SKAP2 Promotes Podosome Formation to Facilitate Tumor-Associated Macrophage Infiltration and Metastatic Progression

Masamitsu Tanaka1, Shintaro Shimamura1,2, Sei Kuriyama1, Daichi Maeda3, Akiteru Goto3, and Namiko Aiba1

Abstract

Tumor-associated macrophages (TAM) play complex and pivotal roles during cancer progression. A subset of metastasis-associated macrophages accumulates within metastatic sites to promote the invasion and growth of tumor cells. Src kinase-associated phosphoprotein 2 (SKAP2), a substrate of Src family kinases, is highly expressed in macrophages from various tumors, but its contribution to the tumor-promoting behavior of TAMs is unknown. Here, we report that SKAP2 regulates podosome formation in macrophages to promote tumor invasion and metastasis. SKAP2 physically interacted with Wiskott-Aldrich syndrome protein (WASP) and localized to podosomes, which were rarely observed in SKAP2-null macrophages. The invasion of peritoneal macrophages derived from SKAP2-null mice was significantly reduced compared with wild-type macrophages, but could be rescued by the restoration of functional SKAP2 containing an intact tyrosine phosphorylation site and the ability to interact with WASP. Furthermore, SKAP2-null mice inoculated with lung cancer cells exhibited markedly decreased lung metastases characterized by reduced macrophage infiltration compared with wild-type mice. Moreover, intravenously injected SKAP2-null macrophages failed to efficiently infiltrate established tumors and promote their growth. Taken together, these findings reveal a novel mechanism by which macrophages assemble the appropriate motile machinery to infiltrate tumors and promote disease progression, and implicate SKAP2 as an attractive candidate for therapeutically targeting TAMs. Cancer Res; 76(2); 358–69. ©2015 AACR.

Introduction

Macrophage density correlates with poor prognosis in various cancers. Tumor-associated macrophages (TAM) originate from circulating monocytes that are recruited to the tumor site and programmed by tumor-derived factors including macrophage colony stimulating factor (M-CSF), IL4, and IL10 (1, 2). These cytokines switch cells to tumor-supportive M2-polarized macrophages. Tumor-supporting functions of TAMs include stimulation of tumor cell growth and the creation of favorable conditions for tumor cell invasion and metastatic spread (3–4). Activated M2 macrophages polarized with IL4 are more invasive in three-dimensional (3D) environments, while classically activated M1 macrophages polarized with TNFα are less motile (5). However, molecular mechanisms that regulate tumor infiltration by macrophages are not well understood.

Macrophages form podosomes, which are involved in cell adhesion and extracellular matrix (ECM) degradation (6–8). Podosomes are comprised of individual conical F-actin bundles perpendicular to the cell surface surrounded by a ring containing cell adhesion proteins linked to actin (6, 9). Podosomes possess a proteolytic activity that can be observed when macrophages are layered on coverslips coated with a fluorescent matrix protein. Furthermore, transformed cells form podosome-like structures called invadopodia. Therefore, podosomes are thought to be invasive structures.

