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Journal of Cellular Biochemistry 113:2523–2531 (2012)

Journal of Cellular Biochemistry

Repulsive Guidance Molecule B (RGMB) Plays Negative Roles in Breast Cancer by Coordinating BMP Signaling

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ABSTRACT

Repulsive guidance molecules (RGMs) coordinate axon formation and iron homestasis. These molecules are also known as co-receptors of bone morphogenetic proteins (BMPs). However, the role played by RGMs in breast cancer remains unclear. The present study investigated the impact of RGMB on functions of breast cancer cells and corresponding mechanisms. RGMB was knocked down in breast cancer cells by way of an anti-RGMB ribozyme transgene. Knockdown of RGMB resulted in enhanced capacities of proliferation, adhesion, and migration in breast cancer cells. Further investigations demonstrated RGMB knockdown resulted in a reduced expression and activity of Caspase-3, accompanied with better survival in RGMB knockdown cells under serum starvation, which might be induced by its repression on MAPK JNK pathway. Up-regulations of Snai1, Twist, FAK, and Paxillin via enhanced Smad dependent sigaling led to increased capacities of adhesion and migration. Our current data firstly revealed that RGMB may act as a negative regulator in breast cancer through BMP signaling. J. Cell. Biochem. 113: 2523–2531, 2012.

KEY WORDS: RGM; BMP; BREAST CANCER; APOPTOSIS; FOCAL ADHESION; EMT; SMAD; MAPK

Repulsive guidance molecule B (RGMB), also known as Dragon (turned "on" in the "DRG"), was first identified as a gene regulated by DRG11 which is a homeobox transcription factor expressed in embryonic dorsal root ganglion (DRG) and dorsal horn neurons [Samad et al., 2004]. RGMB is a myelin-derived inhibitor of axon growth in the central nervous system [Liu et al., 2009] and contributes to the patterning of the developing nervous system [Samad et al., 2005]. RGM family comprises RGM-A, -B and -C. RGMs are GPI-linked membrane proteins that contain an N-terminal signal peptide, a C-terminal propeptide, and a conserved RGD motif. They share 50–60% sequence homology and all have a conserved von Willbrand factor (vWF) type D domain. RGMs are involved in embryonic development and tissue repair of the nerve system (RGMA and RGMB), and maintenance of iron homeostasis (RGMC) [Monnier et al., 2002; Babitt et al., 2005].

In 2005, RGMB was the first member of the RGM family to be identified as a BMP co-receptor [Samad et al., 2005], whilst BMPs have been implicated in breast cancer and bone metastasis [Babitt et al., 2005; Ye et al., 2007, 2009a, in press]. BMPs are involved in both promotion and inhibition of breast cancer progression and played an important role in TGF-β induced epithelial–mesenchymal transition (EMT), which contributes to the metastatic process [Buijs et al., 2007; Alarmo et al., 2008, 2009; Takahashi et al., 2008; Cassar et al., 2009]. There are two signal pathways BMPs go through [Babitt et al., 2005], the Smad dependent or independent pathway. In the Smad dependent signal transduction, TGF-β signals via Smad2/3, while BMP signals through Smad1/5/8. The Smad-independent pathway is a chain activation, which is triggered by BMP receptors, followed by a cascade of activation, that is, MAP4K (ILP)-MAP3K (TAK1)-MAPK (JNK, ERK1/2, P38). RGMs, which are involved in BMP Smad-dependent or independent (MAPK) pathways might have impact on breast cancer through regulation of BMP pathways or target genes. In the present study, we further examined the role played by RGMB in regulating functions of cancer cells, and its implication in the signal transduction of BMPs.

MATERIALS AND METHODS

MATERIALS AND CELL LINES

Polyclonal goat anti-RGMB antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All the primers used are shown in Table I and were synthesized by Invitrogen Inc. (Paisley, UK).

Grant sponsor: Cancer Research Wales; Grant sponsor: Breast Cancer Hope Foundation; Grant sponsor: Fong Family Foundation.

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Manuscript Received: 27 September 2011; Manuscript Accepted: 5 March 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 13 March 2012

DOI 10.1002/jcb.24128 • © 2012 Wiley Periodicals, Inc.

TABLE I. Primers Used in This Study

Gene	Sense (5′–3′)	Antisense (5′–3′)
RGMA	TCGACAATAATTACCTGAACG	ACTGAACCTGACCGTACA
RGMB	TTCAGGTTCAAGTGACAAACG	CCTGGAAGTTCTTGAAGATG ACTGAACCTGACCGTACA
RGMB (ribozyme)	CTGCAGGTCATCTGTCACAGC	TCATCTGTCACAGCTTGGTA ACTAGTGTACAGATCAGAAA
RGMC	TTGGTACTGATGAGTCCGTGAGGA AATGACTTCCTCTTTGTCCA	GTTTCGTCCTCACGGACT ACTGAACCTGACCGTACA
c-Myc	TGCTCCATGAGGAGACAC	CATTCCTGCATGTTCTTAAA ACTGAACCTGACCGTACATG
FAK	AACAGGTGAAGAGCGATTAT	ATCCAGACTCTGACCTTT ACTGAACCTGACCGTAC
Paxillin	CAATCCTTGACCCCTTAGA	ACAGTATGATCCC ACTGAACCTGACCGTAC
RhoC	GAGAAGTGGACCCCAGAG	ATTGGAGACACTGGAAGTTTT ACTGAACCTGACCGTACAC
Slug	CTCCAAAAAGCCAAACTACA	TTCATCTTGGCCACGCTC ACTGAACCTGACCGTACA
Twist	AAGCTGAGCAAGATTCAGAC	GAGGATCTCTGGTTGTGGTA ACTGAACCTGACCGTACA
Snai1	ACTATGCCGCGCTCTTTCC	GAGGACCTGGTAGACCGAAGT TCAGCGGGGACATCCTGAGC
GAPDH	GGCTGCTTTTAACTCTGGTA	GACTGTGGTCATGAGTCCTT

Smad-3 inhibitor SIS3 was purchased from Merck Chemicals Ltd. (Nottingham, UK). Recombinant human BMP-7 (rh-BMP-7) was obtained from R&D Systems Europe Ltd. (Abingdon, UK).

