RSK Promotes Prostate Cancer Progression in Bone through ING3, CKAP2, and PTK6-Mediated Cell Survival

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Abstract

Prostate cancer has a proclivity to metastasize to bone. The mechanism by which prostate cancer cells are able to survive and progress in the bone microenvironment is not clear. Identification of molecules that play critical roles in the progression of prostate cancer in bone will provide essential targets for therapy. Ribosomal S6 protein kinases (RSK) have been shown to mediate many cellular functions critical for cancer progression. Whether RSK plays a role in the progression of prostate cancer in bone is unknown. IHC analysis of human prostate cancer specimens showed increased phosphorylation of RSK in the nucleus of prostate cancer cells in a significant fraction of human prostate cancer bone metastasis specimens, compared with the primary site or lymph node metastasis. Expression of constitutively active myristylated RSK in C4-2B4 cells (C4-2B4/RSK) increased their survival and anchorage-independent growth compared with C4-2B4/vector cells. Using an orthotopic bone injection model, it was determined that injecting C4-2B4/RSK cells into mouse femurs enhanced their progression in bone compared with control cells. In PC3-mm2 cells, knockdown of RSK1 (RPS6KA1), the predominant RSK isoform, but not RSK2 (RPS6KA2) alone, decreased anchorage-independent growth in vitro and reduced tumor progression in bone and tumor-induced bone remodeling in vivo. Mechanistic studies showed that RSK regulates anchorage-independent growth through transcriptional regulation of factors that modulate cell survival, including ING3, CKAP2, and PTK6. Together, these data provide strong evidence that RSK is an important driver in prostate cancer progression in bone.

Implications: RSK, an important driver in prostate cancer progression in bone, has promising potential as a therapeutic target for prostate cancer bone metastasis. Mol Cancer Res; 13(2): 348–57. ©2014 AACR.

Introduction

Prostate cancer is the second leading cause of cancer-related death in men in the United States. Mortality from prostate cancer is due mainly to development of metastasis in bone. Prostate cancer has a proclivity to metastasize to bone. One critical feature for metastatic prostate cancer cells to colonize in bone is to survive in the bone microenvironment. The mechanism by which prostate cancer cells are able to survive and progress within the bone microenvironment is not clear. Identification of molecules that play critical roles in the progression of prostate cancer in bone will provide targets for therapy.

Ribosomal S6 protein kinase (RSK) is a family of signal transducing Ser/Thr kinases. Four isoforms, RSK1–4, have been reported in mammalian cells (for review, see refs. 1–4). The best functionally characterized isoforms are RSK1 and RSK2. Each RSK isoform contains two nonidentical kinase domains, one at the N-terminus and one at the C-terminus. Phosphorylation of RSKs at Ser/Thr, which occurs at multiple sites, is required for RSK activation (4) and the N-terminal kinase domain is primarily responsible for substrate phosphorylation (5). RSKs phosphorylate many proteins, both cytosolic and nuclear (2). The many effects of RSKs on various proteins may contribute to the observations that RSKs mediate wide-ranging cellular processes, including proliferation (6–8), migration (9), and invasion (1).

Expression of RSK1 and 2 proteins, analyzed by Western blot analysis, has been previously shown to increase in prostate cancer when the cancer is localized in the primary site (8). However, whether expression of RSKs is increased in bone metastases is unknown, likely due to the lack of suitable RSK antibody for IHC analysis. Clark and colleagues (8) also showed that RSK inhibition decreases the proliferation of cancer cells, including LNCaP and PC3 prostate cancer cells and MCF-7 breast cancer cells, but not normal breast epithelial cells MCF-10A (8). These observations suggest that RSKs are involved in prostate cancer progression. Whether RSKs play a role in prostate cancer bone metastasis is unknown.
In this study, we examined the role of RSKs in prostate cancer bone metastasis. Our studies showed that expression of RSKs in prostate cancer cells increases cell survival and anchorage-independent growth in vitro and enhances prostate cancer progression in bone in vivo.

