Bone metastasis is a severe complication associated with various carcinomas. It causes debilitating pain and pathologic fractures and dramatically impairs patients’ quality of life. Drugs aimed at osteoclast formation significantly reduce the incidence of skeletal complications and are currently the standard treatment for patients with bone metastases. Here, we reported that serum- and glucocorticoid-inducible kinase 1 (SGK1) plays a pivotal role in the formation and function of osteoclasts by regulating the Ca\(^{2+}\) release-activated Ca\(^{2+}\) channel Orai1. We showed that SGK1 inhibition represses osteoclastogenesis in vitro and prevents bone loss in vivo. Furthermore, we validated the effect of SGK1 on bone metastasis by using an intracardiac injection model in mice. Inhibition of SGK1 resulted in a significant reduction in bone metastasis. Subsequently, the Oncomine and the OncoLnc database were employed to verify the differential expression and the association with clinical outcome of SGK1 gene in patients with breast cancer. Our data mechanistically demonstrated the regulation of the SGK1 in the process of osteoclastogenesis and revealed SGK1 as a valuable target for curing bone metastasis diseases.
SGK1 Promotes Bone Metastasis of Breast Cancer

Animals

C57BL/6 mice and BALB/c nu/nu mice were purchased from the Experimental Animal Center of Tongji Medical College (Wuhan, China). The mice were housed in animal care facility of Tongji Medical College at 25°C with 12-hour light/dark cycle and free access to standard chow and water. All animal studies were approved by the Institutional Animal Research Committee of Tongji Medical College. All animal experiments were carried out in accordance with the protocols approved by the Institutional Animal Care and Use Committee.

Intracardiac metastasis model

MDA-MB-231 cells were resuspended in sterile PBS at a concentration of $5 \times 10^5$ cells per 100 μL. We used a marked location midway between the sternal notch and the top of xiphoid process, and slightly left (anatomic) of the sternum. Four-week-old male (BALB/c nu/nu) mice were used for these experiments. A successful intracardiac injection was determined on the basis of the systemic distribution of bioluminescence throughout the animal at 12 hours postinjection. Only mice demonstrating evidence of a successful injection were used for further analyses. Four weeks after inoculation, bone metastatic cells in hind limbs were isolated and cultured in DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). Cultured bone metastatic cells were reincorporated into the left ventricle of the heart. This procedure was repeated four times until no metastases were detected in organs other than bones. These bone metastatic cells in hind limbs, designated MB54-TJGK cells, were isolated and cultured in DMEM.

Cell culture and transfection

MDA-MB-231 and RAW264.7 cell lines were purchased from the Shanghai Institute of Life Sciences Cell Resource Center, Shanghai, China. Cell lines authentication were conducted by China Center for Type Culture Collection (CCTCC). STR data were compared with ATCC and DSMZ, all the locations were exactly matched. BMMs were obtained from femoral and tibial bone marrow of 6- to 8-week-old C57BL/6 mice as described earlier (27). All cell cultures were regularly checked for mycoplasma contamination. BMMs and RAW264.7 were cultured and induced to osteoclast as described previously (15). Scrambled, SGK1-knockdown and Orai1-overexpression shRNA were obtained from ViGene Biosciences, Inc.

Western blot and immunofluorescence analysis

Antibodies against the following targets were purchased from Cell Signaling Technology: IkB kinase α (IKKα), p-IκBα, IkBα, p-IκBα, p65, p-p65, AKT, and phosphorylated AKT (p-AKT). Antibodies against c-Fos, NFATc1, and SGK1 was purchased from Abcam. Rabbit antibodies against the PI3K catalytic subunit p110 and Orai1, the TRAP staining kit and all other reagents were purchased from Sigma-Aldrich. Mouse anti-β-actin and secondary antibodies were obtained from Boster. Protein bands were visualized using a ChemiDoc XR+ System with Image Lab Software (Bio-Rad).