Src kinase–associated phosphoprotein 2 (SKAP2) is a substrate of Src family kinases (SFK) and is highly homologous to SKAP55 (SKAP1; refs. 10, 11). SKAP2 is phosphorylated on tyrosine of Src family kinases (SFK) and is highly homologous to SKAP55 (SKAP1; refs. 10, 11). SKAP2 is phosphorylated on tyrosine residues by SFKs including Fyn, Lyn, and Hck (10), and we previously identified SKAP2 as one of the major tyrosine-phosphorylated proteins in glioblastoma (12). SKAP1 is expressed almost exclusively in T cells, whereas SKAP2 is expressed widely in lymphoid and myeloid cells (10, 11, 13). Both SKAP1 and SKAP2 have a pleckstrin homology (PH) domain and a Src homology 3 (SH3) domain (14). In addition, SKAP2, but not SKAP1, has a coiled-coil domain at its N-terminus for self-dimerization (15). SKAP1 and SKAP2 have been suggested to be involved in cell adhesion by immune cells through their association with integrin and filamentous actin (F-actin; refs. 16–19). However, SKAP2 cannot substitute for SKAP1 in regulating integrin-mediated adhesion of T cells; instead, SKAP2 is required for B-cell adhesion to fibronectin, suggesting that they are not redundant (19–21). SKAP2 is recruited to engaged integrins together with SFKs, Adap, and Sirps, which is required for integrin-dependent cytoskeletal rearrangements, such as actin ruffle formation, leading to migration and chemotaxis of macrophages (22).
The current study examined the significance of SKAP2 in macrophages during cancer progression. SKAP2 is tyrosine phosphorylated in the presence of cancer cells. SKAP2 was required for podosome formation and invasiveness in macrophages, an effect that was dependent on SKAP2 tyrosine phosphorylation and interaction with WASP, an actin nucleation–promoting factor (23). SKAP2 physically interacted with WASP, and recruitment of SKAP2 to podosomes together with WASP played an essential role in macrophage invasion. Consequently, SKAP2-dependent macrophage invasion promoted infiltration of macrophages into tumors and further enhanced tumor growth and metastatic spread. These results suggest that SKAP2 may become a useful target for inhibiting TAM-mediated cancer progression.

Materials and Methods

Cell lines and preparation of peritoneal macrophages

RAW264.7 macrophage cell line and MDA-MB-231 human breast cancer cell line were obtained from the ATCC cell bank, and Lewis lung carcinoma (LLC) lung cancer cell line and B16BL6 melanoma cell line, established from C57BL/6 mice, were obtained from RIKEN BRC cell bank. 58As9 cell line was derived from a patient with scirrhous gastric carcinoma and was provided by the group that established it (24). All these cell lines were cultured in DMEM containing 10% FBS. To collect mouse peritoneal macrophages (5 × 10⁶ cells) from C57BL/6 mice by lavage 4 days after intraperitoneal injection of cytokines, peritoneal macrophages were treated with IL4 and M-CSF (20 ng/mL, each) for 2 days, then used in experiments.

Mice

SKAP2−/− mice were obtained from Mutant Mouse Regional Resource Centers (B6.129S-Skag2tm1Eac/J, ref. 19). These mice were bred under specific pathogen-free conditions at the Animal Research Laboratory Bioscience Education Research Center of Akita University (Akita, Japan). All animal experimental protocols were approved by the Committee for Ethics of Animal Experimentation, and the experiments were conducted in accordance with the guidelines for animal experiments at Akita University (Akita, Japan). Dissected lungs from ten mice per group were fixed, sliced into 200 μm sections using a LinearSlicer, and then observed under a confocal microscope.

Specimens from cancer patients

Gastric cancer and lung adenocarcinoma specimens were obtained from patients who had undergone resection of primary gastric tumors or pneumonectomy or lobectomy. None of the patients had undergone preoperative radiation or chemotherapy. All samples were collected from the surgical pathology files at Akita University Hospital (Akita, Japan) between 2008 and 2014 (gastric cancer) or 2005 and 2011 (lung cancer), and tissues were obtained with the informed consent of the patients.

Statistical analysis

Statistical significance was calculated using Student t test. P values < 0.05 were considered statistically significant. All other methods are available in Supplementary Materials and Methods.

Results

SKAP2 is highly expressed in cancer stromal macrophages and is tyrosine phosphorylated upon contact with cancer cells

The expression of SKAP2 and its homolog SKAP1 was examined in mouse peritoneal macrophages and the RAW264.7 macrophage by RT-PCR. Peritoneal macrophages obtained from C57BL/6 mice and RAW264.7 cells expressed SKAP2 at a high level. On the other hand, these cells did not express detectable level of SKAP1, as expected (Fig. 1A). Therefore, SKAP2 is predominantly expressed in these macrophages. We further confirmed that almost 100% of isolated peritoneal macrophages expressed SKAP2 and the pan-macrophage marker F4/80 by immunofluorescence (Fig. 1B and C).