The MDA-MB-231 and MCF-7 cell lines used in this study were acquired from the American Type Culture Collection (ATCC, Rockville, MD). The cells were routinely maintained in DMEM-F12 medium supplemented with 10% fetal bovine serum and antibiotics.

RNA ISOLATION AND RT-PCR

RNA isolation was carried out using the ABgene Total RNA Isolation Reagent (TRIR) kit (ABgene, Surrey, UK). Reverse transcription was performed using 0.5 μg RNA for each sample and iScript™ cDNA Synthesis Kit (BIO-RAD). PCR was then carried out at the following conditions: 94°C for 5 min, followed by 35 cycles of 94°C for 40 s, 55°C for 40 s, 72°C for 1 min, and a final extension of 7 min at 72°C.

REAL-TIME QUANTITATIVE PCR (Q-PCR)

The assay was based on the Amplifuor technology and primers were designed using Beacon Designer software which included complementary sequence to universal Z probe (Intergen Inc., Oxford, UK), as previous reported [Parr et al., 2004; Jiang et al., 2005]. GAPDH was used as housekeeping gene. The primers sequences are provided in Table I.

ESTABLISHMENT OF MDA-MB-231 AND MCF-7 RGM KNOCKDOWN VARIANTS

RGMB expression was knocked down in MDA-MB-231 and MCF-7 cells using anti-human RGMB hammerhead ribozyme transgenes which were designed based on the secondary structure of RGMB mRNA using Zuker's RNA mFold program. The transgenes were generated through touchdown PCR and cloned into pEF6/His TOPO mammalian expression plasmid vectors (Invitrogen Inc.). Control empty plasmid vectors and the ribozyme transgenes were then transfected into MDA-MB-231 and MCF-7 cells by electroporation. The cells were selected for 2 weeks with 5 μg/ml blasticidin before further use.

IN VITRO CELL GROWTH ASSAY

The cells were seeded into a 96-well plate at a density of 3,000 cells per well, and incubated for periods of up to 5 days. The results were shown as growth rate, which was calculated by normalization against the base absorbance measurements at Day 0. The protocol has been previously described by Jiang et al. [1998].

IN VITRO CELL MATRIX ADHESION ASSAY

Forty-five thousand five hundred cells were seeded onto the 96-well plate coated with Matrigel basement membrane in 200 μl of normal medium and incubated for 40 min. Adherent cells were then fixed in 4% formaldehyde (v/v) in BSS before being stained in 0.5% crystal violet solution (w/v). Absorbance was determined following a previously reported method [Parr et al., 2001].

IN VITRO CELL INVASION ASSAY

Transwell inserts (upper chamber) with 8 μm pores were coated with 50 μg/insert of Matrigel. 2×10^4 cells were added to each well. After 72 h, cells that had migrated through the matrix and adhered to the other side of the insert were fixed and stained with 0.5% (w/v) crystal violet following a method previously reported [Parr et al., 2001].

IN VITRO CELL WOUNDING/MIGRATION ASSAY

A wounding/migration assay was also used to assess the migratory properties of the breast cancer cells [Davies et al., 1999]. Cells were grown upon reaching confluence the monolayer of cells was scraped with a 21G needle. The wound was tracked and recorded using a CCD camera attached to a Lecia DM IRB microscope (Lecia GmbH, Bristol, UK) through taking pictures every 15 min over a 90-min period. The distance the wound front migrated was measured.

PROTEIN EXTRACTION, SDS–PAGE AND WESTERN BLOT ANALYSIS

Protein was extracted following a previously reported method [Ye et al., 2008]. Equal amounts of each sample were loaded onto 8% polyacrylamide gel and following SDS–PAGE, the proteins probed with the specific primary (1:200), and the corresponding peroxidise-conjugated secondary antibodies (1:1,000). The protein bands were visualized using the SupersignalTM West Dura system (Pierce Biotechnology).

APOPTOSIS ANALYSIS USING FLOW CYTOMETRY

Apoptosis assay was performed using a Vybrant® Apoptosis Assay Kit (Invitrogen Inc.) which contains fluorescent conjugated annexin V (FITC annexin V) and Propidium Iodide (PI) solution. This was repeated three times and the number of apoptotic cells including both early (Q4) and late apoptotic/necrotic cells (Q2) in each sample was determined using the FlowMax software package following a previously described method [Ye et al., 2008].

STAINING OF APOPTOTIC CELLS

The cells were incubated either with DMEM media containing 10% FCS or without serum for a period of 48 h, before both the adherent cells and those floating in the culture medium were harvested and washed in PBS. The cell pellet was then re-suspended in 20 ng/ml Hoechst33258 (Sigma–Aldrich) solution and incubated in darkness for 15 min. The cells were centrifuged and re-suspended with BSS before being counted and documented under a fluorescent microscope. A percentage for apoptotic cells was then calculated based on the cellular morphology changes occurring during apoptosis, including blebbing, loss of cell membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, and chromatin condensation.

CELL CYCLE ANALYSIS USING FLOW CYTOMETRY

For the cell cycle analysis, the cells were fixed with 75% ice-cold ethanol, and stored at −20°C. The cells were then resuspended in 1 ml PI staining solution (0.1% Triton X-100, 0.05 mg/ml propidium iodide, and 1 mg/ml RNase A), followed by an incubation of 15 min at 37°C, the cells were analyzed in the flow cytometer. This was repeated and the average percentages of cells in G0/G1, S, and G2/M phases were calculated following a previously described procedure [Jiang et al., 1998].