For IF analysis of cultured cells, BMMs were fixed with 4% paraformaldehyde for 15 minutes and then blocked with 5% bovine serum albumin and 0.1% Triton X-100 in PBS for 30 minutes at room temperature. Immunostaining was performed using the appropriate primary and secondary antibodies. Nuclei were counterstained with DAPI. Images were captured using a fluorescence microscope.

Quantitative real-time RT-PCR

Total RNA was extracted from cultured cells and cDNA was synthesized with the Easy Script First-Strand cDNA Synthesis Super Mix Kit (TransGen Biotech). Real-time PCR was performed using Power SYBR Green PCR Master Mix (TransGen Biotech). The specific primers used for PCR amplification were as follows (forward; reverse): SGK1, F 5'-TGTGAAGTCCCTCTGTTGGA-3' and R 5'-CCACTTCTGACCGCCGTTC-3'; TRAP, F 5'-GATGCCAGGCA-CAAGGGTT-3' and R 5'-CATACAGGGGTGTTGGCA-3'; NFA1c, F 5'-CAAGGCGGCTGACGGG-3' and R 5'-GAGAAAGACTCATACAC-3' and R 5'-TGAGCTTAGGGCGAGATT-3'; MMP9, F 5'-CTGCGAGCGACGACTAAG-3' and R 5'-CTCGGGAGTCTTCAGAG-3'; GAPDH, F 5'-ATCCACTCTTCCA-3' and R 5'-CTGCGAGCGACGACTAAG-3'.

Datasets of mRNA expression and clinical information

Follow-up data of 3,951 patients from Kaplan–Meier plotter (kmplot.com/analysis/) and the GEO database were included in this study, which was divided into two groups according to the expression of SGK1 (28). Expression data of SGK1 was drawn from the Oncomine dataset, which embodies large quantities of high-throughput data of various disease.

Statistical analysis

Results are presented as the means ± SD. Every experiment had a sample size greater than three and was independently performed three times, unless otherwise stated. Multigroup comparisons of the means were carried out by one-way ANOVA test with post hoc contrasts by Dunnett multiple comparisons test. Cumulative survival time was calculated by the Kaplan–Meier method and analyzed by the log-rank test.

Results

Suppression of SGK1 inhibits RANKL-induced osteoclastogenesis and impairs osteoclast function in vitro

We evaluated the expression of SGK1 during RANKL-induced osteoclastogenesis by quantitative real-time RT-PCR and immunoblotting. Upon RANKL treatment, the mRNA and protein levels of SGK1 were significantly increased (Fig. 1A and B; Supplementary Fig. S1A). We treated bone marrow mononuclear cells (BMM) once a day with different concentrations of GS650394, an SGK1 inhibitor, in the presence of RANKL (100 ng/mL) and M-CSF (30 ng/mL) for 7 days (29). Osteoclast formation were inhibited in a dose-dependent manner (Fig. 1C and D). Subsequently, the potential toxicity of GS650394 was evaluated by measuring the proliferation of BMMs via the Cell Counting Kit-8 (CCK-8) after treating the cells with different concentrations of GS650394. However, the proliferation of BMMs was not markedly altered by GS650394 at concentrations lower than 2 μmol/L (Fig. 1E).