**Materials and Methods**

**Materials**

C4-2B4-LT and PC3-mm2-LT, expressing luciferase and red fluorescence protein Tomato, were generated as described previously (10, 11). The authenticity of PC3-mm2 and C4-2B4 cell lines was confirmed by fingerprinting. pGIPZ lentiviral human PTK6 shRNA was from Thermo Scientific. RSK1, pRSK(T359/S363), CKAP2, b-actin antibodies were from Santa Cruz Biotechnology. Antibodies against total RSK (RSK1/RSK2/RSK3), p38-MAPK (D13E1), phospho p38-MAPK (Thr180/Tyr182) (D3F9), SAPK/JNK (S6G8), p-SAPK/JNK (Thr183/Tyr185) (81E11) were from Cell Signaling Technology. Antibodies against PTK6 and ING3 were from Proteintech. The myrRSK plasmid was kindly provided by Dr. John Blenis (Harvard Medical School).

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded human prostate cancer specimens from primary tumor (20 cases), lymph node metastasis (19 cases), and bone metastasis (20 cases) were obtained from M.D. Anderson Cancer Center (MDACC) Prostate Cancer Tissue Bank through an institutional-approved IRB protocol. Immunohistochemistry using pRSK(T359/S363) antibodies (Santa Cruz Biotechnology) was performed using procedures described previously (11). The staining was defined as positive when >10% of the tumor cells in the specimen were immunoreactive.

**Generation of C4-2B4 cells overexpressing myrRSK**

cDNAs encoding myrRSK (12) was inserted into bicistronic retroviral vector pBMN-I-GFP. C4-2B4/RSK cells were generated from C4-2B4-LT LT cells transduced with retrovirus generated from pBMN-RSK-GFP vector and selected by FACS. C4-2B4-LT cells transduced with empty vector (C4-2B4/vector) were generated similarly.

**Western blotting analysis**

Protein concentration was determined by Coomassie Plus assay. Proteins were separated in SDS-PAGE and immunoblotted as indicated.

**Cell proliferation and soft agar colony assay**

Cell proliferation was determined by viable cell counting. The soft agar colony assay was performed as described by Giancotti and Ruoslahti (13).

**Intrabone injection, bioluminescence imaging, and μCT**

The luciferase-expressing prostate cancer cells were injected into the right femurs of male SCID mice. Tumor growth was monitored weekly using bioluminescence imaging (BLI) with an IVIS Imaging System (Xenogen). Femurs and tibias were fixed in formaldehyde, and specimens were imaged on an Explore Locus RS pre-clinical in vivo scanner (GE Medical Systems). Images were reconstructed and analyzed in MicroView (Parallax Innovations, Inc.).

**Knockdown of RSK1 or RSK2 in PC3-mm2 cells**

Lentivirus-expressing shRNAs were generated by cotransfecting RSK1 shRNA or RSK2 shRNA plasmids with pCMV-dR8.2 dvpr and pCMV-VSVG packaging plasmids into 293FT cells. PC3-mm2-LT cells transduced with pLKO or pLKO-shRSK1 vectors were selected by 5 μg/ml puromycin to generate PC3-plKO and PC3-shRSK1, respectively. RSK2 knockdown PC3-mm2 cells were generated similarly using pGIPZ-shRSK2. To generate PC3-shRSK1/2 double knockdown cells, PC3-shRSK1 cells were infected with lentivirus from pGIPZ-shRSK2 and the cells selected by FACS through GFP expressed from pGIPZ.

**Reverse transcription and quantitative PCR analysis**

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen). Quantitative real-time RT-PCR (qRT-PCR) was performed, using GAPDH as a control. The PCR primer sequences are listed in Supplementary Table S1.

**Generation of C4-2B4/RSK and C4-2B4/vector cells overexpressing ING3 and CKAP2**

cDNAs encoding human ING3 (accession number: NM_019071.2) and CKAP2 (accession number: BC136332.1) were cloned by PCR and used to generate retroviral vectors. The primer sequences used are listed in Supplementary Table S1. C4-2B4/RSK and C4-2B4/vector cells infected with retroviruses containing ING3 or CKAP2 cDNA were selected by 400 μg/mL G418.
Knockdown PTK6 in C4-2B4/RSK cells

Lentivirus-expressing PTK6 shRNAs were generated as described above and used to infect C4-2B4/RSK cells.