The expression of SKAP2 was next examined in macrophages within human cancer tissues. Human gastric cancer tissues were immunologically stained with anti-SKAP2 antibody. Staining of SKAP2 was rarely detected in cancer cells (1/28 cases), but it was detected at various levels in stromal cells infiltrating cancer tissues in all cases (28/28 cases; Fig. 1D, top). When samples containing dense SKAP2-positive stromal cells were further analyzed by coimmunostaining with antibodies specific for SKAP2, CD206, and F4/80 (Fig. 1D, bottom), SKAP2 expression by CD206− and CD206+ macrophages was similar (72% and 68%, respectively; Table 1).

Next, changes in SKAP2 status in macrophages were examined upon coculture with cancer cells. Phosphorylation of SKAP2 in macrophages increased upon coculture with all cancer cell lines examined (Fig. 1E). Moreover, increased SKAP2 expression and tyrosine phosphorylation were detected upon exposure to conditioned medium from MDA-MB-231 cells (Fig. 1F). Taken together, these data suggest that SKAP2 expression is not elevated by M2 polarization of macrophages at least in cases of gastric cancer. However, coculture experiments suggest that tyrosine phosphorylation of SKAP2 increases upon contact with various cancer cells.

To determine the function of SKAP2 in macrophages during tumor progression, SKAP2 knockout mice were prepared. The total number of macrophages in SKAP2−/− mice was not largely altered in these mice (Supplementary Fig. S1A). Cytokines
Table 1. Summary of immunohistochemical analysis of gastric cancer specimens

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<th>Marker Combination</th>
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*Immunofluorescence staining was performed in four selected cases that contained highest number of SKAP2-positive stromal cells. The number of positive cells for each marker is summarized in the bottom.
Figure 2.
SKAP2 is necessary for podosome formation in macrophages. A–C, peritoneal macrophages (PM) from wild-type mice (A and B) or SKAP2−/− mice (C) were immunofluorescently stained for SKAP2 and paxillin. B, the fluorescence intensity was measured along the arrow and plotted. D, percentage of cells containing podosomes (n = 100). E and F, wild-type (E) or SKAP2−/− (F) peritoneal macrophages were immunostained for WASP and F-actin. G, immunofluorescence analysis of peritoneal macrophages on gelatin-coated plates (red, SKAP2; magenta, F-actin). H, percentage of cells causing degradation of the gelatin matrix (n = 100). D and H, *, P < 0.05, Student t test. Results from three independent determinations are shown as means ± SD.

Scale bar, 5 μm.
Figure 3.
SKAP2 interacts with WASP and alters its localization. A, RAW264.7 cells stably expressing EGFP-tagged WASP were immunofluorescently stained with SKAP2. Scale bar, 5 μm. B, SKAP2 physically interacts with WASP. RAW264.7 cells were lysed and immunoprecipitated (IP) WASP followed by immunoblotting (IB) SKAP2 (left), or IP SKAP2 followed by IB WASP (right). C, diagram of recombinant GST fusion SKAP2 fragments (top). Protein extract from COS-1 cells transfected with HA-WASP were pulled down with GST alone, the GST-PH domain or the SH3 domain of SKAP2, and then immunoblotted with an anti-HA antibody (bottom, left). Immunoblot of GST fusion proteins using an anti-GST antibody is shown at the bottom. (Continued on the following page.)
SKAP2 Controls Podosomes in Macrophages

Next, the intracellular localization of SKAP2 in macrophages was evaluated. When peritoneal macrophages pretreated with M-CSF and IL4 were plated on collagen-coated coverslips, SKAP2 localized to small rings at the ventral surface, which are characteristic structures of podosomes (Fig. 2A). Under higher magnification, SKAP2 was localized to the inner podosome structure, which was surrounded by a ring containing paxillin (Fig. 2B). On the other hand, podosomes were rarely observed in SKAP2−/− macrophages (Fig. 2C and D). These results were confirmed by immunostaining macrophages for F-actin and WASP. Wild-type, but not SKAP2−/− peritoneal macrophages, contained F-actin and WASP-positive podosomes (Fig. 2E and F).