To control the possible off-target effects of GS650394, we knocked down SGK1 in RAW264.7 cells using small interfering RNAs (siRNAs; Supplementary Fig. S1B). Moreover, three shRNA lentiviruses tested (sh1, sh2, sh3) were selected to create stable SGK1 knockdown in both BMMs and RAW264.7 cells. The protein level of SGK1 was reduced as shown in Fig. 1F and Supplementary Fig. S1C. Upon RANKL treatment, osteoclast formation was significantly inhibited in cells with either siRNA or shRNA-mediated knockdown of SGK1 (Fig. 1G and H; Supplementary Fig. S1D–S1F). Therefore, SGK1 activity is upregulated during RANKL-induced osteoclastogenesis, and the
Figure 1. SGK1 is upregulated during osteoclast differentiation, and inhibition of SGK1 suppresses osteoclast differentiation and function. A and B, BMMs were cultured in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) and were collected at the indicated time points to analyze SGK1 expression via quantitative real-time RT-PCR and Western blotting. Immunoblots were quantified using ImageJ software. Experiments were performed in triplicate. *P < 0.05; ***P < 0.001 (Dunnett t test) versus day 0 group. C, BMMs (1.5 × 10⁵ cells/well) were incubated in complete medium containing M-CSF, RANKL, and varying concentrations of GSK650394 (0, 0.5, 1, and 2 μmol/L) for 7 days. Then, the cells were fixed and stained for TRAP. D, TRAP-positive multinucleated (>3 nuclei) osteoclasts were counted in each well of a 96-well plate, and the results were displayed in a histogram. Data are presented as the means ± SDs of three independent experiments. **P < 0.01 (Dunnett t test) versus the vehicle-treated group. E, BMMs (4 × 10⁴ cells/well) were cultured with M-CSF (30 ng/mL) and treated with varying concentrations of GSK650394 (0, 0.5, 1, 2, and 5 μmol/L) for 5 days. Cell proliferation was assessed by the Cell Counting Kit-8. Data are presented as the means ± SDs of three independent experiments. F, SGK1 protein expression in BMMs was assessed by Western blotting. ACTB was used as a loading control. Immunoblots were quantified using ImageJ software. Experiments were performed in triplicate. G, BMMs cells were seeded in 96-well plates at a density of 1.0 × 10⁴ cells/well, transfected with SGK1-specific shRNAs and cultured in the presence of RANKL (100 ng/mL) for 5 days. TRAP staining was performed. H, TRAP-positive cells with >3 nuclei were counted. Data are presented as the means ± SDs of three independent experiments. ***P < 0.001 (Dunnett t test) versus the vector group. ****P < 0.0001 (Dunnett t test) versus the vector group. I, BMMs cultured in 96-well plates were treated with M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 5 days and then seeded in Corning Osteo Assay Surface and incubated with different concentrations of GSK650394 for 72 hours. J, Bone resorption area was measured. Results shown are the percentage of eroded area of the total area. Data are presented as the means ± SDs of three independent experiments. **P < 0.01 (Dunnett t test) versus the vehicle-treated group.
suppression of SGK1 inhibits RANKL-induced osteoclastogenesis in vitro.

Given that osteoclast formation was impaired in SGK1-knockdown osteoclast precursors, we next investigated the effect of GSK650394 on bone resorption. Mature osteoclasts were seeded in calcium phosphate-coated plates in the presence of RANKL and M-CSF and treated with different concentrations of GSK650394 for 3 days. Compared with that in control cells, the formation of resorption pits in vitro was significantly reduced in GSK650394-treated osteoclasts (Fig. 1I and J). The luciferase reporter gene assays also suggested that GSK650394 treatment inhibited NF-κB luciferase reporter in BMMs (Supplementary Fig. S2A). Thus, impaired SGK1 activity inhibits the resorption function of mature osteoclasts.

SGK1 modulates the NF-κB and PI3K/AKT pathways in osteoclastogenesis

The NF-κB and PI3K/AKT transcription factor families are both downstream of TRAF6, which play pivotal roles in osteoclastogenesis. SGK1 is known to modulate transcription by upregulating NF-κB activity through the phosphorylation and activation of IKKα, whereas in the PI3K/AKT pathway, SGK1 is highly homologous to AKT and is a critical downstream mediator of PDK1 (24). Specific inhibitors of the PI3K/AKT pathway result in the reciprocal activation of SGK1 (20). To explore the potential mechanisms by which SGK1 modulates osteoclastogenesis, we analyzed the expression of proteins associated with osteoclast formation in BMMs. Western blot analysis showed that the inhibition of SGK1 via GSK650394 dramatically suppressed the RANKL-induced phosphorylation and degradation of IKKα, IkBα, and p-IκBα approximately 60 minutes after stimulation with RANKL (Fig. 2A; Supplementary Fig. S2A). Moreover, regarding to the AKT/PI3K pathway, GSK650394 treatment decreased the levels of p-AKT and the PI3K catalytic subunit p110 (Fig. 2B; Supplementary Fig. S2B). Therefore, the suppression of SGK1 inhibits RANKL-induced NF-κB and PI3K/AKT activation.