IMMUNOPRECIPITATION OF PHOSPHORYLATED PROTEINS

The cells were starved of serum for 2 h and then subject to corresponding treatment. The cells were lysed in SDS-free lysis buffer. The phosphorylated protein was immunoprecipitated using anti-phosphorylated serine/threonine antibody and A/G protein conjugated agarose beads, and then denatured in sample buffer prior to a separation on SDS–PAGE. This technique was modified from previously described method [Davies et al., 1999].

IMMUNOCHEMICAL STAINING

Cells were seeded at a density of 20,000 cells per well in 200 μl of normal medium, on a 16-well chamber slide (Lab-Tek, Nagle Nunc Int., IL). The cells were incubated for 24 h or subjected to any treatment before fixation in 4% formaldehyde. The cells were incubated with the primary antibody at a concentration of 1:100 for 1 h followed by labeling with a specific secondary antibody of 1:200 and DAB chromogen.

STATISTICAL ANALYSIS

Normally distributed data was analyzed using the two sample *t*-test while non-normally distributed data was analyzed using the Mann–Whitney and Kruskal–Wallis tests. Statistic significance was considered as $P < 0.05$.

RESULTS

KNOCKDOWN OF RGMB IN BREAST CANCER CELLS

To investigate the role of RGMB in breast cancer, specific ribozyme transgenes targeting RGMB were utilized to knockdown the expression of RGMB in MDA-MB-231 cells ($MDA^{\Delta RGMB}$). As shown in Figure 1A, the expression of RGMB mRNA was eliminated from MDA-MB-231 cells by the ribozyme transgene, in comparison to the level of expression seen in the wild type (MDA^{WT}) cells and MDA-MB-231 empty plasmid control ($MDA^{pEF/His}$) cells. A reduction of RGMB protein expression was also evident in $MDA^{\Delta RGMB}$ cells using Western blot analysis and the immunocytochemical staining (Fig. 1B,C).

INFLUENCE ON CELLULAR FUNCTIONS BY KNOCKDOWN OF RGMB

The influence of RGMB knockdown on cell proliferation was examined using in vitro growth assay. Cell growth was significantly increased in $MDA^{\Delta RGMB}$ cells at Day 3, compared to MDA^{WT} ($P = 0.02$) and $MDA^{pEF/His}$ ($P = 0.01$). The effect was more remarkable at day 5 in $MDA^{\Delta RGMB}$ cells, $P < 0.01$ versus MDA^{WT}, and $MDA^{pEF/His}$ (Fig. 2A).

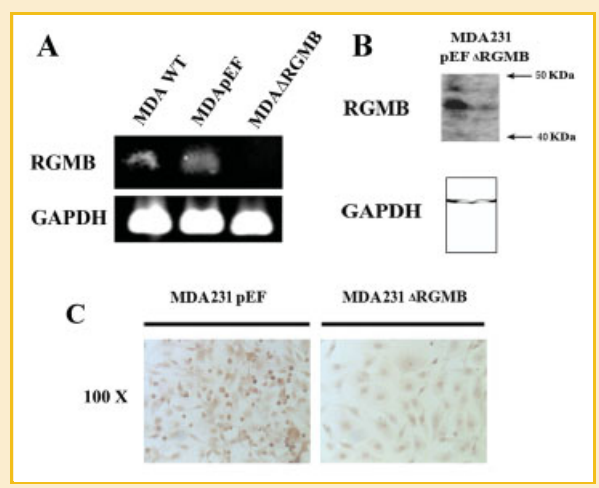

Fig. 1. Knockdown of RGMB in MDA-MB-231 cells. A: RGMB transcripts were substantially reduced in MDA-MB-231 cells by RGMB ribozyme trangenes. B: Reduced RGMB protein expression was confirmed using Western blot. The size of RGMB protein shown in the result is approximate 47 kDa. C: Immunochemical staining also showed much weaker staining of RGMB in the RGMB knockdown cells.

