in addition to the variant v8-v10 some other variants were also increased in the cell colonies that formed in soft agar for both PC3 and DU145 cells (Fig. 5D). These results indicate that the increase of CD44 variants is compatible with the capability of cancer cells to re-colonize in soft agar, and suggest that RBM3 attenuates cancer stem cell-like features by, at least in part, inhibiting splicing of CD44.

It has been shown that CD44 undergoes sequential proteolytic cleavages by matrix metalloproteases (MMPs) including MMP9. This results in shedding of the ecto-CD44 that in turn regulates cell-extracellular matrix interaction, and release of the CD44 intracellular domain (CD44-ICD) that translocates to the nucleus and activates transcription of genes such as cyclin D1 (31, 32). Indeed, the expression pattern of cyclin D1 is consistent with that of CD44s in cell colonies that formed in soft agar or in cells cultured at 32°C, indicating a potential correlation between CD44s function and transcription of cyclin D1 (Supplementary Fig.S4A-C). Further, as shown in Figure 5E and F, addition of a MMP9 inhibitor significantly decreased the shedding of ecto-CD44 as well as the transcription of cyclin D1 in PC3-RBM3 cells but not in PC3-GFP cells.
(Fig.5E,F). Given that the CD44v8-v10 is exclusively expressed in PC3-GFP cells, this result alludes to the possibility that CD44v8-v10 attenuates the cleavage of CD44 mediated by MMP9. In support of this conjecture, treatment with recombinant MMP9 significantly increased the expression of cyclin D1 in the LNCaP cells that over-express CD44s alone but not in the cells that over-express both CD44s and CD44v8-v10 (Fig.5G,H and Supplementary Fig. S4D).

**CD44s is negatively related to the cancer stem-like features of PCa**

To investigate further the significance of expressions of CD44s and CD44v in PCa, the soft agar clonogenic assay was performed in LNCaP cells, which are lack of endogenous expression of either CD44s or CD44v. Surprisingly, CD44s over-expression significantly inhibited the clonogenic capability of LNCaP cells, while co-expressing CD44v8-v10 with CD44s restored this capability (Fig. 6A). In support, the expression of CD44s was found to be decreased in metastatic PCa as compared to localized tumors (Fig. 6B), and its expression was lower in the tumors of patients with recurrence than those without recurrence (Fig. 6C), although the expression of CD44v8-v10 did not show any correlation with tumor progression (Supplementary Fig. S5), which may due to the potential expressions of other CD44 variants in tumor samples. Taken together, these data suggest that CD44s is negatively correlated with cancer stem cell-like features and accordingly with the progression of PCa.

**Discussion**

While accumulating evidence suggests that RBM3 plays an important role in multiple cellular processes during development and during stress response to a variety of stresses including hypothermia (11, 12), the role of RBM3 in cancer remains equivocal. For example, while RBM3 is thought to be a proto-oncogene that is up-regulated in many cancers where it appears to protect
cancer cells from mitotic catastrophe (19) or apoptosis (33), several studies also demonstrate that high RBM3 expression in cancer cells, especially in the nucleus, predicts a better prognosis in a variety of cancers, including PCa (34-40). This negative correlation between RBM3 expression and tumor progression does not seem to support its oncogenic function but it is in agreement with the present results demonstrating decreased RBM3 expression in metastatic PCa compared to primary cancer and, more importantly, that over-expression of RBM3 in PC3 cells greatly attenuates the stem cell-like feature of this aggressive cell line.