Suppression of SGK1 inhibits RANKL-induced c-Fos and NFATc1 expression and downregulates the expression of osteoclast maker genes

To determine whether the suppression of SGK1 inhibits the expression of c-Fos and NFATc1, which are essential transcription factors in osteoclast formation, we analyzed the expression of these two factors by Western blot analysis. As shown in Fig. 2C and Supplementary Fig. S2B, the protein levels of c-Fos and NFATc1 were upregulated during RANKL-induced osteoclast differentiation, which can be prevented efficiently by GSK650394 treatment.

We then tested whether GSK650394 suppresses the expression of osteoclast marker genes such as tartrate-resistant acid phosphatase (TRAP), MMP9, NFATc1, and cathepsin K (CK); notably, TRAP, MMP9, and CK are target genes of NFATc1 (30). As shown in Fig. 2D and E, GSK650394 inhibited the mRNA expression of MMP9 and CK during late stages of osteoclastogenesis and inhibited TRAP and NFATc1 at both early and late stages of osteoclastogenesis, respectively.

Activation of Orai1 abolishes the inhibition of osteoclastogenesis by GSK650394

Orai1 is a SOCE channel on membrane, which regulates Ca^{2+} oscillations and NFATc1 transactivation during osteoclastogenesis (31). Immunofluorescence (IF) and Western blot analysis showed that the inhibition of SGK1 decreased Orai1 protein expression during osteoclastogenesis (Fig. 3A, C, and D). Ca^{2+} imaging analysis confirmed essentially complete inhibition of SOCE in BMMs treated with GSK650394 compared with control (Fig. 3E). We therefore speculated that Orai1 might mediate the effect of SGK1 on osteoclast. To elucidate the role of Orai1 in the SGK1-mediated inhibition of osteoclast formation, we overexpressed Orai1 in BMMs (Fig. 3B). Scrambled or Orai1 overexpressed BMMs were treated with or without GSK650394 in the presence of RANKL (75 ng/mL) for 5 days, respectively. As shown in Fig. 3F and G, the inhibitory effects of GSK650394 on osteoclast differentiation were markedly alleviated in Orai1 overexpression group, indicating that Orai1 is a key downstream mediator of the terminal differentiation of osteoclasts by SGK1 (Supplementary Fig. S2C).

GSK650394 prevents ovariectomy-induced bone loss

To investigate the effects of SGK1 on bone loss, we analyzed the bone mass of femurs in ovariectomized (OVX) mouse model. The result revealed that the reduction of intrabecular bone volume/tissue volume (BV/TV) and trabecular number (Tb.N) in the OVX mice was dramatically relieved by GSK650394 treatment (50 mg/kg; Fig. 4A and B). Furthermore, we performed TRAP staining in femoral sections. The number and size of osteoclasts in OVX mice were significantly higher than those in sham-operated mice. In response to GSK650394 treatment, the OVX mice demonstrated dramatically reduced ovariectomy-induced osteoclast activity (Fig. 4C). Histomorphometric analyses confirmed that the osteoclast surface/bone surface (Oc.S/Bs) ratio was strikingly higher in OVX mice than in sham-operated mice (Fig. 4D). Furthermore, compared with those of untreated OVX mice, these osteoclastic parameters were significantly decreased in GSK650394-treated OVX mice (Fig. 4E).

In addition, compared with OVX group GSK650394 treatment significantly suppress the ovariectomy-induced elevation in type I collagen cross-linked C-terminal telopeptide (CTX-I) and RANKL levels in serum (Fig. 4E). Moreover, we tested the levels of OPG by ELISA. The results are similar (Fig. 4E). Therefore, compared with that in the untreated OVX mice, the RANKL/OPG ratio was markedly suppressed in the GSK650394-treated OVX mice (Fig. 4E).