Results

p-RSK expression is increased in human prostate cancer specimens

To examine whether RSKs are activated during prostate cancer progression, we performed IHC staining of paraffin-embedded human prostate cancer specimens. RSK isoforms are composed of two distinct kinase domains, both of which are activated by phosphorylation. Dalby and colleagues (14) showed that one of the mechanisms for RSK activation involves ERK-mediated phosphorylation of two adjacent conserved residues (T359 and Ser363 in RSK1) in the linker region of RSK, and S363 phosphorylation directly activates the N-terminal kinase domain. We used anti-pRSK(T359/S363) antibody to immunostain human prostate cancer specimens that represent various stages of prostate cancer progression from primary prostate cancer to lymph node and bone metastasis. In primary prostate cancer, 19 of 20 primary tumors were negative with anti-pRSK(T359/S363) staining (according to 10% cutoff value; Fig. 1A). In the lymph node metastases specimens, 18 of 19 were negative (Fig. 1B). In contrast, in specimens from bone metastases, eight of 20 cases stained positive (Fig. 1C and Supplementary Fig. S1). The differences in pRSK(T359/S363) expression between bone metastasis and lymph node metastasis or primary tumors are significant ($P = 0.003$; Fig. 1D). In addition, the majority of pRSK(T359/S363) in bone metastasis specimens are localized in the nucleus (Fig. 1C, inset; Supplementary Fig. S1), consistent with the reports by Zhao and colleagues (15) and Chen and colleagues (16) that RSKs undergo translocation to the nucleus following growth factor stimulation. These observations suggest that RSKs are activated in a significant fraction of metastatic prostate cancer cells in bone and may play a role in the progression of prostate cancer in bone.

RSK expression increases anchorage-independent growth of C4-2B4 cells

To examine the role of RSK in prostate cancer bone metastasis, we expressed myrRSK, a constitutively activated RSK (12), in
C4-2B4 cells, which is an androgen-independent subline derived from LNCaP (17). To allow for in vivo BLI, myrRSK in a bicistronic retroviral vector (pBMN-myrRSK-GFP) was transduced into C4-2B4-LT cells that stably expressed luciferase and Tomato red fluorescence protein, also introduced by bicistronic vector (pBMN-Luc-Tomato). C4-2B4-LT cells expressing both luciferase and myrRSK (C4-2B4/RSK) were selected by FACS for cells expressing both GFP and Tomato (Fig. 2A, left). Control C4-2B4/vector cells were generated by transducing C4-2B4-LT with pBMN-I-GFP vector. Western blot analyses showed that overexpression of RSK in C4-2B4 cells not only increased the levels of total RSK but also the phosphorylation of RSK at T359/S363, consistent with the expression of a constitutively activated RSK (Fig. 2A, right).

Next, we examined the effects of RSK on C4-2B4 proliferation as RSKs are known to regulate cell-cycle progression through modulation of protein factors that play a role in G1–S, or G2–M transition (for review, see ref. 2). In prostate cancer, studies by Clark and colleagues (8) showed that treatment of LNCaP and PC3 with the RSK inhibitor SL0101 led to inhibition of prostate cancer cell proliferation. We found that there is no significant difference in cell proliferation between C4-2B4/RSK and the control C4-2B4/vector cells when the cells were cultured in media containing 10% (Fig. 2B) or 5% (Fig. 2C) FBS, indicating that expression of myrRSK in C4-2B4 cells does not affect their proliferation under standard culture condition. However, when these cells were cultured in media containing lower concentrations of FBS, i.e., 1.0% or 0.1%, the number of C4-2B4/RSK cells is significantly higher compared with C4-2B4/vector cells (Fig. 2C). These observations suggest that expression of myrRSK confers a survival advantage for C4-2B4 cells in low serum growth conditions.

Next, we examined whether the RSK-mediated survival can lead to increased anchorage-independent growth in soft agar. When plated in soft agar, none or very few colonies, if any, were observed in C4-2B4/vector cells (Fig. 2D), whereas a significant number of colonies were observed in C4-2B4/RSK plates (Fig. 2D). Thus, myrRSK expression confers anchorage-independent growth of C4-2B4 cells.

Overexpression of constitutively activated RSK in C4-2B4 increases C4-2B4 tumor progression in bone

One critical feature for metastatic prostate cancer cells to progress in bone is to survive in the bone microenvironment. It is possible that myrRSK-mediated anchorage-independent growth of C4-2B4 cells may enhance C4-2B4 tumor growth in bone. To examine the effect of myrRSK on C4-2B4 tumor growth in bone, the C4-2B4/vector and C4-2B4/RSK cells were injected into femurs of SCID mice, and the tumor growth in bone was monitored by bioluminescence. The bioluminescence signals were significantly higher in C4-2B4/RSK tumors compared with those in C4-2B4/vector tumors (Fig. 2E). Quantification of the bioluminescence showed that expression of myrRSK led to more...
a 10-fold increase in tumor growth compared with vector-transfected cells (Fig. 2F). Together, these results indicate that increased expression of activated RSK in C4-2B4 cells enhances their growth in bone, likely through an increase in cell survival.

