Big Mitogen-Activated Protein Kinase 1/Extracellular Signal-Regulated Kinase 5 Signaling Pathway Is Essential for Tumor-Associated Angiogenesis

Masaaki Hayashi, 1 Colleen Fears, 1 Brian Eliceiri, 2 Young Yang, 3 and Jiing-Dwan Lee 4

1 Department of Immunology, The Scripps Research Institute; 2 Cancer Biology Division, La Jolla Institute for Molecular Medicine, La Jolla, California; and 3 Johnson and Johnson Pharmaceutical Research and Development, San Diego, California

Abstract

Although big mitogen-activated protein kinase 1 (BMK1) has been shown to be critical for embryonic angiogenesis, the role of BMK1 in tumor-associated neovascularization is poorly understood. Exogenous tumors were established in BMK1+/−, BMK1floxflox , or BMK1floxflox mice carrying the Mx1-Cre transgene. Induced deletion of host BMK1 gene significantly reduced the volumes of B16F10 and LL/2 tumor xenografts in BMK1floxflox mice by 63% and 72%, respectively. Examining the tumors in these induced BMK1-knockout animals showed a significant decrease in vascular density. Localized reexpression of BMK1 in BMK1-knockout mice by administration of adenovirus encoding BMK1 restored tumor growth and angiogenesis to the levels observed in wild-type mice. These observations were further supported by in vivo Matrigel plug assays in which vascular endothelial growth factor- and basic fibroblast growth factor–induced neovascularization was impaired by removing BMK1. Through screening with the Pepchip microarray, we discovered that in BMK1-knockout endothelial cells, phosphorylation of ribosomal protein S6 (rpS6) at Ser235/236 was mostly abrogated, and this BMK1-dependent phosphorylation required the activity of p90 ribosomal S6 kinase (RSK). Immunofluorescent analysis of tumor vasculature from BMK1-knockout and control animals revealed a strong correlation between the presence of BMK1 and the phosphorylation of rpS6 in tumor-associated endothelial cells of blood vessels. As both RSK and rpS6 are known to be important for cell proliferation and survival, which are critical endothelial cell functions during neovascularization, these findings suggest that the BMK1 pathway is crucial for tumor-associated angiogenesis through its role in the regulation of the RSK-rpS6 signaling module. (Cancer Res 2005; 65(17): 7699-706)

Introduction

Four major mitogen-activated protein (MAP) kinase pathways have been identified in mammals: extracellular signal regulated kinases (ERK1/2), c-jun-NH2-kinases (JNK1-3), p38s (p38α, β, γ, and δ), and BMK1 MAP kinases. Like the other MAP kinases, the BMK1 pathway is activated by a wide variety of mitogens, including epithelial growth factor, nerve growth factor, lysophosphatidic acid, phorbol ester, and stress stimuli such as H2O2, UV irradiation, and laminar flow shear stress. In the last few years, genetically modified mice targeting these signaling pathways have been generated (1). Mice deficient in BMK1 developed cardiovascular defects, which resulted in embryonic lethality around 10.5 days postcoitum (2–5). In these mice, vasculogenesis did occur but the resulting vasculature failed to mature. Endothelial cells lining the vessels and in the developing heart displayed a disorganized, rounded morphology (2–4). Impaired angiogenesis was observed in both the embryo proper and the extraembryonic tissue including yolk sac and placenta (3–5). Although failure of heart tube going to rightward looping has been reported (3), this phenotype in conventional BMK1-knockout (BMK1-KO) mutants seems to be a secondary phenomenon of the dying embryos caused by the endothelial defect, as mice lacking the BMK1 gene specifically in cardiomyocytes developed normally whereas endothelial-specific ablation of BMK1 in animal resulted in cardiovascular defects similar to that observed in the conventional knockout mice (2). In addition to the functional importance of BMK1 during embryonic angiogenesis, BMK1 is also indispensable for the maintenance of vascular integrity in adult mice, as induced ablation of BMK1 in adult mice led to lethality after 3 to 4 weeks due to the endothelial failure. However, the apparent existence of a vascular network in mutant embryos and the relatively long period before death in adult mice after BMK1 ablation suggest that endothelial cells lacking BMK1 may still have some angiogenic potential.