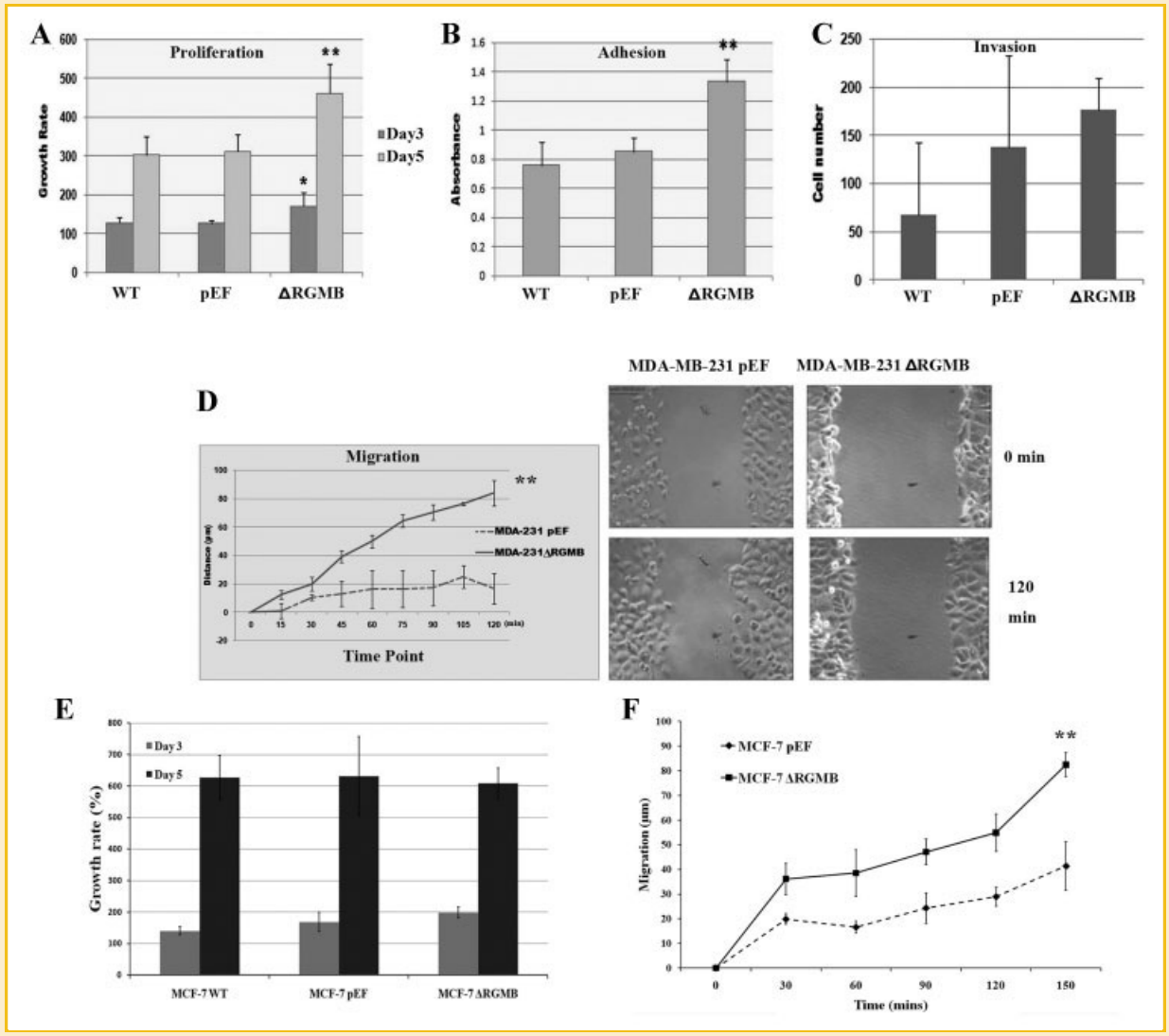

Fig. 2. Effect of RGMB knockdown on functions of breast cancer cells. A: The growth rate was increased after the loss of RGMB in MDA231 cells at both Day 3 and Day 5. B: The adhesive capacity was markedly increased in RGMB knockdown cells. C: No significant difference of invasion was seen in RGMB knockdown cells compared to the controls. D: The migration speed of MDA231 cells was markedly increased after RGMB knockdown. The right shows the pictures taken under microscope. The left shows the distance of the cells moved after wounding measured every 15 min. E: Effect on in vitro growth of MCF-7 by knockdown of RGMB. F: Knockdown of RGMB in MCF-7 resulted in a reduction of cell migration, $P < 0.01$ compared with the control. The error bars displayed the standard deviation at each time point. Each function assays were performed minimum three times, shown are representative data. $^{*}P < 0.05$, $^{**}P < 0.01$.

An enhanced adhesive capacity was seen in $MDA^{\Delta RGMB}$ cells. The absorbance of adhered $MDA^{\Delta RGMB}$ cells was significantly increased, $P < 0.01$ when compared to MDA^{WT} and $MDA^{pEF/His}$ cells (Fig. 2B).

The invasiveness of MDA-MB-231 cells was also determined using an in vitro invasion assay after knockdown of RGMB. The loss of RGMB had no significant effect on the invasiveness of MDA-MB-231 cells. Although the number of cells invaded for $MDA^{\Delta RGMB}$ (176.33 ± 33.08) was increased, compared to $MDA^{pEF/His}$ (138.67 ± 94.51), the increase did not reach statistical significance ($P = 0.54$; Fig. 2C).

A migration assay was utilized here to examine the effects on cell motility of RGMB knockdown. The migrating speed of $MDA^{\Delta RGMB}$ cells was obviously faster compared to the control cells, $P < 0.01$ (Fig. 2D).

Additionally, we examined the impact of RGMB knockdown on in vitro growth and migration of an–other breast cancer cell line, MCF-7, which expressed RGMB. No obvious effect was seen on the cell growth over a period of 5 days. However, knockdown of RGMB resulted in a reduction of migration of MCF-7 cells, which is consistent with the finding in MDA-MB-231 cells (Fig. 3E,F).

EFFECT ON APOPTOSIS AND CELL CYCLE OF MDA-MB-231 CELLS BY KNOCKDOWN OF RGMB

The proportion of apoptotic cells was analyzed using flow cytometry (Fig. 3A). Apoptotic index refers to total apoptotic population including both early apoptotic cells (Q2) and late apoptotic cells (Q4). There was only a small proportion of apoptotic cells seen in both $MDA^{\Delta RGMB}$ cells (6.47%) and $MDA^{pEF/His}$ (8.39%) control cells

