Pivotal Role of the Lipid Raft SK3–Orai1 Complex in Human Cancer Cell Migration and Bone Metastases

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Abstract

The SK3 channel, a potassium channel, was recently shown to control cancer cell migration, a critical step in metastasis outgrowth. Here, we report that expression of the SK3 channel was markedly associated with bone metastasis. The SK3 channel was shown to control constitutive Ca2+ entry and cancer cell migration through an interaction with the Ca2+ channel Orai1. We found that the SK3 channel triggers an association with the Orai1 channel within lipid rafts. This localization of an SK3–Orai1 complex seemed essential to control cancer cell migration. This suggests that the formation of this complex in lipid rafts is a gain-of-function, because we showed that none of the individual proteins were able to promote the complete phenotype. We identified the alkyl-lipid Ohmline as a disrupting agent for SK3–Orai1 lipid raft localization. Upon Ohmline treatment, the SK3–Orai1 complex moved away from lipid rafts, and SK3-dependent Ca2+ entry, migration, and bone metastases were subsequently impaired. The colocalization of SK3 and Orai1 in primary human tumors and bone metastases further emphasized the clinical relevance of our observations. Targeting SK3–Orai1 in lipid rafts may inaugurate innovative approaches to inhibit bone metastases. Cancer Res; 73(15): 4852–61. ©2013 AACR.

Introduction

The emerging concept of ion channels as key regulators of cancer expansion (for review; 1–3) has several implications, including the potential of their chemical targeting for cancer treatment. Therefore, a precise understanding of the mechanisms underlying the role of ion channels in cancer cells is paramount. We have recently shown that SK3 (KCa3.3 gene), a potassium channel of the small conductance Ca2+-activated potassium (KCa) channel family (4), is a mediator of cancer cell migration (5, 6). The physiologic expression of the SK3 channel was first studied in central neurons where it has a fundamentally role in regulating neuronal excitability (7). This channel is not restricted to neuronal tissues (8), and was found to be expressed in smooth muscle, where it regulates smooth muscle tone (9–11). Interestingly, the SK3 channel is expressed in tumor breast biopsies and melanoma cells, but its expression was not observed in nontumor breast tissues and primary cultures of melanocytes (6, 12). The lack of effect of SK3 channel expression on cell proliferation (12) led us to investigate whether this specific role in cell migration conferred this channel a role in metastases development. Indeed, the formation of secondary tumors from primary sites seems to be a multistep process in which tumor cell migration is a critical event.

In this report, we show a role for SK3 in bone metastases, which is the first report establishing an ion channel as a control factor for bone metastases development. SK3 action proved to be mediated through an association with Orai1, a voltage-independent Ca2+ channel. The SK3–Orai1 complex regulates a constitutive Ca2+ entry, calpain activation, and cell migration. At the cellular level, the SK3–Orai1 complex was localized in lipid rafts. The alkyl-lipid Ohmline disrupted SK3–Orai1 complexes from lipid rafts and impaired SK3-dependent Ca2+ entry, migration, and bone metastases, qualifying this lipid as a potential platform for drug development. Finally, the colocalization of SK3 and Orai1 in primary human tumors and bone metastases from clinical samples emphasized the clinical consistency of these observations. This is the first report showing that the deregulation of an ion channel complex by a lipid could control metastases.
Materials and Methods

Cell lines

Human breast cancer cell line MDA-MB-435s was purchased from the American Type Culture Collection (LGC Promochem) and was grown as already described (6). A recent study suggested that the MDA-MB-435s cell line originated from breast tissue (26). This cell line was transduced by a retrovector containing the luciferase gene and with a lentivector containing either an interfering short hairpin RNA (shRNA) specific to SK3 (SK3− cells) or a nontargeting shRNA (SK3+ cells) as previously validated (13). No difference of luciferase expression and activity has been observed between SK3+ and SK3− cells (see Supplementary Fig. S1B). HEK293 and 518A2 cells are described in Supplementary Methods.