Pathologic angiogenesis, such as tumor-associated angiogenesis, is distinct from physiologic angiogenesis in many aspects. The neovascularization triggered by tumors is recognized as being distinctively aberrant: disordered not only architecturally (as a maze of dilated, saccular, tortuous microvessels) but also histologically (lacking support cells such as pericytes) and hemodynamically (being leaky, with intermittent, static, and even reverse blood flow; refs. 6, 7). As the BMK1 pathway is critical for physiologic neovascularization, we examined whether this cascade plays a role in vasculature development in tumors. By inducing the deletion of host BMK1 gene in BMK1floxflox mice bearing xenograft tumors, we found that BMK1 pathway was required for tumor growth due to its essential role in the development of tumor vasculature. Pepchip microarray with total cellular proteins from BMK1-deficient endothelial cells showed that phosphorylation of rpS6 in these cells is significantly reduced. Further investigation showed that rpS6 phosphorylation was also attenuated in the BMK1-KO endothelial cells of tumor vasculature. These data suggest that the BMK1 pathway is critical for tumor-associated angiogenesis, possibly through its action on rpS6 phosphorylation in endothelial cells.

Materials and Methods

In vivo tumor growth and angiogenesis. We have previously reported a mouse model (conditional BMK1 knockout) in which ablation of the BMK1
gene can be induced by polyinosinic-polycytidylic acid (pIpC) injection (2). In this animal model, the copy number of the BMK1 floxed allele can be a factor influencing the clinical outcome. That is, BMK1<sup>flox</sup><sup>-/-</sup>, Mx1-Cre(+) mice started dying as early as 9 days after Cre induction following three pIpC injections, whereas the earliest onset of death for BMK1<sup>flox</sup><sup>+/+</sup>, Mx1-Cre(+) mice was at 19 days postinduction. These findings suggest that besides the gene copy number, the total numbers of pIpC injections may also alter the clinical outcome of BMK1<sup>flox</sup><sup>/+</sup>, Mx1-Cre(+) mice. Indeed, reducing the pIpC injection to a single dose significantly delayed the onset of death of BMK1<sup>flox</sup><sup>/+</sup>, Mx1-Cre(+) mice. In our preliminary experiment, all mice were apparently healthy until 4 weeks and died between 5 and 7 weeks after pIpC injection, whereas mice which received three pIpC injections became sick at 2 to 3 weeks. Cre expression, an indication of BMK1 deletion, was detectable in endothelial cells under the condition of a single administration of pIpC (Fig. 1A). Under this experimental condition, we sought the possible involvement of BMK1 in tumor angiogenesis. Eight- to twelve-week-old mice were first injected i.p. with 250 μg pIpC. Five days later, 1 × 10<sup>6</sup> tumor cells of B16F10 melanoma or LL/2 Lewis lung carcinoma (American Type Culture Collection, Manassas, VA) were inoculated s.c. into the right flank region of the mice. All studies were approved by The Scripps Research Institute Animal Care and Use Committee and complied with NIH guidelines. Tumor growth was monitored every other day with calipers. Tumor volume was calculated by the equation, $V = (L \times W^2) \times 0.5$ (V: volume, L: length, W: width). For immunohistochemical studies, B16F10 and LL/2 tumors were resected from mice 14 and 16 days after tumor implantation, respectively.

In vivo matrigel angiogenesis assay. Matrigel (Growth Factor Reduced, BD Biosciences, San Jose, CA) at 4°C was mixed with or without 100 ng/mL vascular endothelial growth factor (VEGF; PeproTech, Rocky Hill, NJ) or 400 ng/mL basic fibroblast growth factor (bFGF; PeproTech) in a final volume of 0.5 mL. The Matrigel mixture was then injected s.c. into the right flank region of pIpC-induced BMK1<sup>flox</sup><sup>/+</sup> mice with or without the Mx1-Cre transgene 5 days after pIpC injection. The plugs were removed 7 days after Matrigel implantation, fixed in Bouin’s solution for 24 hours, and then embedded in paraffin. All tissues were sectioned, mounted onto slides, and stained with Masson’s trichrome. In another group, plugs were homogenized in Drabkin’s reagent (Sigma-Aldrich, St. Louis, MO). After centrifugation, the supernatants were filtered through 0.22-μm filters and the hemoglobin content was measured at the wavelength of 540 nm following the instructions of the manufacturer.