SGK1 serves as a promising therapeutic target for bone metastasis

To further verify the effect of GSK650394 on the formation of bone metastases, we used a model based on the intracardiac inoculation of tumor cells (32). A bone-seeking clone (MBS4-TJGK) of the human breast cancer cell line MDA-MB-231 was established by repeated intracardiac inoculation of tumor cells (32). A bone-seeking clone (MBS4-TJGK) of the human breast cancer cell line MDA-MB-231 was established by repeated sequential intracardiac inoculation of the cells into nude mice and further culture of metastatic cells obtained from bone metastases in vitro. After four cycles, MBS4-TJGK metastasize exclusively to bones. Then, MBS4-TJGK cells were inoculated intracardially. Nude mice were intraperitoneally injected with GSK650394 (50 mg/kg, five times per week) for 4 weeks. Bioluminescence imaging showed that compared with the DMSO-treated group, hind limb and spine tumor burden in mice was significantly decreased in the GSK650394-treated group (Fig. 5A and B). Micro-CT imaging confirmed that the mice treated with GSK650394 exhibited a reduction in bone lesions (Fig. 5C).

Because SGK1 is also highly expressed in heart and gastrointestinal tract, we then evaluate the toxicity of GSK650394. Mice were euthanized 3 weeks after injection and plasma biochemistry analyses were performed. The indicators for heart and liver injuries, as well as kidney functions did not change significantly at the concentration of 50 mg/kg (Fig. 5D and E). However, when lifted dose to 75 mg/kg, lactic dehydrogenase (LDH-L) dropped in treated group mice (Fig. 5D).
These results suggest that intraperitoneal injection of GSK650394 in our model did not lead to reductions in hepatic and renal function but may have off-target influence on myocardial cells damaging with high dose.

Then, direct effect of GSK650394 treatment on MDA-MB-231 cell lines was tested by flow cytometry and CCK-8 (Fig. 5F and G). Apoptotic and necrosis cells were detected using FITC-Annexin V/propidium iodide (PI). Cells incubated with GSK650394 at the concentration of 5 μmol/L showed higher apoptotic rate among other groups. The proliferation of MDA-MB-231 cells were altered by GSK650394 in a dose-dependent manner.

Validation of the role of SGK1 from database
Comparing the mRNA expression level of SGK1 in normal breast tissues and breast cancer tissues (Fig. 6A), we found SGK1 is significantly increased in breast cancer (33, 34, Perou and colleagues). It validated our results of RT-PCR. To further determine the clinical significance of SGK1, we examined whether SGK1 expression was associated with the clinical outcome of breast cancer patients (Fig. 6B). The survival analysis based on large population included in the KM plotter showed no significant prognostic performance of SGK1 on breast cancer patients (HR = 0.92; 95% CI, 0.82–1.02; P = 0.120). But in other datasets GSE2603 (HR = 2.24; 95% CI, 1.05–4.79; P = 0.033) and GSE12276 (HR = 1.74; 95% CI, 1.30–2.34; P < 0.001) we found high expression of SGK1 could predict poor prognosis, indicating the potential oncogenic effect of SGK1 (35, 36).

Discussion
Ca²⁺ signaling is essential for diverse biological processes and is known to be involved in osteoclast formation and function (37–43). Ca²⁺ release from intracellular stores and Ca²⁺ entry across the cell