Immunohistochemistry

Cells were fixed in 10% formalin, included in gel, and embedded in paraffin. Murine tissues were fixed in 10% formalin and embedded in paraffin, with a mild decalcification for bone tissues. Tissue microarrays (TMA) were constructed from human formalin-fixed tissues obtained from 177 primary prostate cancers and 37 bone metastases specimens, including 15 prostate cancer metastases and 22 breast cancer metastases. Normal prostate and breast tissues were also included in the TMA. All primary prostate cancers were of the acinar type, with 59 having a Gleason score of 6, 106 with a Gleason score of 8 and 107 with a Gleason score of 9-10. Among the 20 breast cancer bone metastases, 16 expressed estrogen receptors, 11 progesterone receptors, and 3 were positive for HER2; 5 tumors were triple negative. For each tumor, 4 cores (0.6-mm diameter) were included in the TMAs. All primary prostate cancers were of the acinar type, with 59 having a Gleason score of 6, 106 with a Gleason score of 8 and 107 with a Gleason score of 9-10. Among the 20 breast cancer bone metastases, 16 expressed estrogen receptors, 11 progesterone receptors, and 3 were positive for HER2; 5 tumors were triple negative. For each tumor, 4 cores (0.6-mm diameter) were included in the TMAs.

Electrophysiology

All experiments were conducted using whole-cell recording configuration of the patch-clamp technique, as previously described (6, 12) and as described in Supplementary Methods.

Intracellular Ca2+ measurements

Cells were loaded in Petri dishes for 45 minutes at 37 °C with the ratiometric dye Fura2-AM (5 μmol/L). Then, cells were trypsinized, washed with Opti-MEM Reduced Serum Medium, GlutaMax (Life-Technologies), and centrifuged (800 × g for 5 minutes). Immediately after centrifugation, cells were resuspended at 1 × 10^6 cells in 2 mL PSS Ca2+-free solution. Fluorescence emission was measured at 510 nm with an excitation light at 340 and 380 nm (Hitachi FL-2500). See Supplementary Fig. S3A for the validation of constitutive Ca2+ entry protocol used.

Western blot, reverse transcriptase qPCR, and calpain activity assay

Western blot experiments were conducted as described (6). The antibodies and the materials and methods used are described in Supplementary Methods.

Cell proliferation and migration assays

Cell proliferation and cell migration were determined as described elsewhere (6, 12, 28) and are specified in Supplementary Methods.

Experimental and spontaneous metastasis models

Mice (Janvier laboratories) were bred and housed at Inserm U892 (Nantes-University) under the animal care license no. 44565. For experimental metastases, 6-week-old female NMRI nude mice were used. Unanesthetized mice were placed into a plastic restraining device, and 0.75 × 10^6 MDA-MB-435s (SK3+/SK3−) cells were injected into the lateral tail vein in 100 μL of serum-free Dulbecco’s Modified Eagle Medium (DMEM) through a 25-gauge needle. For the mammary fat-pad (MFP) model, female NMRI/nude mice, 3- to 4-weeks old, were used. Mice were anesthetized by intraperitoneal 100 mg/kg ketamine plus 10 mg/kg xylazine administration and a right fat pad was cleared. Subconfluent SK3− and SK3+ cells were harvested, washed in PBS, and 2 × 10^6 cells were injected in a volume of 50 μL of DMEM without serum into the cleared fat-pad. Tumor volumes were calculated using the formula: length × width × depth. For MFP-metastases in vivo assays with Ohmline, SK3− cells were incubated with 1 μmol/L Ohmline or with vehicle (0.6% ethanol/0.4% dimethyl sulfoxide) for 24 hours and injected into the cleared fat pad. Mice were treated 3 times a week for 15 weeks with Ohmline at 15 mg/kg or with vehicle administered intravenously. Primary tumors were removed when the volume reached 400 mm3. In control animals, we have not observed adverse effects upon Ohmline administration (no compartmental, weight growth abnormalities, or liver and heart toxicities were observed after necropsy; ref. 13). This absence of side effects is explained by the low and nontoxic concentration of Ohmline used and the selective effect of this lipid on SK3 channel. The materials and methods used for bioluminescence imaging (BLI) are described in Supplementary Methods.

Membrane fractionation, immunofluorescence, and incorporation of Ohmline in tissues

The materials and methods used are described in Supplementary Methods (29–31).