**Computer-assisted morphometric analysis of vasculature.** For the analysis of tumor vessels, representatives sections obtained from five tumors from each cell clone were photographed with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI), and morphometric analyses were done using Scion Image software (Frederick, MD). Three random fields were examined at ×10 magnification, and the number of vessels per square millimeter and average vessel size were determined. For the analysis of angiogenesis in Matrigel implants, sections from three plugs per group were examined and the total number of pixels of microvessels from five 10× fields was calculated and averaged.

**Statistics.** Tumor volume and the area and size of CD31-positive vessels were compared by using two-sided unpaired t test. Differences were considered statistically significant at $P < 0.05$.

**Cell culture, chemicals, and plasmid.** The mouse lung capillary endothelial cells (MLCEC) and mouse adult fibroblasts (MAF) were generated from BMK1<sup>flox</sup><sup>/+</sup> mice carrying SV40-T transgene and maintained as previously described (2). Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex BioScience (Rockland, ME) and were cultured using EGM-2 BulletKit (Cambrex BioScience). The plasmid encoding dominant-negative RSK1 (K112/464R) was a gift from Dr. Blenis, Department of Cell Biology, Harvard Medical School, Boston, MA.

**PepChip kinase screening.** PepChip kinase, a microarray containing 1,152 synthetic kinase substrate peptides, was purchased from Pepscreen systems (Lelystad, Netherlands). MLCECs and MAFs were infected with either empty adenovirus (Ad-EV) or recombinant adenovirus encoding Cre recombinase (Ad-Cre) for 12 hours in six-well plates (1 × 10<sup>5</sup> particles per cell). Two days after infection, cells were lysed in 25 mmol/L HEPES (pH 7.6), 50 mmol/L NaCl, 0.1 mmol/L EDTA, 0.5% Triton X-100, 0.1 mmol/L sodium orthovanadate, and 1 mmol/L phenylmethylsulfonyl fluoride, and the cleared supernatants were used for the screening according to the instructions of the manufacturer. The microarrays were scanned by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) with a resolution of 50 μm. Evaluation of the data was done using ImageQuant software. Normalization of signal intensity of each peptide was done according to the instructions of the manufacturer.

**Antibodies.** The antibodies against the phosphorylated forms of rpS6 (Ser235/236 and Ser240/244), p70S6K (Thr389), p90RSK (Thr359/Ser363), Akt (Ser473), MAP/ERK Kinase (MEK)1/2 (Ser217/221), ERK1/2 (Thr202/ Tyr204), JNK (Thr183/Tyr185), and p38 (Thr180/Tyr182) in conjunction with the antibodies against these molecules, regardless of their phosphorylation status.

**Results.** The expression of Cre recombinase after pIpC induction was detected along the aortic inner wall by immunohistochemical staining using an anti-Cre antibody. A, representative image showing the expression of xenograft tumors. A, time schedule of tumor xenograft and immunohistochemical analysis of the Cre expression in aorta. Mice carrying the Mx1-Cre transgene with the genotypes of BMK1<sup>+/+</sup> (WT), BMK1<sup>flox</sup><sup>++</sup> (BMK1-HT), and BMK1<sup>flox</sup><sup>-/-</sup> (BMK1-KO) were injected i.p. with 250 μg pIpC to induce deletion of the floxed BMK1 gene, followed by the implantation of B16F10 melanoma or LL/2 Lewis lung carcinoma cells 5 days after pIpC injection. Tumor volume was measured every other day. The expression of Cre recombinase after pIpC induction was detected along the aortic inner wall by immunohistochemical staining using an anti-Cre antibody. B, representative image showing the growth of xenograft LL2 tumors at the time of sacrifice. B16F10 (C) or LL/2 tumors (D) grown in BMK1-WT, BMK1-HT, and BMK1-KO mice were measured at the indicated day after initial B16F10 or LL/2 cell injection. Points, mean (n = 8 in each group); bars, SD. **, $P < 0.01$, compared with WT and heterozygous mice.
status, were purchased from Cell Signaling (Beverly, MA). The anti-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal anti-HA and anti-FLAG antibodies were purchased from Covance (12CA5; Princeton, NJ) and Stratagene (M2; La Jolla, CA), respectively. The anti-BMK1 antibody was previously described (9).