To compare the proteolytic activity of wild-type and SKAP2−/− macrophages, they were layered on cover slips coated with fluorescently labeled gelatin. Rings of gelatin degradation with accumulation of F-actin and SKAP2 were observed in wild-type peritoneal macrophages (Fig. 2G). The proportion of cells with apparent degradation of the gelatin matrix was decreased by SKAP2 knockout (Fig. 2H). These results suggest that SKAP2 is essential for podosome formation and matrix degradation by macrophages.

SKAP2 physically interacts with WASP and regulates its localization in podosomes

The macrophage cell line RAW264.7 was used to confirm that SKAP2 is required for podosome formation. Immunostaining for SKAP2 revealed localization of SKAP2 in podosome rings in RAW264.7 cells (Fig. 3A, left). Among several molecules involved in podosome, we focused on WASP, a well-known actin assembly factor containing a proline-rich domain that may interact with SH3 domain of SKAP2 (Fig. 3B). As a positive control, SKAP2 mutant and WASP was significantly reduced (Fig. 3C, right). These results indicate that the SH3 domain of SKAP2 binds to WASP.

To assess the effect of SKAP2 on the localization of WASP in RAW264.7 cells, SKAP2 was knocked down with a miR (SKAP2-miR), and then rescued by transfection with miR-resistant SKAP2 tagged with HA at the C-terminus (resSKAP2; Fig. 3D). In SKAP2-miR RAW264.7 cells, EGFP-WASP was diffusely localized in the cytoplasm, while it was detected in podosomes in RAW264.7 cells rescued with SKAP2 (Fig. 3E and F). Moreover, expression of membrane-tethered WASP containing myristoylation and palmitoylation signal (myr-palm WASP) partially restored podosome formation in SKAP2-deficient RAW264.7 cells (Fig. 3G and H).

In addition, accumulation of SKAP2 and EGFP-WASP was detected in punctate structures in monolayers protrusions of RAW264.7 cells, which resemble invadopodia observed in cancer cells (Supplementary Fig. S2A). Large podosome rosettes were occasionally observed upon stimulation with M-CSF and PMA (Supplementary Fig. S2B). These structures were also rarely detected in SKAP2-miR RAW264.7 cells.

SKAP2 is required for macrophage invasion in vitro

On the basis of the observation that SKAP2 localizes to podosomes and is required for podosome formation, we hypothesized that depletion of SKAP2 prevents macrophage invasion, which may affect infiltration of tumors by macrophages. To evaluate the migration of macrophages towards cancer cells, 3D gel invasion assays were performed. First, cancer cells were embedded in an ECM gel, and peritoneal macrophages pretreated with M-CSF and IL4 were plated separately on top of the gel. The area of invading peritoneal macrophages in the gel was then quantified (Fig. 4A). The breast cancer cell line MDA-MB-231 was selected for use in this assay because its conditioned medium activated SKAP2. After 5 days of incubation, significant numbers of wild-type peritoneal macrophages invaded into gels containing MDA-MB-231 cells, while invasion by SKAP2−/− peritoneal macrophages was greatly reduced (Fig. 4B and C). In addition, wild-type, but not SKAP2−/− peritoneal macrophages invaded gels containing only conditioned medium from MDA-MB-231 cells (Supplementary Fig. S2C), suggesting that some soluble factors released by cancer cells attract peritoneal macrophages.