Knockdown of RSK1 or RSK2 expression decreases anchorage-independent growth of PC3-mm2 cells

We further determined the effect of RSK knockdown on prostate cancer progression in bone. Four RSK isoforms, RSK1–4, have been reported in mammalian cells. Among them, RSK1 and RSK2 have been studied more extensively. Thus, we determined their roles in prostate cancer growth in bone. PC3-mm2 cells that were derived from prostate cancer bone metastasis and exhibit robust tumor growth in bone were selected for the study. Lentiviral shRNAs were used to knockdown RSK1 or RSK2, individually or together. The lentiviral vector pLKO, with a puromycin selection marker, was used to knock down RSK1 and the lentiviral vector pGIPZ, with both puromycin and GFP selection markers, was used to knock down RSK2. PC3-mm2 cells with knockdown of both RSK1 and RSK2 were generated by selecting for the expression of shRSK1 through puromycin resistance and selecting for the expression of shRSK2 through fluorescent activated cell sorting for GFP. As a result, the PC3-mm2 RSK1/2 double knockdown cells were resistant to puromycin (for shRSK1), and positive for GFP (for shRSK2) and Tomato (for luciferase; Fig. 3A). Western blotting using antibodies specific to RSK1 or RSK2 was used to confirm knockdown of RSK1 or RSK2 in these cell lines. As expected, the levels of RSK1 or RSK2 proteins were significantly reduced compared with pLKO or pGIPZ vector-transfected cells (Fig. 3B). To determine the proportion of RSK1 or RSK2 in total RSK in PC3-mm2 cells, Western blot of total RSK was performed on these RSK knockdown cells. As shown in Fig. 3B, RSK1 is the major RSK in PC3-mm2 cells as knockdown of RSK1 led to a significant reduction (78%) of total RSK, whereas knockdown of RSK2 only led to a modest (8%) decrease in total RSK. Western blotting with anti-pRSK antibodies showed that knockdown of RSK also resulted in decreases in the phosphorylation of RSK at T359/S363 (Fig. 3B).

We further examined the effect of RSK knockdown on PC3-mm2 cell survival. Similar to C4-2B4 cells, we found that knockdown of RSKs in PC3-mm2 cells had no effect on cell proliferation when the cells were grown in medium with 10% serum (Fig. 3C, left). However, knockdown of RSK1 or both RSK1/2 led to a decrease in cell survival when cultured in 1% serum condition (Fig. 3C, right). Decreases in colony formation in soft agar were also observed in PC3/shRSK1 and PC3/RSK1/2 cells compared with controls (Fig. 3D). The differences in the anchorage-independent growth of RSKs knockdown PC3-mm2 cells are not nearly as much as that observed in myrRSK-overexpressing C4-2B4 cells, suggesting that RSK1 is one of many factors that contribute to the anchorage-independent growth of PC3-mm2 cells.

Knockdown of RSK on PC3-mm2 tumor progression in bone

Next, we examined the effects of RSK knockdown on the growth of PC3-mm2 cells in bone. PC3-mm2 cells transduced with vector (pLKO, pGIPZ) or with single or double RSK1 and RSK2 knockdown were injected into mouse femurs. Tumor growth was monitored weekly by BLI, and the tumor growth at 3 weeks was
shown in Fig. 4A. Quantification of bioluminescence signals demonstrated that knockdown of RSK1 in PC3-mm2 cells led to a significant inhibition of their progression in bone, whereas RSK2 knockdown did not (Fig. 4B). Knockdown of both RSK1 and RSK2 generated a reduction similar to knockdown of RSK1 alone (Fig. 4B). These results show that RSK1 is largely responsible for RSK-mediated PC3-mm2 progression in bone, likely due to the higher levels of RSK1 in PC3-mm2 cells (Fig. 3B).