Topical administration of adenovirus. Recombinant adenovirus encoding BMK1 (Ad-BMK1) or βGal (Ad-βGal) was injected separately into the skin site of tumor implants of plpC-induced BMK1flox/flox, Mx1-Cre(+) and BMK1flox/flox mice once a week (1 x 10¹⁰ viral particles/100 µL) for a total of three administrations.

Results

The BMK1 pathway in host BMK1-floxed mice is essential for the growth of B16F10 melanoma and LL/2 Lewis lung tumor xenografts. BMK1+/+, BMK1flox/+ and BMK1flox/flox mice [hereinafter referred to as wild-type (WT), BMK1-heterozygous (BMK1-HT), and BMK1-knockout (BMK1-KO), respectively] carrying the Mx1-Cre transgene were injected with plpC followed by inoculation of B16F10 melanoma cells or LL/2 Lewis lung carcinoma cells using the timeline denoted in Fig. 1A. The tumors which developed in BMK1-KO mice were significantly smaller than those in WT and BMK1-HT mice (Fig. 1B). The reduced tumor growth in BMK1-KO mice was observed from 6 to 8 days after implantation to the day tumor excision was done (Fig. 1C and D). At excision, the mean B16F10 tumor size was 63% and 18% smaller in BMK1-KO and BMK1-HT mice, respectively, than that in WT mice (Fig. 1C, and the mean LL/2 tumor size was 72% and 22% smaller in BMK1-KO and BMK1-HT mice, respectively, than that in WT mice by 16 days (Fig. 1D). Because only the host BMK1 gene, but not the tumor BMK1 gene, was deactivated by plpC treatment, the retarded tumor development observed in BMK1-HT and BMK1-KO mice indicated that the host BMK1 pathway is critical for supporting the growth of tumor xenografts.

The vascular development within B16F10 and LL/2 tumors was significantly suppressed in BMK1 knockout mice. As the BMK1 pathway is involved in embryonic angiogenesis, we wondered whether this pathway is also involved in development of tumor vasculature, which may account for the reduced tumor growth observed in BMK1-KO mice. We compared vessel density and vessel morphology between the tumor sections from WT and BMK1-KO mice by immunohistochemical staining with an antibody against CD31, an endothelial cell marker. The vascularization in tumors from BMK1-KO mice (Fig. 2C and D) was significantly reduced compared with tumors from WT mice (Fig. 2A and B). Quantification of the vascularization by counting the CD31-positive vessels revealed that the vascular density in the B16F10 melanoma and LL/2 tumors of BMK1-KO mice was decreased (Fig. 2F). Within the tumors, the overall number of vessels, regardless of their size, was lower in BMK1-KO mice, but more significantly, the number of larger vessels was greatly diminished in these mice (Fig. 2F). Thus, retarded tumor growth in BMK1-KO animals correlated with a decreased number and with significantly smaller blood vessels within these tumors.