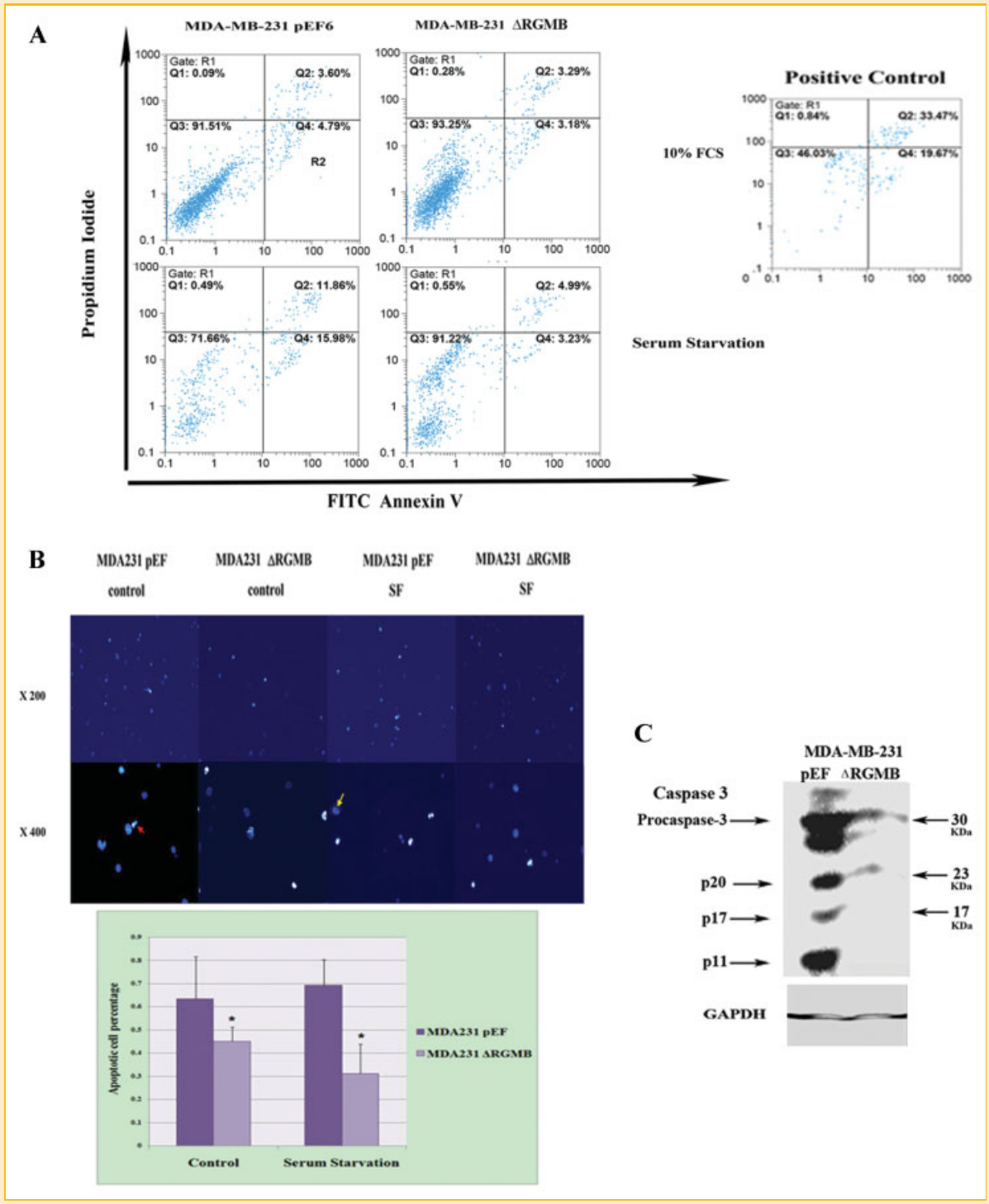

Fig. 3. RGMB knockdown protected MDA-MB-231 cells from apoptosis. A: The apoptotic population in MDA-MB-231 cells was analyzed using flowcytometry. There was a markedly smaller population of cells undergoing apoptosis in the RGMB knockdown cells (8.22%) after 48 h serum starvation, compared to the control cells supplement with 10% FCS (27.84%). The positive control (top right) shows hydrogen peroxide induced apoptosis. B: Analysis of apoptotic cells in MDA-MB-231 cells using Hoechst staining. Yellow arrow heads pointed to the normal cells; red arrow heads pointed to the cells undergoing apoptosis. There was a significant reduction in the proportion of apoptotic cells in MDA$^{\Delta RGMB}$ (0.45 ± 0.06) cells compared to the control cells (0.63 ± 0.18), under normal condition ($P = 0.04$). This reduction was more obvious in the serum free induced apoptosis (0.31 ± 0.13 vs. 0.69 ± 0.11, $P = 0.000$). Representative data were shown of three independent repeats. Error bars represent standard error of mean. SF: Serum Starvation. C: The protein level of caspase-3 in was reduced in RGMB knockdown cells. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

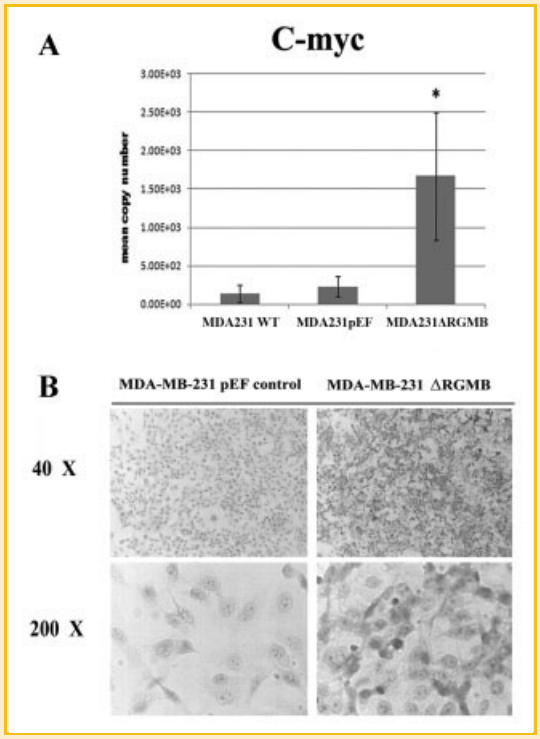

Fig. 4. Effect on expression of c-Myc by RGMB knockdown in MDA-MB-231 cells. A: The result shows the mRNA level of c-Myc is significantly increased by the loss of RGMB (mean ± SD 1,670 ± 831), compared to the wild type (140 ± 112; $P = 0.03$) and pEF (230 ± 129; $P = 0.04$). B: lummnocytochemical staining confirms the c-Myc proteins were markedly increased in RGMB knockdown cells compared to the control cells. Shown are representative results from three independent experiments.