Figure 2.
GSK650394 inhibits RANKL-induced activation of the NF-κB, PI3K pathways, and suppresses the expression of c-Fos/NFATc1 and osteoclast marker genes. A, GSK650394 inhibits RANKL-induced phosphorylation of NF-κB. BMMs were starved in 0.5% FBS in α-MEM for 12 hours before treatment and then pretreated with or without GSK650394 (2 μmol/L) for 24 hours followed by RANKL (100 ng/mL) stimulation for the indicated times. Cell lysates were extracted for immunoblotting with the indicated antibodies. Immunoblots were quantified using ImageJ software. Experiments were performed in triplicate. B, Effect on PI3K activation. BMMs were treated as described above, and total cell protein was fractionated for immunoblotting with the indicated antibodies. Immunoblots were quantified using ImageJ software. Experiments were performed in triplicate. C, GSK650394 inhibits RANKL-induced protein expression of c-Fos and NFATc1. BMMs treated with or without GSK650394 (2 μmol/L) were cultured in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) and collected at the indicated time points for the analysis of c-fos and NFATc1 protein expression. Immunoblots were quantified using ImageJ software. Experiments were performed in triplicate. D and E, GSK650394 suppresses RANKL-induced mRNA expression of TRAP, NFATc1, CK, and MMP9. BMMs were treated with or without GSK650394 (2 μmol/L) for 2 or 5 days. Total RNA was isolated and analyzed by quantitative real-time RT-PCR. Data represent the means ± 5Ds. *, P < 0.05; **, P < 0.01 (Tukey test).
membrane both accomplish Ca\(^{2+}\) in cytoplasm. SOCE, a major Ca\(^{2+}\) influx pathway in most nonexcitable cell types, is mediated by the Ca\(^{2+}\) release-activated Ca\(^{2+}\) channel, which consist of Orai1, 2, or 3 binding to their regulators STIM 1 or 2 (31). Previous work has identified SGK1 as a powerful stimulator of several ion channels including Orai1. Some clinical studies have been undertaken to corroborate the relevance of SGK1 in diseases such as hypertension and cancer. The mechanism can be related to coordinate with different signaling pathways include FoxO1 and NF-\(\kappa\)B to target organ inflammation (44). However, it was unknown...
whether SGK1 affects osteoclast differentiation and bone resorption via Ca\(^{2+}\) channel through Orai1 until now.

In our study, we observed SGK1 expression was upregulated upon the initiation of osteoclastogenesis. Both pharmacologic inhibition and knockdown of SGK1 by shRNA suppressed osteoclast differentiation and function. In addition, Orai1 protein abundance appeared as well as the significant decrease of Ca\(^{2+}\) influx after GSK650394 treatment (Fig. 3A–E). Moreover, overexpressing Orai1 in BMMs counteracted the suppressive effect of GSK650394 on osteoclast formation. On the basis of these data, we concluded that SGK1 expression is closely related to osteoclastogenesis and has a strong effect on Orai1. To further confirm SGK1 inhibition on ovariectomy-induced bone loss in vivo, we used GSK650394 to prevent bone loss in OVX mouse model. We observed attenuated ovariectomy-induced bone loss in GSK650394 treated group, which was consistent with results in vitro. In the previous work, SGK1 was showed to be evolved in nuclear translocation and activation of NF-κB (24, 45, 46). SGK1 could phosphorylate IKKα, which lead to IKKβ activation. The IKK complex and the NF-κB pathway would be activated similarly, which were confirmed in our study. In addition, we demonstrated that Orai1 established an previously unknown link between SGK1 and osteoclast genesis.

SGK1 expressed heavily in several tumor cells, including colon cancer, myeloma, medulloblastoma, prostate cancer, and ovarian tumors (47, 48). Some research has shown that SGK1 contributes to the glucocorticoid-induced resistance of breast cancer cells to...
Figure 5. GSK650394 prevents bone metastasis in an intracardiac inoculation model. A, Representative images of whole-body in vivo bioluminescence imaging (BLI) of mice at day 30 after the intracardiac injection of MBS-TJGK4 cells. B, Quantitation of bioluminescence images at day 30 (n = 6). Statistically significant differences between the treatment and control groups are indicated as \(^*\) \(P < 0.0003\) (Student t test). C, Representative micro-CT images of tibia from the DMSO- and GSK650394-treated groups. D, The myocardial cells injury after intraperitoneal injection of DMSO, GSK650394 (50 mg/kg), or GSK650394 (75 mg/kg) were determined by blood chemistry tests. LDH1, lactic dehydrogenase isoenzyme 1; LDH-L, lactic dehydrogenase; CK-MB, creatine kinase-MB. \(^*\) \(P < 0.05\) (Dunnett t test) versus the vehicle-treated group. E, The hepatic and renal function of mice after intraperitoneal injection of DMSO, GSK650394 (50 mg/kg), or GSK650394 (75 mg/kg) were determined by blood chemistry tests. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CR, creatinine. F, Apoptosis was analyzed using an Annexin V/PI Staining Kit. GSK650394 concentrations of 1, 2, and 5 \(\mu\)mol/L were used. G, MDA-MB-231 cells were treated with varying concentrations of GSK650394 (0, 1, 2, and 5 \(\mu\)mol/L) for 5 days. Cell proliferation was assessed by the Cell Counting Kit-8. Data are presented as the means ± SDs of three independent experiments.
chemotherapy, and SGK1 silencing could increase the toxicity of chemotherapeutic drugs. On the basis of the Oncomine database, we found the expression of SGK1 was significantly upregulated in different types of breast cancer compared with normal breast tissue. However, the result of further survival analysis in breast cancer varies from different database. To investigate the exact role of SGK1 in prognosis of breast cancer, further research should be conducted in specific population cohort with confounding factors adjusted.