Vascular endothelial growth factor– and basic fibroblast growth factor–induced angiogenesis in Matrigel plugs was abrogated in BMK1 knockout mice. VEGF and bFGF are angiogenic growth factors secreted by a number of different types of cancers including melanoma, breast, pancreas, and lung carcinoma (10–13). Because we showed that the BMK1 pathway is critical for neoangiogenesis associated with lung carcinoma and melanoma (Fig. 2), we wondered whether BMK1 has a role in VEGF- and bFGF-induced angiogenesis. To investigate this, plpC-induced BMK1flox/flox mice with or without the Mx1-Cre transgene (hereafter referred to as BMK1-KO and control mice, respectively) were implanted with Matrigel 5 days after plpC injection. As shown in Fig. 3, few host endothelial cells migrated into the Matrigel implants in the absence of growth factors, regardless of their genotype. However, in the presence of either VEGF or bFGF, endothelial cells of control mice extensively migrated into the Matrigel implants and generated a vessel network as judged by the containment of erythrocytes. The aggressive angiogenesis resulted in absorption of the Matrigel plugs and led to partial destruction of the implants (Fig. 3A and B). In contrast, in BMK1-KO mice, although endothelial cells migrated into the Matrigel implants, they stayed at the periphery and failed to form functional vessels. Quantification of the blood vessels in the Matrigel implants revealed a significant drop in BMK1-KO mice compared with those in control littermates (Fig. 3C and E). Functional vessel formation within the implants was estimated by measuring the hemoglobin content within the excised implants. We found 82% and 87% reductions of hemoglobin content in BMK1-KO mice compared with that in control mice with plugs containing VEGF and bFGF, respectively (Fig. 3D and F). These results indicated that the BMK1 cascade was critically required for both VEGF- and bFGF-induced angiogenesis.
BMK1 is critical for the Ser235/236 phosphorylation of rpS6 in endothelial cells, but not in fibroblasts. We previously showed that BMK1 was critical for angiogenesis through the role of BMK1 in endothelial cells. Therefore, studying the molecular actions of BMK1 in endothelial cells should help us understand the BMK1-dependent regulatory mechanism(s) for tumor-associated neovascularization. To decipher the molecular role of BMK1 in endothelial cell, we established two cell lines from BMK1<sup>flox/flox</sup> mice derived from either MLCECs or from MAFs (2). These two cell lines were infected with recombinant adenovirus encoding Cre recombinase (Ad-Cre) to remove the BMK1 gene or infected with empty virus (Ad-EV) as a control.

Protein extracts from these treated cells were separately used in in vitro kinase reactions on a Pepchip microarray for analyzing the phosphorylation profiles of kinase substrates in these cells with or without the presence of BMK1 protein. As the MLCEC and MAFs were isolated from BMK1<sup>flox/flox</sup> mice carrying a temperature-sensitive SV40 large T-antigen, it was possible that the presence of SV40 T-antigen may affect the experimental outcome. To eliminate this possibility, protein extracts from MLCECs or MAFs grown at 37°C, a nonpermissive condition for SV40 T-antigen, were used in these experiments. Among 1,152 synthetic peptides on the microarray coding for known phosphoacceptor sites of natural proteins, we found that peptides containing the Ser235/236 of rpS6 showed considerable reduction in phosphorylation in BMK1-depleted MLCECs, but not in control MLCECs (Table 1; Fig. 4A). Moreover, peptides that encode serine residues other than Ser235/236 of rpS6 did not show altered phosphorylation after BMK1 ablation in MLCECs (Table 1). The level of Ser235/236 phosphorylation of rpS6 between BMK1-depleted MAFs and control MAFs, however, was about the same. These results were further confirmed by immunoblot analysis using an antibody against phosphorylated Ser235/236 of rpS6 (Fig. 4B). As shown in Fig. 4B, the level of rpS6 phosphorylation was significantly reduced in BMK1-deleted MLCECs grown at 37°C compared with that in control MLCECs. Interestingly, activation of the Raf-MEK1/2-ERK1/2 MAP kinase pathway was observed in MLCECs without BMK1, suggesting that this increased activity of ERK1/2 was not enough to compensate for the abrogated rpS6 phosphorylation induced by BMK1 deficiency (Fig. 4B). Moreover, introduction of exogenous BMK1 in BMK1-depleted MLCECs by infecting these MLCECs with recombinant adenovirus encoding BMK1 restored the phosphorylation of rpS6 to the level of control MLCECs (Fig. 4C). These results suggest that BMK1 is critical for rpS6 phosphorylation at Ser235/236 in endothelial cells. As rpS6 is a ribosomal protein in the translational apparatus, and phosphorylation of rpS6 is known to stimulate or facilitate the activation of protein synthesis, it is very likely that the BMK1 pathway regulates endothelial cell function by controlling the translational apparatus in these cells.