under normal culture condition, that is, in DMEM supplemented with 10% FCS. However, a marked difference in apoptosis between these cells was seen after 48 hours serum starvation. MDA-MB-231$^{\Delta RGMB}$ cells had a far smaller proportion of apoptotic cells (8.22%) compared to that of control cells (27.84%), which had more apoptotic cells induced by serum starvation (Fig. 3A). This was further confirmed by counting apoptotic cells after Hoechst staining, in which MDA-MB-231$^{\Delta RGMB}$ cells under serum starvation demonstrated a significant decrease in apoptotic population, $P < 0.05$ compared to the control (Fig. 3B). Flow cytometry was also employed to investigate the effect on cell cycle. No obvious effect on cell cycle was seen in the MDA-MB-231 cells after knockdown of RGMB (data not shown).

As apoptosis was shown to be inhibited by knockdown of RGMB, the total Caspase-3 protein, an executioner caspase indicative of end-stage apoptosis, was found to be markedly reduced in RGMB knockdown cells compared to the control cells (Fig. 3C). As seen from Figure 3C, the subunits of Caspase-3, P11, and P17 have almost disappeared, which are the indicators of activated caspase-3 enzyme. The loss of RGMB resulted in a decrease in both protein expression and activation of caspase-3.

The present study further examined expression of the genes related to cell death in these transfected cells. Interestingly, c-Myc, a gene found to be implicated in apoptosis and cell cycle was significantly increased in MDA-MB-231 human breast cancer cells after the loss of RGMB, both at the mRNA and protein levels. As shown in Figure 4, the transcript level of c-Myc was significantly increased after the knockdown of RGMB (1,670 ± 831copies), $P < 0.05$ compared to the control cells (140 ± 112 for WT and 230 ± 129 for pEF). The immunocytochemical staining of c-Myc was confined in the cytoplasm and nucleus of the breast cancer cells, which was stronger in the MDA$^{\Delta RGMB}$ cells compared to the MDA$^{\Delta pEF/His}$ cells (Fig. 4A,B).

INFLUENCE ON SMAD-DEPENDENT BMP SIGNALING BY RGMB KNOCKDOWN IN MDA-MB-231 CELLS

As RGMs have been indicated in BMP signaling, we investigated phosphorylation of Smad-1 and Smad-3 in the transfected cells. We found that RGMB knockdown slightly promoted Smad-3 activation in MDA-MB-231 cells in exposure to rh-BMP-7 (40 ng/ml, 30 min; Fig. 5A,B). More interesting, the phosphorylation of Smad-1 in RGMB knockdown cells was markedly increased compared to control cells particularly when Smad-3 signaling was blocked by a specific small inhibitor SIS3 (3 μmol/ml, 30 min; Fig. 5B). This indicated that BMP-7 activated both Smad-1/5/8 and Smad-2/3 pathways, which were enhanced by the knockdown of RGMB. When the Smad-3 pathway was blocked, RGMB knockdown facilitated BMP signaling through the Smad-1 dependent pathway.

In addition to Smad-dependent BMP signaling, the Smad-independent pathway was also investigated here, where the ERK, p38, and JNK pathways were all included. Activation of ILP, TAK, and JNK by phosphorylation at serine/threonine residues was eliminated by knockdown of RGMB in MDA-MB-231 cells (Fig. 5).

KNOCKDOWN OF RGMB AND EXPRESSION OF FAK AND PAXILLIN IN MDA-MB-231 CELLS

The loss of RGMB resulted in up-regulated expression of FAK and Paxillin, at both mRNA and protein levels (Fig. 6). After treatment with rh-BMP-7, the protein levels of these two molecules were to some degree increased, while the up-regulation in MDA$^{\Delta RGMB}$ cells was enhanced. However SIS3 did not elicit additional effect on both expression and activation of FAK, while an increased level of Paxillin was seen in MDA$^{\Delta RGMB}$ cells when blocking Smad3 signaling. Phosphorylated FAK showed a similar pattern to total FAK. This suggests that BMP-7 might trigger the downstream signaling pathway which played a positive role in the regulation on FAK expression, and the regulation of BMP target genes (FAK and Paxillin) mediated by RGMB in MDA-MB-231 cells is unlikely through Smad-3 activation.

INFLUENCE OF RGMB ON THE MOLECULAR EVENTS LEADING TO EMT

Knockdown of RGMB significantly increased the motility of the MDA-MB-231 breast cancer cell line, one of the hallmarks of EMT. Further experiments showed that increased mRNA levels of EMT regulators; Snai1, Twist, Slug, and RhoC in MDA$^{\Delta RGMB}$ cells compared to the control (Fig. 7A).

Snai1 plays an important role in EMT, and has been shown to be up-regulated by TGF-β, possibly via Smad2/3 [Miyazono, 2009], to investigate whether the Smad-3 signaling pathway of BMP