As a translation enhancer (18, 24) RBM3 expression may facilitate the high rate of cell proliferation and protein synthesis in early tumor development; however, it may not be required by or even impede tumor progression, which needs several additional properties such as migration, invasion, and re-colonization in the new microenvironment. This is evident by the present results showing that RBM3 expression drives differentiation and attenuates the stem cell-like features of highly aggressive cancer cells (PC3). In fact, disseminated tumor cells (DTCs) that localize and take up residence in the bone marrow niche in PCa are characterized by a non-proliferative status namely, quiescence or dormancy (41). Indeed, in the present study, the colonized cells in soft agar showed a dramatically decreased expression of cyclin D1 along with the decreased expression of RBM3, indicating a blocking of cell proliferation. It is speculated that the capability of slowing-down to quiescence might be critical for survival and re-population of tumor cells in a new microenvironment. Thus, it seems plausible that the up-regulation of RBM3 is involved in the early development of PCa, while its down-regulation again is important for the subsequent steps in tumor progression.
As far as we are aware, this is the first report to demonstrate that RBM3 significantly inhibits splicing of the CD44v8-v10 variant in cancer. CD44 is commonly expressed as a wide variety of isoforms that mainly arise from alternatively spliced variants, which is particularly important for CD44 functions in tumor cells (42). Among the various isoforms, CD44v8-v10 has been reported to be the predominant form that is expressed in a variety of cancers (43-45). Indeed, over-expression of this isoform enhances bladder cancer progression potentially by decreasing the interaction of CD44 with HA (46). On the other hand, CD44s has been demonstrated an inhibitory role during metastasis in PCa (47); this, however, does not require the binding of PCa cells to HA (48). The regulation of CD44-ICD on gene transcription has only been explored more recently (31, 32). In the present study, we found that CD44v interfered with the tumor suppressing function of CD44s by blocking its cleavage and potentially the consequent transcription of cyclin D1, which is a CD44-ICD-induced gene. This leads to the involvement of CD44v in enhancing cancer stem cell-like characteristic in PCa cells (Fig. 7). Importantly, RBM3 that is a stress-response gene was shown to impair the splicing of CD44v8-v10 and therefore tune up the balance between the expression of CD44 variants and standard isoform, which may likely be the mechanism by which RBM3 regulate the stem cell-like properties of cancer cells in attempt to adapt the change of microenvironment. Indeed, as shown in the present study, cancer cells that are capable of re-colonizing in soft agar showed much enhanced splicing of CD44 when compared with cells those growing under 2D condition. The modulation of alternative splicing appears to be an important adaptive response of the stem cell to the change of environment. This may likely be the case during cancer evolution as well. If so, modulating RBM3 activity to affect variant splicing may represent a novel therapeutic strategy to curtail the evolutionary capability of cancer cells.
Acknowledgements

We thank Dr. John T. Isaacs, Professor of Chemical Therapeutics Program and Professor of Oncology and Urology at the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins for providing the CD133+/− PrEC cells.
References


Figures legends

**Figure 1.** RBM3 expression in human prostatic tissues and cells. (A), mRNA expression of RBM3 in human adult and fetal tissues. Data are mean ± s.d. (n = 4), *P < 0.05. P, adult prostate; FP, fetal prostate; T, adult testis; FT, fetal testis. (B), normal prostatic epithelial PrEC cells were sorted by FACS according to the CD133 expression on cell surface. The RBM3 expression was detected by RT-PCR. Values are mean ± s.d. (n = 5), ***P < 0.001. (C), mRNA levels of RBM3 was determined in human normal and prostate cancer tissues by RT-PCR. Normal, normal prostate tissue (n = 19); OC-PCa, organ-confined prostate cancer (n = 79); Met-PCa, metastatic prostate cancer (n = 23). Experiment was repeated for three times. Bars indicate median values. (D), representative images of RBM3 immunohistochemical staining in paraffin-imbedded, human prostate cancer tissues. In left panel, arrow heads indicate cancer area; arrows indicate adjacent normal glands. Right panel is the higher magnification image of the middle panel. Scale bars, 100 μm.

**Figure 2.** RBM3 over-expression attenuates the stem cell-like features of cancer cells. (A), PC3 cells and DU145 cells were cultured at 43°C for 4h or treated with Paclitaxel at 100 nM for 48h, and then allowed to recover for one month. RBM3 expression was detected by RT-PCR in the re-growing cancer cells that survived from these treatments. Data are mean ± s.d. (n = 3), *P < 0.05. (B), mRNA levels of RBM3 were determined in the cancer cells when forming colonies in soft agar or growing routinely in 2-D culture. Data are mean ± s.d. (n = 3), *P < 0.05. (C), representative dishes from clonogenic assay in soft agar. Five thousand PC3-RBM3 or PC3-GFP cells were cultured in 0.3% agar in growth medium for 2 weeks. (D), left panels, representative individual colonies in soft agar observed under light microscope (upper panels) or fluorescence microscope (lower panels). Scale bars, 100 μm. Right panel, number of visible colonies in soft agar.
agar formed from control and RBM3-overexpressing clones. Data are mean ± s.d. (n = 4). *P < 0.05 in comparison to G#1; #P<0.05 in comparison to G#2. (E), left panels, representative prostasphere formed in the Prostasphere formation assay. Scale bars, 100 μm. Right panels, number of prostasphere per 96-well plate formed from control and RBM3-overexpressing clones. Data are mean ± s.d. (n = 4). Note that there is no prostasphere formed from RBM3-overexpressing clones. In all panels: G#1, PC3-GFP-clone #1; G#2, PC3-GFP-clone #2; R#1, PC3-RBM3-clone #1; R#6, PC3-RBM3-clone #6.