Statistics

Data were expressed as median with quartile or mean ± SEM (N, number of experiments; n, number of cells). Statistical analyses were made using the unpaired Student t test or the Mann–Whitney test. For comparison between more than 2 means, we used Kruskal–Wallis one-way ANOVA followed by Dunn test. Differences were considered significant when P < 0.05 (SigmaStat, Systat Software and Minitab software, Minitab Inc.).

Results and Discussion

The SK3 channel controls bone metastasis development

To investigate the role of SK3 in metastases development, we engineered luciferase SK3-positive, MDA-MB-435s breast cancer-derived cells. Using specific shRNA knockdown of the KCNN3 gene product, we obtained SK3− cells; control cells...
receiving a random shRNA remained SK3\(^+\). Compared to SK3\(^+\) cells, SK3\(^-\) cells displayed almost no outward current, their plasma membrane was more depolarized, and they exhibited a lower migration capacity although their proliferation was not affected (Fig. 1A and Supplementary Fig. S1). Next, we investigated SK3 function using a cancer cell xenograft model in NMRI/nude mice (Fig. 1B). Silencing of the SK3 channel led to a lower composite metastatic score, based on the number of metastases per mouse and on the intensity of the bioluminescent signal per metastasis (Supplementary Fig. S2A; ref. 13). Interestingly, this lower score essentially reflected a lower bone metastases development in SK3\(^-\)–grafted mice compared to SK3\(^+\)-grafted mice (Fig. 1B). Conversely, the lung bioluminescent signal intensity was not significantly different between SK3\(^-\) and SK3\(^+\)-grafted mice (Supplementary Fig. S2B). At week 9, bone metastases were detected in 83% (10 of 12) of the mice injected with SK3\(^+\) cells but only in 36% (4 of 11) of the mice injected with SK3\(^-\) cells (Fig. 1B, middle). Moreover, the intensity of the bioluminescent signal was significantly different between SK3\(^-\) and SK3\(^+\)-grafted mice (Fig. 1B, bottom). Consistent with in vivo observations, both the frequency of bone metastases (100% vs. 54%) and the intensity of the bioluminescent signal, detected ex vivo at necropsy, were lower in mice injected with SK3\(^-\) as compared to SK3\(^+\) cells (Supplementary Fig. S2C).

These observations did not examine the impact of SK3 channel on the primary tumors in relation to metastatic development. Other channels, such as hEag1 (voltage-gated potassium channel), IKCa (intermediate conductance KCa channel) or TRPV2 (transient receptor potential V2) have been reported to influence the volume of subcutaneously xenografted tumors, by acting on their proliferation and/or migration capacities (14–16). Because ectopic tumor models could not accurately reflect the metastatic potential of tumor cells, we used an orthotopic mammary-tumor model known to support the development of metastases in several tissues. We grafted SK3\(^+\) or SK3\(^-\) cells into the mammary fat pad (MFP) of NMRI/nude mice (17, 18). SK3 channel suppression did not influence primary tumor growth, and the proliferation index (Ki67 staining) was identical in the 2 groups of mice (Fig. 1C). Importantly, SK3\(^+\) tumors were still positive for SK3 staining, whereas SK3\(^-\) tumors remained negative (Fig. 1C), confirming the stability of the SK3 phenotype following grafting. Metastases occurred in both groups and were mainly observed in bones and lungs. However, the bioluminescent signal was weak in bones (Fig. 1C) but not in lungs of SK3\(^-\)-grafted mice (Supplementary Fig. S2D). This suggested that SK3 channel expression in cancer cells affected their ability to form metastases in bone but not in lung.