We next examined whether restoration of SKAP2 expression in SKAP2−/− peritoneal macrophages rescued their invasive ness. Interaction between the PH domain and dimerization domains of SKAP2 causes autoinhibition, which is relieved by PI3 binding (15). SKAP2−/− macrophages were transduced by adenoviral infection with wild-type SKAP2, an inactive mutant showing disrupted PI3 binding (R140M), an active mutant that impairs autoinhibitory DM1/PH domain interactions (D129K), or a mutant in which the major tyrosine phosphorylated by SIKs was substituted (Y260F) or a mutant unable to bind WASP (W336K). Cancer cell–induced gel invasion by peritoneal macrophages was at least partially rescued by addi-
Figure 4.
SKAP2 is required for macrophage invasion. A, schematic representation of the experiment. B and C, MDA-MB-231 cells (DiO labeled, green) were embedded in gels in Transwell with 0.4 μm pores. DiI labeled (red) peritoneal macrophages (PM; B, wild-type; C, SKAP2−/−) were then overlaid onto the gels. After incubation for 5 days, gels were fixed and sectioned. D–I, SKAP2−/− peritoneal macrophages were infected with an adenovirus containing wild-type SKAP2 (wt), activated mutant (D129K), inactive mutant (R140M), tyrosine substituted mutant (Y260F), or SH3 domain mutant (W336K), and expression of each construct was evaluated by immunoblotting (D). These cells were used in the gel invasion assay (E–I). Scale bar, 200 μm. J and K, the invasion index of each peritoneal macrophage mutant was calculated and expressed as the ratio of mutant cells to wild-type peritoneal macrophages (J) or R140M peritoneal macrophages (K). L, percentage of cells containing podosomes was summarized as in Fig. 2D. Results from three independent experiments are shown as means ± SD. *P < 0.05. α-tub, α-tubulin; Adeno v, adenovirus.
Figure 5.
SKAP2 induces coordinated invasion of macrophages and cancer cells. A, schematic representation of the experiment. B, SKAP2 expression was examined in the indicated cells by immunoblotting. C and D, MDA-MB-231 cells (C) or LLC cells (D) alone were assayed. E and F, representative pictures of invasion by peritoneal macrophages (PM) and MDA-MB-231 cells (E, 7 days incubation) or RAW264.7 cells and LLC cells (F, 5 days). Macrophages were pretreated with M-CSF and IL4. Scale bar, 100 μm. G, the invasion index for each experiment was defined as the ratio of the objective cells to wild-type peritoneal macrophages or RAW264.7 cells. Results from three independent experiments are shown as means ± SD. *, P < 0.05. α-tub, α-tubulin.
IL4, while SKAP2 alone was increased by treatment of the cells with M-CSF and expressed SKAP2 (Fig. 5B). When these cells were subjected to immunostaining for F-actin, podosomes were frequently observed in peritoneal macrophages expressing D129K and wild-type SKAP2, but not in R140M, Y206F, and W336K SKAP2 (Fig. 4L). These results suggest that SKAP2 is essential for migration of macrophages toward cancer cells, and that tyrosine phosphorylation and the interaction between WASP and SKAP2 may be involved in this pathway.

SKAP2 promotes coordinated invasion by macrophages and cancer cells

We previously observed that stromal cells including cancer-associated fibroblasts (CAF) promote invasion by cancer cells (25). To evaluate coordinated invasion by macrophages and cancer cells, another gel invasion assay was performed. Peritoneal macrophages and cancer cells were labeled with distinguishable fluorescence dyes and placed on top of a gel with a concentration gradient of serum (Fig. 5A). In addition to the pair of peritoneal macrophages and MDA-MB-231 cells, the same analysis was performed using RAW264.7 macrophages and LLC cells. Neither of these cancer cell lines expressed SKAP2 (Fig. 5B).