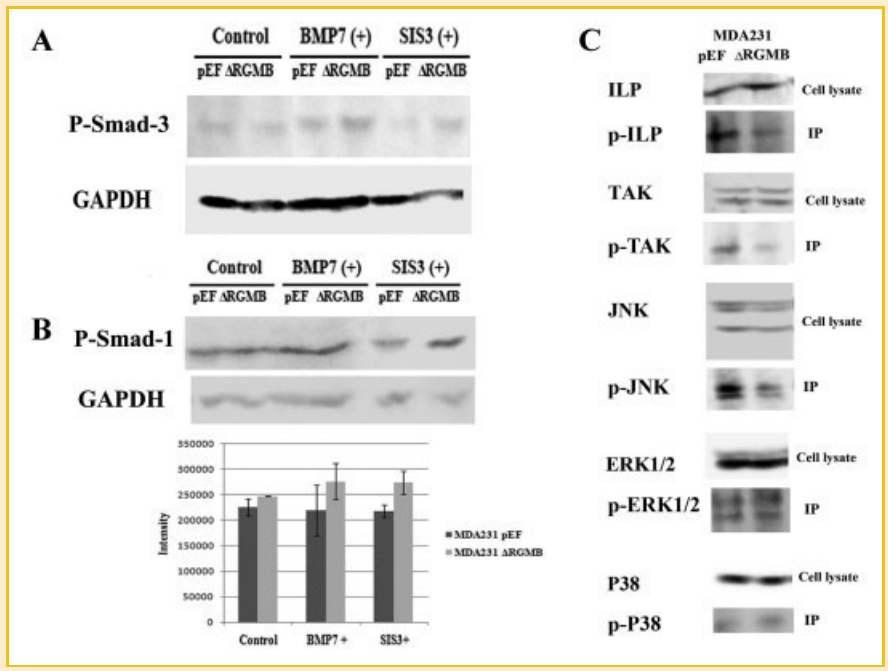

Fig. 5. RGMB and BMP signaling. A: An enhanced activation of Smad-3 was in RGMB knockdown cells exposed to rh-BMP-7. After the phosphorylation of Smad-3 was blocked, the RGMB knockdown partially maintains Smad-3 signaling pathway. B: The activated Smad-1 level was slightly up-regulated in RGMB knockdown cells and this is enhanced after BMP7 treatment and Smad-3 being blocked. Bar graph shows the intensity quantification normalized against GAPDH. C: Effect on Smad independent pathway, MAPK pathway by RGMB knockdown. The total proteins of these MAPK signaling molecules are not affected by RGMB knockdown. A reduction of serine/threonine phophorylation was seen in ILP, TAK, and JNK after the loss of RGMB, shown as p-ILP, p-TAK, and p-JNK.

participated in this, the phosphorylation of Smad-3 was blocked in the RGMB transfected cells. However, the mRNA level of Snai1 was increased by blocking Smad-3 signaling (Fig. 7B). Together with the activation profile of Smads induced by RGMB knockdown (Fig. 5), it was suggested that Snai1 was activated mainly by a Smad-1 dependent BMP signaling pathway in MDA-MB-231 cells, and the activation could be further enhanced by RGMB knockdown after smad-3 was blocked.

DISCUSSION

The role played by RGMB in regulating functions of breast cancer cells was for the first time examined in the current study. We demonstrated that the knockdown of RGMB promoted MDA-MB-231 cell proliferation, adhesion and migration capacity in vitro, but had little effect on invasion. In another breast cancer cell line MCF-7, RGMB knockdown resulted in an increased migration, but

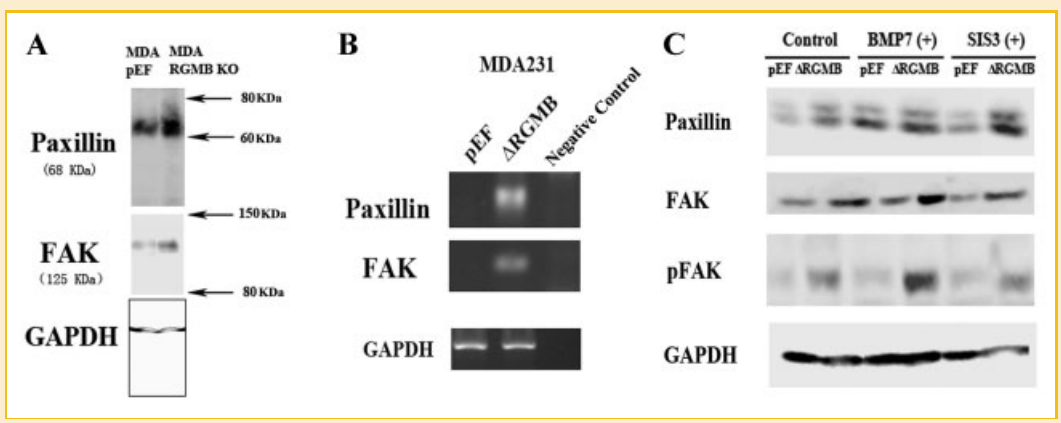

Fig. 6. RGMB knockdown influenced expression and activation of FAK and Paxillin. A: Paxillin and FAK protein levels were up-regulated in RGMB knockdown cells. The arrows on the right show the position of ladders. B: The conventional PCR result confirmed the RGMB knockdown induced the expression of FAK and Paxillin from mRNA level. C: The up-regulation of the expression of FAK and Paxillin and phosphorylated FAK (P-FAK) by RGMB knockdown was partially enhanced after BMP7 treatment, while remained at similar level after Smad-3 pathway was blocked.

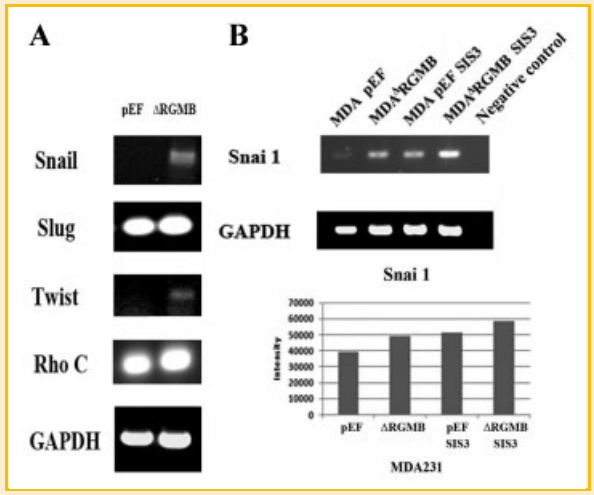

Fig. 7. Expression of EMT regulators altered by RGMB knocking down in MDA231. A: Snai1, Slug, Twist, and RhoC were all to some degree up-regulated in MDA231 RGMB knockdown cells compared to the pEF control. B: The RT-PCR showed the Snai1 was up-regulated after RGMB was knocked down when calculating the intensity (right), which is enhanced after Smad-3 was blocked by SIS3.