**External Ca\(^{2+}\) elevation upregulates SK3 channel activity and activates Ca\(^{2+}\) entry promoting calpain activation and cell migration**

These findings suggest that SK3 channel might contribute to/or facilitate bone metastases. As an interaction with the bone microenvironment could influence SK3 activity and because this channel is Ca\(^{2+}\)-sensitive (5), we evaluated the effect of external Ca\(^{2+}\) concentrations in modulating SK3 channels. In active bone resorptive lacunae, osteolysis arose in legs and rachis of the 2 metastases models (Fig. 2A), extracellular Ca\(^{2+}\) concentrations could be as high as 8 to 40 mmol/L, whereas in the vicinity of unaltered bone surface, it is normally closer to 2 mmol/L (19). In vitro, changing the extracellular Ca\(^{2+}\) concentration from 2 to 5 mmol/L led to an increase in the migration of SK3\(^+\) cells, an effect not observed for SK3\(^-\) cells (Fig. 2B). Because we showed that SK3 channel control cancer cell migration by hyperpolarizing the plasma membrane of cancer cells (12), we tested the effect of increasing external Ca\(^{2+}\) concentration on the membrane potential of wild-type MDA-MB-435s cells. Figure 2C shows the outward potassium currents recorded using a ramp protocol from -70 to +70 mV: within 2 minutes, the amplitude of potassium currents increased, leading to a shift of the membrane potential toward more negative values (membrane hyperpolarization). The apamin-sensitive current carried by the SK3 channel was assessed in cells incubated in PSS solution with 2 or 5 mmol/L extracellular Ca\(^{2+}\) (Fig. 2C). Increasing external Ca\(^{2+}\) concentrations more than doubled the amplitude of SK3 currents, leading to a 20 mV membrane hyperpolarization (Fig. 2C). Interestingly, we noticed that SK3 hyperpolarization promoted Ca\(^{2+}\) entry and, thus, elevated intracellular Ca\(^{2+}\) concentration by increasing the Ca\(^{2+}\)-driving force (Fig. 2D).

Hence, a physiologic 2 mmol/L extracellular Ca\(^{2+}\) concentration would activate the SK3 channel, which could be overactivated by higher extracellular Ca\(^{2+}\) concentrations. Of note, activated SK3 channels increased the activity of the Ca\(^{2+}\)-sensitive protease calpain (Fig. 2E), a factor contributing to many aspects of cell migration, such as cell spreading, membrane protrusion, chemotaxis, and adhesion complex formation and turnover (20). In addition, the proteolysis of the calpain target talin is promoted by SK3 expression and is increased by A23187 and/or by high external Ca\(^{2+}\) concentrations (Fig. 2E), conditions that increase intracellular Ca\(^{2+}\) concentrations. Because calpain activation is a critical step leading to adhesion complex turnover and cell migration (20), we can hypothesize that at least part of the role of SK3 in migration could be attributed to by calpain activation.

**SK3 action is mediated through its association with the Orai1 channel, forming a lipid-raft SK3–Orai1 complex**

We next aimed at identifying the Ca\(^{2+}\)-channel involved in Ca\(^{2+}\) entry. The voltage-independent Ca\(^{2+}\)-channel Orai1, and its regulator STIM1, have been shown to be store-operated channels (SOC) in breast cancer cells and have been implicated in cancer cell migration (21) and calpain activation (22). Orai1 knockdown totally abolished SK3-dependent cell migration (Fig. 3A). The suppression of STIM1 had no effect on MDA-MB-435s cell migration (Fig. 3A) in contrast to the MDA-MB231 breast cancer cell line (21) that did not express SK3 protein (6). These results suggest a role for Orai1 channels in constitutive SK3-dependent Ca\(^{2+}\) entry, independently of STIM1 (see Supplementary Fig. S3A for the validation of the constitutive Ca\(^{2+}\) entry protocol used). Consistently, the inhibition of Orai1, either by siRNA, shRNA (with 2 different sequences) or by using 2-APB, totally abolished SK3-dependent constitutive Ca\(^{2+}\) entry and the increase of cancer cell migration observed.
SK3–Orai1 Channel Complex Promotes Bone Metastasis