First, gel invasion by wild-type peritoneal macrophages alone was increased by treatment of the cells with M-CSF and IL4, while SKAP2−/− peritoneal macrophages were less invasive under the same conditions (Supplementary Fig. S2D). Treatment of RAW264.7 macrophages with M-CSF also enhanced gel invasion (data not shown). When MDA-MB-231 or LLC cells were separately plated on the gel, very few invaded into the gels (Fig. 5C and D). Vertical sections of gels containing mixtures of wild-type peritoneal macrophages and MDA-MB-231 cells revealed that the two cell types invaded the gel at significant levels, while mixtures of SKAP2−/− peritoneal macrophages and MDA-MB-231 cells did not invade effectively (Fig. 5E).

Add-back of SKAP2 into SKAP2−/− peritoneal macrophages rescued invasion by both macrophages and MDA-MB-231 cells (Fig. 5E, Ad SKAP2). Similar results were obtained with RAW264.7 macrophages and LLC cells (Fig. 5F). SKAP2 expression in RAW264.7 cells regulated their invasion as well as the invasion of LLC cells. These results indicate that SKAP2 is specifically required for coordinated invasion by macrophages and cancer cells. On the other hand, conditioned medium from wild-type peritoneal macrophages alone did not increase the invasion of cancer cells into the gels (data not shown), suggesting that direct interaction between cancer cells and peritoneal macrophages is required to induce cancer cell invasion.

SKAP2 promotes metastasis of cancer cells

During metastatic spread of cancer, macrophages infiltrate in the tumor and promote its growth and spread. To examine the significance of SKAP2 in cancer metastasis, LLC cells were injected into the tail veins of wild-type or SKAP2−/− mice. To evaluate macrophage accumulation in tumors, LLC cells stably expressing IL6 were generated. IL6, a major inflammatory chemokine, promoted the chemotactic migration of peritoneal macrophages in vitro (Supplementary Fig. S3A). However, overexpression of IL6 did not increase proliferation or invasion by LLC cells (Supplementary Fig. S3B and S3C). Lung metastasis of LLC cells increased upon expression of IL6, and many more macrophages were observed in metastases from IL6-overexpressing LLC tumors (Supplementary Fig. S3D). While many large tumor nodules were detected in lungs of wild-type mice 14 days after injection of LLC IL6 cells, metastatic tumor nodules were greatly reduced in SKAP2−/− mice (Fig. 6A, D, and G). When the number of macrophages infiltrating metastatic nodules was counted by immunostaining with an F4/80 antibody, a statistically significant difference was observed between wild-type and SKAP2−/− mice (Fig. 6B, C, E, and F and Supplementary Fig. S3E). The mean number of macrophages in the same tumor area was around 3.5 to 7.0 times higher in wild-type mice than in SKAP2−/− mice (Fig. 6H).

SKAP2-deficient macrophages do not infiltrate metastatic tumor nodules

We next examined whether exogenously injected macrophages infiltrate tumors via SKAP2. To test this, LLC IL6 cells were first intravenously injected into wild-type mice. These mice were then injected with DiI-labeled wild-type or SKAP2−/− peritoneal macrophages that had been pretreated with M-CSF and IL4, and distribution of injected peritoneal macrophages in the lungs was examined 7 days later (Fig. 6I). Tumors in lungs from mice that were injected with wild-type peritoneal macrophages were larger and more numerous than those from mice that were injected with SKAP−/− peritoneal macrophages or those that were not injected with peritoneal macrophages (Fig. 6K and L). When exogenously injected peritoneal macrophages were visualized in the lungs, total number of DiI-labeled wild-type peritoneal macrophages was higher than that of SKAP−/− peritoneal macrophages (Supplementary Fig. S3F).

In particular, wild-type peritoneal macrophages accumulated in metastatic cancer foci, whereas fewer SKAP−/− peritoneal macrophages were located in metastatic foci, although expression of IL6 receptor was not changed (Fig. 6I, M and N). These results suggest that SKAP2 regulates the infiltration of macrophages into tumors.