PC3-mm2 is known to induce strong osteolytic bone lesions. Thus, we also evaluated the effects of RSK1 and RSK2 on PC3-mm2–induced bone changes. Analysis of tumor-bearing femurs by X-ray showed that PC3-pLKO and PC3-pGIPZ cells induced strong osteolytic lesions (Supplementary Fig. S2). Non–tumor-bearing femurs do not show radiographic changes. Knockdown of RSK1 or both RSK1 and RSK2 showed a reduction of the osteolytic lesion compared with PC3 vector-injected femurs (Supplementary Fig. S2). Knockdown of RSK2 showed moderate reduction of the osteolytic response (Supplementary Fig. S2). To better define the effect of RSK knockdown on tumor-induced bone changes, the femurs were subjected to μCT analysis (Fig. 4C) and the bone volumes determined (Fig. 4D). When compared with PC3-pLKO or PC3-pGIPZ–bearing femurs, tumor-bearing femurs with PC3-shRSK1 or PC3-shRSK1/2 cells had higher bone volumes (Fig. 4D), likely due to the slower growth of PC3-shRSK1 or PC3-shRSK1/2 cells in bone. These observations suggest that knocking down RSK1 or RSK1/2 in PC3-mm2 cells led to a decrease in tumor growth in bone accompanied by a decrease in bone destruction. Together, both overexpression and knockdown approaches support a role of RSK in prostate cancer progression in bone.

Signal pathways regulating RSK-mediated survival in C4-2B4 cells

We then examined signal pathways that regulate RSK-mediated events. RSKs have been shown to affect many cellular functions through multiple signal pathways. As our data above indicated RSK regulated the survival of prostate cancer cells, we focus on signal pathways that may be involved in regulating RSK-mediated survival. Jin and colleagues (18) reported that RSK2 mediates survival of head and neck cancer cells by both transcription-independent, e.g., phosphorylation of p38 MAPK and SAPK/JNK, and transcription-dependent mechanisms, e.g., expression of inhibitor of growth protein 3 (ING3), cytoskeleton-associated protein-2 (CKAP2), and protein-tyrosine kinase (PTK6/Brk). Thus, we examined whether these mechanisms are also involved in RSK-mediated survival of prostate cancer cells. The effect of myrRSK expression on p38 MAPK and SAPK/JNK phosphorylation was examined by Western blot analysis. As shown in Fig. 5A, the phosphorylation of p38 MAPK and SAPK/JNK was decreased significantly in C4-2B4/RSK cells compared with C4-2B4/vector cells (Fig. 5A), suggesting that inhibition of these proapoptotic factors may be involved in RSK-mediated survival in prostate cancer cells. The effects of RSK expression on ING3, CKAP2, and PTK6 expression were also examined. As shown in Fig. 5B–D (left plots), expression of myrRSK in C4-2B4 cells led to decreases in the message levels of the tumor suppressors ING3 and CKAP2 and an increase in the message for PTK6, an intracellular nonreceptor tyrosine kinase, as determined by RT-PCR. qRT-PCR further showed that expression of myrRSK in C4-2B4 cells led to 65% and 80% decreases in ING3 and CKAP2 messages, respectively, and a 40.0-fold increase in PTK6 message (Fig. 5B–D, middle plots). Western blot showed that the changes in the protein levels correspond to the changes in the levels of messages (Fig. 5B–D, right plots).

ING3 is involved in RSK-mediated C4-2B4 cell survival

We examined whether the decrease in message levels of ING3 is involved in RSK-mediated anchorage-independent growth. ING3 is a tumor suppressor that has been shown to be involved in apoptosis and cell cycle (19). To examine whether decrease of ING3 expression is involved in the RSK-mediated prostate cancer cell survival, we overexpressed ING3 in C4-2B4/RSK cells using a bicistronic retroviral vector with a neomycin selection marker. C4-2B4/vector cells transfected with empty neomycin vector were