exhibited no obvious effect on in vitro cell growth. It suggests that RGMB has an inhibitory control on some traits of breast cancer cells. To elucidate the effect on cell growth by RGMB knockdown, we examined apoptosis, cell cycle and some of the relevant molecules. Our results indicated that knockdown of RGMB promoted survival of breast cancer cells under serum starvation. Caspase-3, which plays a central role in the execution-phase of cell apoptosis was then determined. Caspase-3 is expressed in cells as an inactive precursor from which the p17 and p11 subunits of the mature caspase-3 are proteolytically generated during apoptosis [Fernandes-Alnemri et al., 1994]. RGMB knockdown markedly reduced the expression and activation of caspase-3, including p17, p11, and precursor. In addition, cell cycle assays showed no effect by RGMB knockdown on the cell cycle of MDA-MB-231 cells. This suggests that RGMB knockdown promotes survival of breast cancer cells, at least partially through a down-regulation of Caspases-3 and subsequent suppression of apoptosis.

c-Myc is a very strong proto-oncogene and works as a transcription factor in activating the expression of a great number of genes. It has been linked to the control of cell-cycle progression [Steiner et al., 1995] and to the inducement of apoptosis. It is very often found to be up-regulated in many types of cancers. This up-regulation was enhanced by RGMB knockdown in breast cancer cells, although there was no significant effect shown in cell-cycle analysis. The expression of c-Myc might still contribute to the promotion on cell growth caused by suppression of RGMB expression, as the effect of c-Myc is diverse and the mechanism is yet unclear.

Additionally, in response to both the extrinsic and intrinsic apoptotic stimuli, JNK plays an essential role through its ability to interact and modulate the activities of diverse pro- and anti-apoptotic proteins (induce BAX, inhibit Bcl2, and so on) [Dhanasekaran and Reddy, 2008]. Through the coordinated regulation of the nuclear and mitochondrial events, JNK ensures the efficient execution of apoptosis. The activation of TAK1-MKK4-JNK pathway was proved to result in apoptotic cell death [Yang et al., 2004b]. In our previous study, activation of Smad independent pathway has also been indicated in the inhibitory effect on prostate cancer cells by BMP-10 [Ye et al., 2009b]. Loss of RGMB resulted in an inhibition of JNK pathway with little effect on other MAPK pathways. The repressed JNK pathway might also contribute to the negative regulation on cell apoptosis by RGMB knockdown, as the JNK pathway was further inhibited by RGMB knockdown during apoptosis induced by serum starvation.

FAK and Paxillin are known as integrin- and growth factor-associated tyrosine kinases promoting cell-matrix adhesion. It also has been shown that when FAK was blocked, breast cancer cells become less metastastic due to decreased mobility [Chan et al., 2009]. FAK activity elicits intracellular signal transduction pathways that promote the turn-over of cell contacts with the extracellular matrix, which plays a key important early step in cell migration. FAK also has a role in motility and cell survival. As shown in our study, RGMB knockdown increased FAK and Paxillin expression and activation, leading to an increased adhesion of MDA-MB-231 cells. This might be associated with the regulation on BMP Smad-dependent pathway.

RGMB has been demonstrated as a co-receptor of BMPs, the role of RGM in cancer might be associated with their participation in the BMP signaling pathways [Kanomata et al., 2009], which have been shown to be involved in the development of metastatic breast cancer [Alarmo et al., 2008]. However, the present study has shown that the co-receptor and co-regulation by RGM are probably more complicated. RGMB knockdown promoted signal transduction of Smad-1 and (partially) Smad-3 pathways, which were enhanced by BMP-7 stimulation. Smad-1 dependent pathway appeared to be enhanced when Smad-3 pathway was blocked. On the other hand, signaling through Smad independent pathways, that is, JNK pathway was repressed by RGMB knockdown. In this Smad independent pathway, ILP an adaptor protein links BMP receptors to TAB-TAK1/2, which consequently activate MAPK pathways. RGMB knockdown resulted in a suppression of this pathway. It suggests that RGMB plays vital roles in coordinating the signal transduction of BMPs via Smad dependent and independent pathways.

Breast cancer progression is associated with aberrant epithelial–mesenchymal transition (EMT), a critical step in malignant conversion for cancer cells to acquire aggressiveness. Snai1, Twist, RhoC, Slug are all described as inducers of EMT during tumor progression and invasion [van Golen et al., 2002; Yang et al., 2004a; Bellovin et al., 2006; Kudo-Saito et al., 2009]. RGMB knockdown resulted in increased expression of both Snai1 and Twist. Snai1 has been proved to be induced by TGF-β via Smad-2/3. In MDA-MB-231 cells, the up-regulation of Snai1 was seen in the RGMB knockdown cells, which tended to be enhanced when Smad-3 signaling was blocked by SIS3. The mechanism involved might be similar to that in the regulation of FAK and Paxillin yet to be investigated.

Taken together, knockdown of RGMB promoted growth, survival, adhesion, and migration of breast cancer cells, in which regulation of c-Myc, Caspases-3, Snai1, Twist, FAK and Paxillin via Smad dependent pathway, and suppression of Smad independent pathway JNK were involved. It suggests that RGMB plays negative roles in breast cancer, by coordinating BMP signaling and subsequent downstream cascades and BMP responsive genes thereby to inhibit breast cancer bone metastasis.

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