Because endogenous macrophages may have masked the additional effects of exogenously injected peritoneal macrophages in the above experiment, similar experiments using SKAP−/− mice were performed. Although injection of LLC IL6 cells alone did not cause severe lung metastasis in SKAP−/− mice (Fig. 6D), additional injection of wild-type, but not SKAP−/− peritoneal macrophages, significantly increased the number of large tumor nodules in SKAP−/− mice (Supplementary Fig. S3G). Although almost the same number of wild-type and SKAP−/− peritoneal macrophages was initially observed in lungs 24 hours after injection (Supplementary Fig. S3H), deletion of SKAP2 affected the further accumulation of macrophages in tumors (Supplementary Fig. S3G, right). These results suggest that SKAP2 is required for macrophages to infiltrate tumors. To generalize these conclusions, similar experiments were performed using B16BL6 melanoma cells. Lung metastasis of intravenously injected B16BL6 cells was more severe in wild-type mice than in SKAP−/− mice (Supplementary Fig. S4A). In addition, injected wild-type peritoneal macrophages accumulated in metastatic B16BL6 melanoma tumors and promoted cancer spread, whereas SKAP−/− peritoneal macrophages did not effectively infiltrate the metastatic tumors (Supplementary Fig. S4B).

To examine the proliferative or cytotoxic effects of wild-type and SKAP2−/− macrophages on cancer cells, the lung tumors shown in Fig. 6I and K were immunostained with antibodies specific for Ki-67 or cleaved caspase-3. Intense staining of Ki-67...
SKAP2 promotes metastasis of cancer cells. A–F, LLC IL6 cells (1 × 10^6) were injected intravenously into wild-type (A–C) or SKAP2^−/−^ (D–F) mice. Mice were sacrificed 14 days after injection. Lungs were sectioned and subjected to immunostaining with an anti-F4/80 antibody. Representative pictures of tumors located in the center (B and E) or in the subpleural region (C and F) of the lung. Scale bar, 100 μm. G, number of tumor nodules in the lung (per mouse) was counted and averaged (n = 10 mice, each). H, number of macrophages (MΦ) in the tumor area (per mm^2) was calculated. The results from ten tumors are shown as means ± SD. *, P < 0.05. I, RT-PCR of IL6 receptor in peritoneal macrophages (PM). K, representative photos of dissected lungs. L, the number of lung nodules of different sizes was counted and averaged. M and N, dissected lungs were fixed, sliced, and observed under a confocal microscope. Green, LLC IL6 cells; red, macrophages. Scale bar, 200 μm. KO, knockout.
was observed in mice additionally injected wild-type, but not SKAP2−/−, peritoneal macrophages particularly in the periphery of the tumor where macrophages infiltrated (Supplementary Fig. S5). On the other hand, there was no significant difference in the levels of cleaved caspase-3 staining in these tumors (Supplementary Fig. S6). These results suggest that tumor infiltration by macrophages stimulates cancer cell proliferation and that SKAP2−/− macrophages are not cytotoxic.

Discussion
TAMs have tumor-promoting effects on cancer cells. Here, SKAP2 was identified as essential for podosome formation in macrophages. SKAP2 deficiency suppressed podosome formation and reduced macrophage invasion. Metastasis of cancer cells was significantly reduced in SKAP2-deficient mice, an effect that was dependent, at least partially, on insufficient infiltration of macrophages into tumors. SKAP2-deficient macrophages could not efficiently accumulate in tumors, and were therefore unable to support cancer proliferation and invasion. SKAP2 may only modify macrophage infiltration, and any proliferative effects are independent of SKAP2, because macrophages could not efficiently infiltrate with wild-type and SKAP2−/− macrophages (data not shown).

Tumors contain interstitial cells other than macrophages (e.g., CAFs); however, we detected no or very low SKAP2 expression in CAFs (Supplementary Fig. S7).