Figure 5. Effects of RSK on the phosphorylation of p38-MAPK and SAPK/JNK and the expression of PTK6, ING3, and CKAP2. A, RSK decreases the phosphorylation of p38-MAPK and SAPK/JNK, V, C4-2B4/vector; RSK, C4-2B4/RSK. B to D, RSK downregulates ING3, CKAP2, and upregulates PTK6 expression. Left plots, RT-PCR. Middle plots, qRT-PCR. Right plots, Western blot. "*, <0.05.
used as a control. The C4-2B4/RSK/ING3 and control cells were selected for neomycin resistance (for ING3 vector) as well as GFP positivity (for myrRSK vector). As shown in Fig. 6A, expression of ING3 in C4-2B4/RSK cells restored ING3 to a slightly higher level than that in control C4-2B4/vector cells. C4-2B4/vector cells overexpressing ING3 (C4-2B4/vector/ING3) were also generated to test the effect of ING3 on C4-2B4 cells. When these cells were cultured in medium containing 0.1% FBS for three days, C4-2B4 overexpression decreased C4-2B4/RSK cell numbers (Fig. 6E). Overexpression of CKAP2 in control C4-2B4/vector cells also decreased cell numbers (Fig. 6E), consistent with the function of CKAP2 in cell-cycle inhibition (21–23). Importantly, overexpression of CKAP2 inhibits RSK-mediated anchorage-independent growth in soft agar assay (Fig. 6F). These observations suggest that CKAP2 is also involved in the RSK-mediated prostate cancer survival.

Involvement of PTK6 in RSK-mediated C4-2B4 cell survival

PTK6, which is upregulated by RSK activation in C4-2B4 cells (Fig. 5D), has been shown to mediate a range of cellular processes related to the development or maintenance of malignancy (24, 25). Irie and colleagues (26) reported that PTK6 regulates IGF-1–induced anchorage-independent survival of mammary epithelial cells. In addition, Harvey and colleagues (27) showed that PTK6/Brk enhances breast carcinoma cell survival in suspension. These observations suggest that PTK6 may also play a role in RSK-mediated anchorage-independent growth. To test this, PTK6 in C4-2B4/RSK cells was knocked down by lentiviral vectors expressing PTK6 shRNAs or non-silencing (NS) shRNA. The C4-2B4/RSK/shPTK6 cells (clones #4 and #5) and C4-2B4/RSK/shNS control cells were selected for puromycin resistance (for shRNA vectors) as well as GFP positivity (for RSK vectors). Western blotting using antibodies specific to PTK6 confirmed that C4-2B4/RSK/shPTK6-#4 and #5 clones have significantly reduced PTK6 levels compared with shNS vector-transfected cells (Fig. 7A). In the survival assay with low serum medium, knockdown of PTK6 slightly decreased the RSK-mediated survival in C4-2B4/RSK cells after culturing for more than five days (Fig. 7B). The changes in survival in low serum medium from PTK6 knockdown are not as striking as those observed with ING3 and CKAP2 overexpression. However, knockdown of PTK6 significantly decreased anchorage-independent growth compared with C4-2B4/RSK/shNS control cells (Fig. 7C). On the basis of these observations, we propose that RSK-mediated transcriptional regulation of ING3, CKAP2, and PTK6 plays a role in RSK-mediated C4-2B4 cell survival in vitro, and possibly C4-2B4 progression in bone in vivo (Fig. 7D).

Discussion

Survival in the bone marrow microenvironment is one of the key steps for the metastatic growth of prostate cancer cells in bone. Our studies showed that RSK increases prostate cancer cell survival in vitro and progression in bone in vivo. In addition, we showed that RSK phosphorylation is increased in human prostate cancer bone metastasis specimens compared with those in primary site or lymph node metastasis. Together, our studies provide evidence that RSK is an important driver for the progression of prostate cancer in bone and suggest that RSK is a promising therapy target for prostate cancer bone metastasis.
RSKs mediate different cellular functions in a context-dependent manner (1). RSKs are known to regulate cell-cycle progression (for review, see ref. 2). Studies by Clark and colleagues (8) showed that RSK inhibition decreases the proliferation of cancer cells, including LNCaP and PC3 prostate cancer cells and MCF-7 breast cancer cells, but not normal breast epithelial cells MCF-10A (8), suggesting that cancer cells may exploit RSK-mediated pathways for proliferation.

Although Clark and colleagues (8) showed that treatment of LNCaP and PC3 cells with RSK inhibitor SL0101 led to inhibition of prostate cancer cell proliferation, we found that overexpression of myrRSK in C4-2B4 or knockdown of RSK1 in PC3-mm2 cells mainly affected anchorage-independent growth, but not cell proliferation, when the cells were cultured under standard culture condition. The differences between our studies and those of Clark and colleagues (8) may be due to the difference in cell lines, i.e., LNCaP versus C4-2B4, and the approach, i.e., inhibitor versus gene expression, used in these studies.