![Figure 3](https://example.com/f3.png)

**Figure 3.** Inhibition of VEGF- and bFGF-induced angiogenesis by BMK1 ablation in vivo. Cross sections of matrigel plugs stained with Masson’s trichrome. BMK1<sup>flox/flox</sup> mice with or without the Mx1-Cre transgene were injected i.p. with 250 μg pIpC. Five days later, 500 μL Matrigels were mixed with or without VEGF (final concentration, 100 ng/mL; A) or bFGF (final concentration 400 ng/mL; B), implanted s.c. and then harvested 7 days after implantation. Bar, 10 μm. C and E, quantification of vascular density in cross sections of Matrigel plugs with or without VEGF and bFGF, respectively. D and F, assessment of hemoglobin content in Matrigel plugs with or without VEGF and bFGF, respectively. Columns, mean (n = 3 in each group); bars, SD. **, P < 0.01, compared with VEGF- or bFGF-containing plugs implanted in BMK1<sup>flox/flox</sup>, Mx1-Cre(−) mice.
Table 1. Signal intensity of rpS6 peptide in the Pepchip screening

<table>
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<tr>
<th>Peptide</th>
<th>MLCEC Cre(−)</th>
<th>MLCEC Cre(+)</th>
<th>MAF Cre(−)</th>
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<td></td>
<td>18,966</td>
<td>4,004</td>
<td>11,349</td>
<td>9,897</td>
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NOTE: The total cell lysates from treated MLCECs or MAFs were prepared and used in the in vitro kinase reaction on Pepchip as described in Fig. 4. Autoradiograms of these chips were obtained using PhosphorImager, and then the signal intensity of each peptide was read and quantified using the ImageQuant software as described in Materials and Methods. The signal intensities of seven peptides with sequences identical to the indicated regions of rpS6 are shown.

BMK1-p90 ribosomal S6 kinase signaling pathway regulates the basic fibroblast growth factor–mediated rpS6 phosphorylation at Ser235/236 in human umbilical vein endothelial cells. As the Ser235/236 phosphorylation sites of rpS6 are not proline-directed residues, it is likely that BMK1 regulates these sites through another kinase. Two kinases, p70/85 ribosomal S6 kinase (S6K) and p90 ribosomal S6 kinase (RSK), have been shown to directly phosphorylate rpS6 (14, 15). To investigate the role of the BMK1 pathway in regulating these two rpS6-upstream kinases during bFGF-mediated endothelial cell activation, we examined the effect of BMK1 on the phosphorylation of rpS6 at Ser235/236 during bFGF-induced activation of HUVECs (9). The bFGF-induced activation of BMK1, along with activation of RSK, and the phosphorylation of rpS6 at Ser235/236 were blocked by expression of MEK5A. In contrast, the bFGF-mediated up-regulation of S6K activity and the phosphorylation of rpS6 at Ser240/244 were not affected by the presence of MEK5A (Fig. 5A).

These results suggested that the BMK1 pathway is required for the activation of RSK and the subsequent phosphorylation of Ser235/236 of rpS6 during activation of endothelial cells by bFGF. This notion was further supported by data showing that specific activation of the BMK1 pathway in endothelial cells using the dominant active form of MEK5, MEK5D, stimulated RSK activity and the phosphorylation of rpS6 at Ser235/236 (Fig. 5B). The requirement of RSK activity in BMK1-induced rpS6 phosphorylation at Ser235/236 was shown by blocking BMK1-dependent rpS6 phosphorylation using a dominant-negative form of RSK in endothelial cells (Fig. 5C). Together, these data implied that the BMK1-RSK signaling module is critical for the phosphorylation of rpS6 at Ser235/236 during bFGF-induced activation of HUVECs.

The Ser235/236 phosphorylation of rpS6 was reduced in tumor vessels in BMK1 knockout mice. To examine the phosphorylation state of rpS6 at Ser235/236 during tumor angiogenesis, sections of LL/2 tumors harvested from WT and
BMK1-KO mice were coimmunostained with an antibody against CD31 (an endothelial cell surface marker) and an antibody against phosphorylated Ser235/236 of rpS6. Based on the CD31 staining, blood vessels in tumors from BMK1-KO mice were significantly fewer in number and noticeably smaller in size than the tumor vasculature found in WT mice (Fig. 6A and B). Phosphorylation of Ser235/236 was observed in endothelial cells of tumor vessels in WT mice, but was barely detectable in endothelial cells of tumor vasculature in BMK1-KO tumor (Fig. 6C–F). As the BMK1 gene in the implanted cancer cells is not floxed and thus cannot be deleted by Cre activity induced by plpC (2), it was not surprising to see that phosphorylation of Ser235/236 of rpS6 was similar in tumor cells surrounding the tumor vasculature of both WT and BMK1-KO mice (Fig. 6C and D). These results showed that the reduced phosphorylation of rpS6 at Ser235/236 site coincided with the ablation of BMK1 gene in endothelial cells of tumor vasculature, which implied that the BMK1 pathway regulates tumor angiogenesis, at least in part, through phosphorylation of rpS6 at Ser235/236.