Podosomes comprise an outer ring of integrin-associated proteins such as vinculin, talin, and paxillin, and an inner core containing actin-regulatory proteins such as Arp2/3 and WASP (26–28). SKAP2 physically associated with WASP and colocalized in the inner paxillin-positive podosome ring. Because podosome formation was partially recovered by the forced expression of myr-palm–tagged WASP and because it localized to podosomes in SKAP2-deficient RAW264.7 cells, SKAP2 may be important for recruitment of WASP to podosomes. Tyrosine phosphorylation of SKAP2 and the SKAP2–WASP interaction are also essential for activation of macrophage invasion. For example, treatment of macrophages with M-CSF induces phosphorylation of SKAP2, which promotes its association with actin (17).

SKAP2-mediated macrophage invasion is considered as a combined effect through podosome formation and previously reported actin ruffles, and localization of SKAP2 in punctate structures associated with ruffles was also described (22). In addition to individual podosomes, podosome rosettes, which are organized structures of individual podosomes that mediate cell adhesion, were occasionally observed in RAW264.7 macrophages (29). As podosome rosettes were not detected in SKAP2-deficient RAW264.7 cells, SKAP2 may also be involved in the formation of these adhesive structures.

In this study, IL6-expressing LLC cells were used to increase macrophage accumulation. For example, the expression of M-CSF and IL6 in non–small cell lung cancer correlates positively with infiltration of the tumor by TAMs (30). Prostaglandin E2 and IL6 are associated with the tumor-induced differentiation of TAMs and with chemotherapy resistance (31, 32). In addition, IL6-expressing ovarian cancer cells were associated with dense infiltration by CD163+ myeloid cells, including TAMs (33). Although IL6 promotes tumor cell proliferation and angiogenesis in some cancers (34), we did not observe increased invasion or proliferation of LLC IL6 cells in vitro.

A recent study shows that SKAP1 (SKAP55) deficiency suppresses PD-1 expression by CD8+ CTLs, which increases anti-tumor immunity (35). In addition, expression of PD-1 ligands PD-L1/B7-H1 and B7-H4 in macrophages inhibits antitumor immune responses (36). We confirmed expression of SKAP1 was not altered in the thymus of SKAP2−/− mice and that the level of B7-H1 and B7-H4 in macrophages was not affected by the absence of SKAP2 (Supplementary Fig. S1D). However, the effect of SKAP2 on PD-1 and antitumor immunity remains to be elucidated. In addition, SKAP2 associates with Sirpα (SHPS-1; ref. 22). Sirpα interacts with its ligand, CD47, and regulates mechanics of phagocytosis (37–39). Upon interaction with CD47, Sirpα-mediated signaling disrupts phagocytosis, which prevents tumor cell elimination by macrophages. Although we did not observe any apparent macrophage-mediated phagocytosis of LLC cells or B16BL6 cells in SKAP2−/− mice, the role of SKAP2 in the phagocytosis of malignant cells should be examined further.

Although prevention of metastasis should be evaluated from multiple angles, we found that injection of wild-type macrophages promoted metastasis of both LLC cells and B16BL6 cells in a SKAP2-dependent manner. In future, it is also important to examine whether SKAP2 accelerates tumor progression by regulating the cytokine profile of macrophages. Our results indicate that SKAP2-mediated macrophage infiltration of tumors plays a major role in cancer progression. Consequently, SKAP2 may be a suitable therapeutic target for preventing the cancer-supportive functions of TAM.

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

Authors’ Contributions
Conception and design: M. Tanaka, S. Shimamura
Development of methodology: M. Tanaka, S. Shimamura
Acquisition of data (provided animals, collected samples, provided facilities, etc.): M. Tanaka, S. Shimamura, S. Kuriyama, A. Goto, N. Aiba
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Tanaka, S. Shimamura, S. Kuriyama, D. Maeda
Writing, review, and/or revision of the manuscript: M. Tanaka, S. Shimamura, A. Goto
Study supervision: M. Tanaka

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