Figure 3. (A), RBM3 over-expression impairs tumorigenesis of PC3 cells. Two hundred thousand cells of PC3-RBM3 or PC3-GFP clones were subcutaneously implanted in the right flank of nude mice (n = 10 in each group). Tumor sizes were measured every week. Values are mean ± s.d. (n = 4), *P < 0.05 in comparison to R#1; #P < 0.05 in comparison to R#6. (B), Kaplan-Meir tumor-free survival curves. *P < 0.05 in comparison to G#1; #P < 0.05 in comparison to G#2. (C), representative images of xenografts from PC3-RBM3 and PC3-GFP clones in nude mice. Embedded scales, centimeters. (D), expression of stem cell-related genes was detected in the PC3-RBM3 and PC3-GFP clones using a stem cell-specific PCR array. Genes whose expression levels altered more than 1.5 fold in the clone R#1 compared to the G#1 are presented. (E), the down-regulation of CD44 in PC3-RBM3 clones was validated by RT-PCR. Values are mean ± s.d. (n = 3), *P < 0.05 in comparison to G#1; #P<0.05 in comparison to G#2. (F), CD44 protein expression was detected by Western blot in the PC3-GFP and PC3-RBM3 clones. Actin levels were used as internal controls. (G), CD44 expression (red) was detected by immunofluorescence staining in the PC3-GFP and PC3-RBM3 clones. Cells were counterstained with DAPI to reveal nuclei. Arrowheads indicate focal membrane-associated structures near the end of cellular processes. Scale bars, 50 μm.
Figure 4. RBM3 over-expression inhibits CD44 alternative splicing variants. (A), schematic diagram of the structure of the CD44 gene. (B), CD44v expression was screened in PC3-GFP and PC3-RBM3 clones by the semi-nest PCR using primers described in (A). Arrows indicate a PCR product at a size of around 650 bp. M, DNA marker; N, negative control without cDNA templates. (C), PC3 cells were treated with non-target siRNA (siN) and two siRNAs (siC1, siC2) specific for exon v8-v10 of CD44, and then the variants expression was determined by RT-PCR using general primer c13 and hs3’. The arrow indicates a PCR product corresponding to CD44v8-v10 at a size around 650 bp. (D), CD44 protein levels were detected in PC3-GFP and PC3-RBM3 clones by Western blot after cells were treated with siC1, siC2 or siN. (E), left panel, mRNA levels of CD44v8-v10 and standard CD44 (CD44s) were determined in PC3-GFP and PC3-RBM3 clones by qRT-PCR using the specific primers (primer design is shown in Supplemental Fig. S4B). Right panel, the expression of CD44v8-v10 in relative to CD44s. Values are mean ± s.d. (n = 3), *P < 0.05 in comparison to G#1; #P < 0.05 in comparison to G#2. (F), R#1 and R#6 cells were treated with siRNA that is specific to RBM3 or non-target siRNA (siN) for 48 hr, and mRNA levels of CD44v8-v10 in relative to CD44s were determined by qRT-PCR. Values are mean ± s.d. (n = 3), *P < 0.05. (G), PC3 cells were cultured under 32°C for 0 hr to 144 hr, and mRNA levels of CD44v8-v10, CD44s as well as RBM3 were detected by qRT-PCR. Values are mean ± s.d. (n = 4).