Figure 1. SK3 suppression inhibited bone metastases. A, validation of the MDA-MB-435s cell system expressing the luciferase gene and expressing or not the KCN3 gene. Whole-cell SK3-current recorded on MDA-MD-435s-shRD (SK3+) and MDA-MD-435s-shSK3 (SK3−) cells (top). Representative recordings from at least 5 cells in each group. Validation of SK3 protein extinction in SK3− cells and luciferase expression in SK3+ and SK3− cells (middle). Representative immunoblots from at least 3 different experiments. Cell migration and proliferation in SK3+ analyses of migration 24 hours after seeding. Data were normalized to results obtained with SK3+ cells and luciferase expression in SK3− and SK3+ cells. (bottom). Histogram showing analyses of migration 24 hours after seeding. Data were normalized to results obtained with SK3+ cells. Columns, mean; bars, SEM. Graph showing proliferation rates evaluated by MTT assays, daily, for 4 days. Points, mean; bars, SEM. N, the number of independent experiments. B, SK3 knockdown inhibits bone metastases. Lung and bone metastases observed 9 weeks after tail vein injection of SK3− cells assessed by BLI in vivo and by hematoxylin and eosin staining (a); BLI assessment of bone metastases likelihood in mice (c); intensity of the bioluminescence signal monitored 9 weeks postinjection (d, right); and BLI of representative mice with spinal column metastases (d, left). N, the number of mice. Box plots indicate the first quartile, the median, and the third quartile, squares indicate the mean. C, breast primary tumor growth is not influenced by SK3 channel. Representative SK3 immunostaining in the primary tumor tissues from mice orthotopically grafted with SK3+ and SK3− cells (a). Graph showing mammary tumor growth in SK3− and SK3+–grafted mice (b). K67 staining of primary tumor tissue sections from mice grafted with SK3+ or SK3− cancer cells, 16 weeks post-graft (c). Hematoxylin and eosin sections of bone metastases and BLI quantification of excised legs (d). Box plots indicate the first quartile, the median, and the third quartile, squares indicate the mean. N indicates the number of mice.
at 5 mmol/L Ca\(_{2+}\) concentration (Fig. 3B and Supplementary Fig. S1BC). Thus, our findings revealed a novel signaling pathway in which the SK3-Orai1 complex elicited a constitutive and store-independent Ca\(_{2+}\)-signaling that promoted cell migration. Having shown that Orai1 was necessary for cancer cell migration, we assessed its cellular localization.

Figure 2. External Ca\(_{2+}\) elevation upregulated SK3 channel activity and activated Ca\(_{2+}\) entry promoting calpain activation and cell migration. A, osteolytic lesions in mice receiving SK3+ cells. Representative X-ray scanner of a vertebrae 9 weeks after the injection of cells in the tail vein and X-ray radiography of the hind limbs 16 weeks after the injection of cells in MFP. Osteolytic lesions are indicated by the arrows. B, external Ca\(_{2+}\) elevation promoted SK3-dependent cell migration. SK3+ and SK3− cell migration recorded with 2 and 5 mmol/L external Ca\(_{2+}\) concentration. Data were normalized to conditions obtained with a 2 mmol/L external Ca\(_{2+}\) concentration. C, external Ca\(_{2+}\) elevation increased the amplitude of SK3 currents leading to membrane hyperpolarization. Representative SK3+ whole-cell currents recorded in the presence of 2 mmol/L external Ca\(_{2+}\) concentrations and following the addition of 3 mmol/L external Ca\(_{2+}\) concentrations after 2 minutes (final external Ca\(_{2+}\) concentration = 5 mmol/L). To maintain a constant surface charge, the same concentration of divalent ions in both PSS solutions was used (see Supplementary Methods). Currents were generated by ramp protocol from –100 to –70 mV in 500 ms from a constant holding of –70 mV and with a pCa7. The arrows indicate membrane potential (Em) values. The inset showing apamin-sensitive current amplitude of the current before and after application of 50 nmol/L apamin (a specific SKG blocker) in 2 and 5 mmol/L external calcium concentration. D, the SK3 channel promoted Ca\(_{2+}\) entry. Fluorescence measurement (left) and relative fluorescence to Ca\(_{2+}\) entry (right) in SK3+ and SK3− cells. Data were normalized to conditions obtained with SK3+ cells. E, the SK3 channel promoted calpain activity and talin cleavage. Relative fluorescence analyses of calpain activities, measured with a fluorogenic calpain substrate Ac-LLY-AFC, with or without the calpain inhibitor Z-LLY-FMK, in 518A2 cells expressing or not SK3. Cells were preincubated or not for 5 minutes with the Ca\(_{2+}\)-ionophore A23187 and treated or not with Ca\(_{2+}\) for 30 minutes. Representative immunoblots from 3 different experiments are shown. Columns, means; bars, SEM. N, the number of independent experiments; n, number of cells.
Immunofluorescence analysis showed that SK3 and Orai1 were localized at the plasma membrane (Fig. 3C), and membrane-fractionation experiments specified this localization to lipid rafts (Fig. 3D). Although the SK3–Orai1 complex was always detected in lipid rafts, SK3-silencing experiments totally displaced Orai1 outside of lipid rafts (Fig. 3D). Thus, we concluded that the SK3–Orai1 complex is one of the components of the Ca\(^{2+}\)-signaling microdomain constituted by lipid rafts (23).