Reexpressing BMK1 in BMK1 knockout mouse restored tumor growth, angiogenesis, and phosphorylation of rpS6. To further strengthen the notion that retarded tumor growth in BMK1-KO mice is due to the defect in neovascularization of the tumors, but not due to the other defects caused by systemic deletion of BMK1 in these mice, we topicaly injected recombinant adenovirus encoding BMK1 (Ad-BMK1) or βGal (Ad-βGal) into the skin site of tumor implants of plpC-induced BMK1lox/lox, Mx1-Cre(+), and BMK1−/−, Mx1-Cre(+) mice. The tumors in Ad-BMK1–infected KO mice were significantly larger than those grown in Ad-βGal–infected KO mice, whereas tumors grown in Ad-BMK1–infected WT mice were similar to those grown in Ad-βGal–infected WT mice (Fig. 7A and B). Tumor vasculature in Ad-BMK1–infected KO mice was better developed than that in Ad-βGal–infected KO mice, whereas the development of tumor vasculature in Ad-BMK1–infected WT mice was comparable to that in Ad-βGal–infected WT mice (Fig. 7C). In addition, immunofluorescent analysis of a cross section of the tumor from Ad-BMK1–infected KO mice revealed that the phosphorylation of rpS6, compared with that in Ad-βGal–infected mice, was restored (Fig. 7C). These findings strengthen the notion that the BMK1 signaling pathway is critical for tumor-associated angiogenesis through regulation of rpS6 phosphorylation in endothelial cells.

**Discussion**

It has been shown that the BMK1 signaling pathway plays a key role in supporting the uncontrolled growth of certain human carcinoma by mediating oncogenic signals from deregulated ErbB or Ras and in providing survival signals against chemotherapy to human tumor cells as well (16–18). Moreover, the poor prognosis and bony metastasis of human prostate cancer has been attributed to up-regulation of BMK1 activity (19). However, the role of BMK1 in carcinogenesis is not limited to its contribution to the malignant nature of tumor cells. In this study, we showed that deletion of the host BMK1 gene substantially inhibited the development of tumor vasculature and consequently blocked the growth of exogenous tumors in two tumor xenograft models, indicating that the BMK1 pathway plays a major role in the neovascularization associated with tumors.

Within four mammalian MAP kinase pathways (ERK1/2, JNKs, p38s, and BMK1) identified thus far, two of them, ERK1/2 and BMK1, mainly mediate intracellular signaling from growth factors, and the other two pathways, JNKs and p38s, are largely involved in cellular responses induced by stress stimuli. Deregulation of both MEK1/2-ERK1/2 and MEK5-BMK1 pathways has been strongly implicated in tumorigenesis. In fact, administration of MEK1/2 inhibitors to tumor-bearing mice not only delays tumor growth in tumor xenograft mouse models (20) but also inhibits the metastasis of the cancer (21). In light of these promising results, U0126 and PD184352 are currently in phase I and phase II clinical trials, respectively, for cancer treatment (22). However, recent studies have reported that U0126 and PD184352 can also inhibit MEK5, raising the possibility that
BMK1 Is Required for Tumor-Associated Angiogenesis

Figure 6. Ser235/236 phosphorylation of rpS6 was reduced by BMK1 removal in endothelial cells of tumor vasculature. Fluorescent microscopic images of LL/2 tumor sections from WT (A, C, E, G, and I) and BMK1-KO (B, D, F, H and J) mice were stained with anti-CD31 antibody (red; A and B), anti-Ser235/236 phosphorylated rpS6 antibody (green; C and D), or with 4',6-diamidino-2-phenylindole (blue; E and F). G and H, merged images; I and J, higher magnifications of these images, respectively. Original magnification, ×200.