RSK has been shown to exhibit transcriptional regulation of gene expression. Indeed, we showed that in human prostate cancer bone metastasis specimens, phosphorylated RSK is mainly localized in the nucleus, suggesting a possible role in gene regulation. Studies by Chen and colleagues (16) showed that nuclear RSK plays a role in modulating c-Fos phosphorylation. We found that RSKs transcriptionally regulated the expression of several apoptosis-related genes, including PTK6, ING3, and CKAP2 (18), in C4-2B4 prostate cancer cell line. As PTK6, ING3, and CKAP2 are CREB target genes (18), RSK may regulate these genes through CREB phosphorylation. Interestingly, the functions of ING3 and CKAP2 are p53 dependent, and C4-2B4 has WT p53 (28). We found that knockdown of RSK1/2 in PC3-mm2 cells did not affect the expression of ING3 or CKAP2 (data not shown), likely due to the lack of functional p53 in PC3-mm2 cells (28). How RSK regulates survival of PC3-mm2 cells is not known and requires further analysis.
Our observation that RSKs play a role in prostate cancer bone metastasis suggests that RSKs have potential to be a therapy target for prostate cancer bone metastasis. As Clark and colleagues (8) showed that RSK inhibition preferentially inhibits the proliferation of both androgen-dependent LNCaP and androgen-independent PC3 prostate cancer cells, but not normal breast epithelial cells, these interesting observations also suggest that it is possible to preferentially inhibit RSK activity in cancer cells. RSK has been shown to be a druggable target. Three different classes of RSK inhibitors, SL0101 (29), FMK (30) and BI-D1870 (31), have been shown to be a druggable target. Three different classes of RSK inhibitors with improved affinity, biologic stability, and membrane permeability of SL0101 has been reported (33, 34). Thus, it is likely that a clinically applicable inhibitor for RSK will be available in the near future. Alternatively, it is possible to target downstream mediators of RSK. Further development of inhibitors of RSK activity or RSK-mediated signal pathways will have potential in the treatment of prostate cancer bone metastasis.

Metastatic prostate cancer cells in bone may affect bone homeostasis, resulting in osteoblastic or osteolytic responses. PC3 cells have been shown to generate osteolytic bone lesions. Tumor cells can modulate osteolytic program through various mechanisms. Hall and colleagues (35) showed that knockdown of DKK-1 inhibited RSK-mediated osteolytic response. In contrast, Dutta and colleagues (36) showed that integrin αvβ5 expression promotes an osteolytic program in PC3 cells by upregulating MMP2, without affecting tumor growth in bone. In this study, we found that knockdown of RSK1 in PC3-mm2 cells leads to a decrease in cell survival, resulting in a decrease of osteolytic response. Thus, therapies targeting RSK may also help in reducing tumor-associated bone lesions.

In conclusion, our studies identify a novel role of RSK in prostate cancer bone metastasis through both transcriptional and posttranslational modulation of pathways that regulate anchorage-independent growth. Further development of strategies that inhibit RSK-mediated survival in prostate cancer is warranted.

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

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.-C. Lee, C.-J. Cheng, C.-F. Wu, J.H. Song, G.E. Gallick
Writing, review, and/or revision of the manuscript: Y.-C. Lee, C.-J. Cheng, J.H. Song, G.E. Gallick, L.-Y. Yu-Lee, J. Kuang, S.-H. Lin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Yu, Y.-C. Lee, S.-H. Lin
Study supervision: S.-H. Lin

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References
7. Clark and colleagues (8). Clark and colleagues (8) showed that RSK inhibition preferentially inhibits the proliferation of both androgen-dependent LNCaP and androgen-independent PC3 prostate cancer cells, but not normal breast epithelial cells, these interesting observations also suggest that it is possible to preferentially inhibit RSK activity in cancer cells. RSK has been shown to be a druggable target. Three different classes of RSK inhibitors, SL0101 (29), FMK (30) and BI-D1870 (31), have been shown to be a druggable target. Three different classes of RSK inhibitors with improved affinity, biologic stability, and membrane permeability of SL0101 has been reported (33, 34). Thus, it is likely that a clinically applicable inhibitor for RSK will be available in the near future. Alternatively, it is possible to target downstream mediators of RSK. Further development of inhibitors of RSK activity or RSK-mediated signal pathways will have potential in the treatment of prostate cancer bone metastasis.


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