The alkyl-lipid Ohmline moved the SK3–Orai1 complex outside of lipid rafts and impaired SK3-dependent Ca\(^{2+}\) entry, migration, and bone metastases

To challenge these observations, we used a lipid inhibitor of SK3 channels called Ohmline (13). We previously showed that Ohmline does not displace pore-binding compounds (13) but, like edelfosine and owing to its phospholipid structure, could act on SK3 channels by being incorporated...
Figure 4. The alkyl-lipid Ohmline moved the SK3–Orai1 complex outside of lipid rafts and impaired SK3-dependent Ca\(^{2+}\) entry, migration, and bone metastases. A, Ohmline treatment moved the SK3–Orai1 complex outside of lipid rafts. Immunoblots representing membrane fractionation on a sucrose gradient of cells treated or not with 1 µmol/L Ohmline for 24 hours (left). Representative immunoblots from 2 different experiments. Hypothetical scheme of Ohmline effects on Orai1 and SK3 (right). B, Ohmline treatment reduced the constitutive Ca\(^{2+}\) entry. Fluorescence measurement (left) and relative fluorescence (right) of constitutive Ca\(^{2+}\) entry in cells treated or not with 1 µmol/L Ohmline for 24 hours. Data were normalized to results obtained in cells treated with vehicle. The constitutive Ca\(^{2+}\) entry protocol has been validated in Supplementary Fig. S3A. Columns, means; bars, SEM. N indicates the number of experiments. C, Ohmline treatment reduced the migration of MDA-MD-435s cells. Histograms showing migration of cells treated or not with 1 µmol/L Ohmline for 24 hours in 5 mmol/L external Ca\(^{2+}\) conditions. Columns, mean; bars, SEM. N indicates the number of experiments. D, MFP-tumor model protocol used for Ohmline injections. E, Ohmline treatment abolished bone metastases in MFP-tumor model. Images of nude mice 15 weeks after SK3\(^{+}\) cell injections in MFP and treated either with vehicle or Ohmline at 15 mg/kg (left). Occurrence of lung and bone metastases in mice treated with either Ohmline or vehicle and representative bioluminescent images ex vivo of lung and bone metastases (vehicle condition; middle). N indicates the number of mice. Measurements of Ohmline incorporation in lung and bone tissues (tissues were pooled from 4 different samples) at week 15 (right). F, Ohmline incorporation in the primary tumors has no effect on their growth. Time course of tumor growth recorded in vehicle and Ohmline-treated mice postgraft (left). Measurement of Ohmline incorporation in tumors from treated mice (right). N indicates the number of mice.
into lipid rafts (24). Addition of Ohmline for 24 hours at 1 µmol/L had no effect on SK3 or Orai1 protein expression, but totally delocalized SK3 and Orai1 channels from lipid-raft fractions (Fig. 4A and Supplementary Fig. S4A). Functionally, Ohmline reduced the constitutive Ca²⁺ entry and thus cancer cell migration (Fig. 4B and C), as observed when the SK3 channel is knocked down (see Figs. 2 and 3; ref. 6). Interestingly, identical results were obtained when using 10 times less Ohmline (Supplementary Fig. S4B). As SK3 activity is abolished shortly after Ohmline application (120 seconds; ref. 13), we hypothesized that Ohmline is incorporated in lipid rafts and acts by dissociating or preventing SK3–Orai1 complex cauterization. This indicates that the SK3–Orai1 complex might only function when localized in rafts and that a delocalization of one of the 2 partners is sufficient to suppress SK3-dependent Ca²⁺ entry and SK3-dependent migration.