Figure 7. Reexpressing BMK1 in BMK1-KO mice in the local environment restored tumor growth, angiogenesis, and the phosphorylation of rpS6. Reexpressing BMK1 by the injection of recombinant adenovirus encoding BMK1 (Ad-BMK1) into the skin site of tumor implants in BMK1-KO mice restored the tumor growth of B16F10 melanoma cells (A) and LL/2 Lewis lung carcinoma cells (B). Points, mean (n = 3 in each group); bars, SD. The tumors in Ad-BMK1–infected KO mice were significantly larger than those grown in Ad-β-galactosidase (Ad-β-gal) or BMK1-KO (Ad-BMK1). These sections were stained with anti-CD31 antibody (red, left) or with anti-Ser235/236 phosphorylated rpS6 antibody (green, middle) as in Fig. 6. Right, merged images (yellow). Original magnification, ×200.

and a known physiologic rpS6 kinase during Xenopus (32) and mouse (33) meiotic maturation, RSK has been recognized as the kinase thought to be responsible for the rpS6 phosphorylation at Ser235/236 in the absence of S6K1 and S6K2 (31). This notion has been reinforced by our finding that blocking the activation of RSK significantly reduced the BMK1-mediated phosphorylation of rpS6 at Ser235/236 in endothelial cells.

The functional importance of rpS6 in animals is underscored by the conditional ablation of rpS6 in hepatocytes (34). In these mice, hepatocytes failed to proliferate after partial hepatectomy due to the blockage in ribosome biogenesis and cell cycle progression. Because there is no report on mice with endothelial cell–specific knockout of rpS6, it is unclear whether rpS6 is also indispensable for the growth and proliferation of endothelial cell, which are critical for neovascularization. However, vasculature in tumors from WT mice contains large functional vessels with strong endothelial cell staining of phosphorylated rpS6, whereas the blood vessels in tumors from BMK1-KO mice were poorly formed and

attenuation of MEK5-BMK1 signaling pathway may also contribute to the antitumor effect of these compounds because the BMK1 pathway is required not only for the uncontrolled proliferation of tumor cells but also for the generation of new blood vessels within tumors, as shown herein.

Activation of protein synthesis by growth factors is an essential step for cell cycle progression, and its inhibition causes cell growth arrest in G0-G1 (23, 24). rpS6, one of 30 ribosomal proteins, has the capability of cells (25). Phosphorylation of rpS6 at multiple sites is a critical for neovascularization. However, vasculature in tumors from WT mice contains large functional vessels with strong endothelial cell staining of phosphorylated rpS6, whereas the blood vessels in tumors from BMK1-KO mice were poorly formed and
were characterized by a lower level of rpS6 phosphorylation in endothelial cells. Thus, there is a positive correlation between the phosphorylation of rpS6 at Ser235/236 in the endothelial cells of tumor vessels and the number and size of tumor vasculature, supporting the notion that phosphorylation of rpS6 by the BMK1 signaling pathway in endothelial cells is important for their angiogenic potential in tumor-associated angiogenesis. A considerable number of drugs/antibodies have been developed in an attempt to block tumor angiogenesis. Most of them are focused on extracellular molecules, such as VEGF, FGF, and endothelial-specific receptor tyrosine kinases (35). Drugs designed to inhibit the intracellular signaling pathways, which are critical for endothelial cell growth and survival, are considerably less, possibly because these pathways play critical roles not only in endothelial cells but also in other cell types. It is worth noting that mice lacking BMK1 specifically in several different cell types, such as cardiomyocytes, neuron, hepatocytes, and mammary epithelial cells, all developed normally without any overt adverse phenotype and have the same life span as their control littermates (36). The only cell type, thus far, affected by the loss of BMK1 is endothelial cells. Importantly, we showed herein that the BMK1 pathway is critically involved in the development of tumor vasculature, mostly likely through its role in regulating the RSK-rpS6 pathway in endothelial cells. Therefore, a pharmacologic inhibitor targeting the BMK1 pathway in vivo should affect primarily endothelial cells especially during generation of tumor vasculature, with minimal effect on the normal function of many other cell types.

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Masaaki Hayashi, Colleen Fearns, Brian Eliceiri, et al.


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