Figure 5. CD44v8-v10 impairs standard CD44 (CD44s) function and enhances cancer stem cell-like features. (A), clonogenic assay in soft agar was performed in parental PC3 cells and PC3-GFP clone after treatment with two different siRNAs specific to CD44v8-v10 (siC1, siC2) or with non-target siRNA (siN). Values are mean ± s.d. (n = 3), *P < 0.05 in comparison to siN. (B), left panel, mRNA expression of CD44v8-v10 and CD44s was detected by qRT-PCR in PC3
cells when forming colonies in soft agar or growing routinely in 2-D culture. Right panel, the mRNA levels of CD44v8-v10 in relative to CD44s. Data are mean ± s.d. (n = 3), *P < 0.05. (C), same experiments with (B) in DU145 cells. Data are mean ± s.d. (n = 3), *P < 0.05. (D), mRNA expressions of CD44s and CD44 variants were evaluated in the cells that formed colony in soft agar or that grew under regular 2D culture condition by a semi-nest PCR using the primers hs5’, c13, and hs3’. Arrows indicate the PCR products raised from CD44v8-v10; arrowheads indicate the PCR products raised from CD44s. M, molecular marker; N, negative control. (E), G#2 and R#1 cells were treated with MMP9 inhibitor SB3CT or DMSO for 24 hr, and soluble CD44 in culture media was detected by ELESA. Values are mean ± s.d. (n = 3), *P < 0.05. (F), G#2 and R#1 cells were treated with MMP9 inhibitor SB3CT or DMSO for 24 hr, and mRNA expression of cyclin D1 was detected by qRT-PCR. Values are mean ± s.d. (n = 3), *P < 0.05. (G), LNCaP cells were transfected with both pLenti-III-CD44s (pCD44s) and pLenti-III-CD44v8-v10 (pCD44v), or pCD44s alone, and then treated with or without recombinant human MMP9. The mRNA levels of cyclin D1 were detected by RT-PCR. Values are mean ± s.d. (n = 3), *P < 0.05. (H), in the same experiment described in (G), the protein levels of cyclin D1 were detected by Western blot. The intensity of Western bands was quantitatively evaluated as shown in right panel. Actin was used as protein loading control. Values are mean ± s.d. (n = 3), *P < 0.05.

Figure 6. (A) LNCaP cells were transfected with both pLenti-III-CD44s (pCD44s) and pLenti-III-CD44v8-v10 (pCD44v), or pCD44s alone, or empty vector (empty) for 48 hr. Cells were then subjected to clonogenic assay in soft agar for 4 weeks. Values are mean ± s.d. (n = 3), *P < 0.05. (B), mRNA levels of CD44s were determined in human normal and prostate cancer tissues that are described in Fig. 1. by RT-PCR . (C), mRNA levels of CD44s were compared between
patients with recurrent PCa (Rec, n = 44) and those without recurrent disease (Non-rec, n = 30). Experiment was repeated for three times. Bars indicate mean ± SEM.

**Figure 7.** Proposed model for RBM3 regulation of CD44 variant splicing in cancer cells. RBM3, a stress-response protein impairs the splicing of CD44v8-v10. When cells experience stress, RBM3 is down-regulated. This results in the release of suppression of RBM3 of CD44 splicing, an increase CD44v8-10 expression but a decrease in the expression of CD44s. In turn, CD44v8-v10 interferes with cleavage of CD44s and inhibits CD44-ICD-mediated cyclin D1 transcription, which may cause cell quiescence in response to stress. TF, transcription factor; CD44-ICD, CD44 intracellular domain.
Figure 2

A

![Bar charts showing RBM3/TBP levels in PC3 and DU145 cells.](chart)

**A**

- **Cont**
- **Heat**
- **Tax**

**B**

- **2-D**
- **Agar**

C

**C**

- **G#1**
- **G#2**
- **R#1**
- **R#6**

D

- **G#1**
- **R#6**

**D**

- No. of colony

E

- **G#1**
- **G#2**
- **R#1**
- **R#6**

**E**

- No. of prosptosphere
Figure 4

A. cDNA of CD44 Gene

B. Gel Electrophoresis Images

C. Gel Electrophoresis Images

D. Gel Electrophoresis Images

E. Graphs showing CD44v8/CD44s ratios

F. Graphs showing CD44v8-10/CD44s ratios

G. Graphs showing fold change

(Reproduced, with permission, Iczkowski 2010)
Figure 5

A

PC3

siN  siC1  siC2

PC3-G#2

B

PC3

CD44s/CD44s-TBP

CD44v8-10/CD44s

C

PC3

CD44s/CD44s-TBP

CD44v8-10/CD44s

D

PC3

DU145

E

CD44s/CD44s-TBP

G#2  R#1

F

CD44/CD44s-TBP

G#2  R#1

H

MMP9:  -  +  -  +
pCD44s  pCD44s+pCD44v

cyclin D1

actin
Figure 7
Stress response protein RBM3 attenuates the stem-like properties of prostate cancer cells by interfering with CD44 variant splicing

Yu Zeng, Dana Wodzenski, Dong Gao, et al.

Cancer Res  Published OnlineFirst May 10, 2013.