We next tested Ohmline potency to reduce metastases development in the MFP model (see protocol Fig. 4D). Ohmline incorporation was measured in primary tumors and in bone and lung metastases (Fig. 4E and F and Supplementary Fig. S4C). Despite incorporation, Ohmline had no effect on primary tumor development (Fig. 4F), strengthening the observation that the SK3–Orai1 complex has no role in primary tumor growth (Fig. 1C). Mice treated with Ohmline did not present any sign of bone metastases confirming the crucial role of the SK3–Orai1 complex in bone metastases development (Fig. 4E). Unexpectedly, an effect of Ohmline was also observed on lung metastases (Fig. 4E). Ohmline was shown to inhibit the SK1 channel (13) and might increase SOCs channel activity (21); this might be the mechanism of decreased lung metastases by Ohmline. As SK3 is expressed in the central nervous system, and despite the high concentration of Ohmline in this tissue, we observed no neurological effects. This can be explained by: (i) the absence of lipid rafts SK3–Orai1 complexes in the brain (Orai1 expression being low) or (ii) the organisation of SK3 channels as heteromultimeric complexes involving SK1 or SK2, in contrast to cancer cells where SK1 is not expressed.

**SK3 and Orai1 are expressed in breast/prostate cancer clinical samples**

Because bone is a privileged site for metastases in prostate cancer, we assessed SK3 epithelial expression in clinical samples. Many (60%) of the prostate cancer tumors, both from primary tumors (113 of 177) or bone metastases (9 of 15), showed positive epithelial SK3 staining, with a granular and predominantly membranous profile (Fig. 5A). Identical results were obtained with breast cancer clinical samples (Fig. 5A). To evaluate the clinical value of these observations we analyzed the coexpression of SK3 with Orai1. In human cancer samples, including primary tumors and bone metastases, the expression of SK3 and Orai1 were significantly associated (Quin2 test, P < 0.0001; Fig. 5C). SK3 protein was not expressed in normal tissues in contrast to Orai1 (Fig. 5B), supporting that this is the expression of SK3 in tumor cells that triggers Orai1 to associate with SK3 as a complex in lipid rafts. Note that it is well known that Orai1 protein expression at the cellular level reveals near ubiquitous distribution.

Taken together, our results reveal a hitherto unknown function for SK3 channels in regulating Ca²⁺ entry through Orai1 channels (Fig. 6). In vivo data further suggest a participation of the SK3–Orai1 complex in the migration of cancer cells and their establishment at permissive secondary sites. Intriguingly, the Orai1 partner STIM1 seems not to be involved in this effect, which could reflect a differential role for Ca²⁺ signaling in tumors, one connecting Ca²⁺ entry to proliferation (25) and the other to metastases. Finally, by detecting SK3 channels in human samples, we confirmed the clinical relevance of SK3–Orai1 expression in bone metastases. Hence, the in vivo efficacy of Omhline in preventing and/or treating bone metastases could have a therapeutic application.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Figure 6. Proposed mechanism for SK3–Orai1 role in bone metastases. A, in the absence of SK3, Orai1 is not embedded within lipid rafts and does not promote constitutive Ca\textsuperscript{2+} influx. B, the presence of SK3 triggers SK3–Orai1 to associate within lipid rafts, resulting in plasma membrane hyperpolarization and constitutive Ca\textsuperscript{2+} entry. C, increased external Ca\textsuperscript{2+} concentration observed in osteolytic metastatic sites amplifies Ca\textsuperscript{2+} entry, leading to a positive feedback loop. D, disrupting lipid rafts with the alkyl-lipid Ohmline allows Orai1–SK3 to move away from lipid rafts and abolishes SK3-dependent constitutive Ca\textsuperscript{2+} entry. Thus, SK3-dependent cancer cell migration and bone metastases are counteracted.
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References


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