SGK1-dependent regulation of Orai1 has been shown to play a critical role in regulation of cell proliferation (17). In this study, we investigated the effect of SGK1 on bone metastasis in breast cancer using an intracardiac injection-induced model of bone metastasis in mice. Our results demonstrated the effects of GSK650394 in the inhibition of breast cancer bone metastasis. Besides the effects on the cell proliferation, more application of SGK1 inhibition has been explored.

On the basis of this study, SGK1 is a powerful regulator in osteoclastogenesis and bone metastasis. SGK1 inhibitors might be a promising therapeutic approach target for various pathologic conditions including bone metastasis. Because we are just exploring the effect of SGK1 on osteoclastogenesis, the possible osteoblastic effect also needs to be considered. Previous study has confirmed that SGK1 could mediate osteoinductive effects of phosphate in the vascular tissue and trigger vascular calcification. SGK1 leads to IkBα phosphorylation and NF-κB activation, which results in osteo-/chondrogenic transdifferentiation of VSMCs. Further study needs to be done in the future (49–51).

There are several limitations to our study. Because SOCE is not the only calcium entry pathway, the calcium influx could be through t-type calcium channel, TRP channels, and so on. We used a selective SOCE-specific inhibitor BTP2 and monitored calcium influx after intracellular store depletion (52). The drug abolished the Ca2+ transient amplitude but cannot be fully rescued by Orai1 overexpression (Supplementary Fig. S1B). Also, upregulation of Orai1 is not the only way SGK1 modify Ca2+ activity. SGK1 can also have effects on activation of K+ channel or Ca2+-permeable TRP channels which could hyperpolarize the cell membrane and influence the Ca2+ channels. Our results only focused on Orai1, not on the whole family of Orai. It is also possible that different levels of other isoforms, Orai2 and Orai3, are present in the cells in both groups (53, 54). It highlights the need for future deeper studies to analyze the differences and connections among the three. Another limitation is that the effects of the inhibitor is not specific for bone metastasis, but also impair the survival of tumor cells. Although in our experiment, GSK650394 did not show a

Figure 6.
Validation of SGK1 from database. A, SGK1 mRNA expression was significantly higher in breast malignancy tissues (IDBC, LBC) compared with normal breast tissues based on the Oncomine database. **P < 0.01; *P < 0.05 (Dunnett t test) versus normal group. B, Overall survival curves of SGK1 according to its expression based on Kaplan-Meier plotter and datasets GSE2603 and GSE12276 from the GEO database.
direct effect on breast cancer apoptosis (Fig 5F), high levels of SGK1 have been found as compensation upon PI3K or AKT pathway and contribute to the resistance to AKT inhibitors in breast cancer cells, whereas loss of SGK1 profoundly impacts thyroid cancer cell proliferation and survival despite intact PI3K and AKT activity (50, 55, 56). Therefore, the effect of GSK650394 to bone metastasis may be composed of complex factors.

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

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Molecular Cancer Therapeutics

Serum- and Glucocorticoid-inducible Kinase 1 is Essential for Osteoclastogenesis and Promotes Breast Cancer Bone Metastasis

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