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14. ABSTRACT

The majority of breast tumors express the estrogen receptor α (ERα), which plays important roles in breast cancer pathogenesis and progression, and hormonal therapies, such as tamoxifen, are the first line of adjuvant therapy (1, 2). Unfortunately, half of these patients will ultimately fail therapy due to de novo or acquired resistance. Moreover, patients with ER, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2, also known as ErbB-2) triple negative breast cancer (TNBC), which is aggressive with high recurrence, metastatic, and mortality rates (3), do not respond to hormonal therapies and have limited treatment options. Epidemiological and clinical studies indicate that obesity, which is now endemic, increases breast cancer risk and is associated with worse prognosis (4), which may be due in part to the high frequency of TNBC and ineffectual hormonal therapy (5). However, the links between obesity and breast cancer are not understood and is the focuse of our study. As hormonal therapy is so effective with relatively few side effects, the possibility of reversing hormonal unresponsiveness is an appealing treatment approach. Our study will lead to novel therapies that will overcome the overarching challenges of developing safe and effective drugs for treating obesity-promoted cancers and TNBC and will identify the bioactive sphingolipid metabolite, sphingosine-1-phosphate (S1P), produced by sphingosine kinases (SphK1 and SphK2), as a critical factor that links obesity and chronic inflammation to drive breast cancer growth and metastasis.

15. SUBJECT TERMS-

Nothing listed

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1. INTRODUCTION

Approximately 15% of American women will be diagnosed with breast cancer, the 2nd leading cause of cancer death among women. Breast cancer death rates have been dropping due to advances in detection and better treatments, particularly hormonal therapies for the ~70% of breast cancers that express estrogen receptors (ER) and progesterone receptors (PR). Aromatase inhibitors, which prevent the production of estrogen, and direct ER antagonists, such as tamoxifen, are especially effective against ER positive tumors, and have less side effects than traditional chemo- and radio-therapies. The human epidermal growth factor receptor 2 (HER2), upregulated in 10 to 15% of breast cancers, can also be targeted with an inhibitory monoclonal antibody (Herceptin). However, hormonal therapies are ineffective in the 15 to 20% of tumors that are ER/PR/HER2 negative, termed triple negative breast cancer (TNBC). These tumors are more aggressive and metastatic, leading to significantly worse prognoses. In addition to de novo resistance, acquired resistance to these types of therapies is a major problem. Therefore, it is critical to that we understand the other signaling pathways that contribute to breast cancer progression and metastasis in order to develop new approaches for treatment of breast cancer. Our previous studies have demonstrated that the bioactive sphingolipid metabolite, sphingosine-1-phosphate (S1P), may be an attractive target for TNBC treatment as it regulates numerous physiological and cellular processes that are important for breast cancer, including cell proliferation and survival, migration and invasion important for metastasis (Maczis et al., 2016), inflammation that can paradoxically drive tumorigenesis, and angiogenesis that provides cancer cells with nutrients and oxygen. In particular, one of the enzymes that produces S1P, sphingosine kinase 1 (SphK1), is commonly upregulated in breast cancer cells and has been linked to increased cancer progression and poor prognosis, possibly leading to resistance to certain anti-cancer therapies. Recently, we found that the other sphingosine kinase isoform, SphK2, also plays an important role in breast cancer progression and metastasis. We believe that our current studies can lead to novel therapies that will overcome the overarching challenges of developing safe and effective novel drugs for treating obesity-promoted cancers, metastatic breast cancer, as well as TNBC, by identifying the bioactive sphingolipid metabolite, S1P produced by SphK1 and SphK2, as a critical factor that drives breast cancer proliferation and metastasis.

2. KEY WORDS

sphingosine-1-phosphate, sphingosine kinase, FTY720 (fingolimod, Gilenya), triple negative breast cancer, ERα, obesity, histone deacetylase, inflammation, tamoxifen resistance

3. ACCOMPLISHMENTS

3.1. Major Goals of the Project

Our project has three major aims.

Aim 1. Determine the role of SphK1 and S1P in obesity promoted chronic inflammation and tumor progression and decipher the molecular links between the SphK1/S1P/S1PR1 axis and persistent NF-kB and STAT3 activation.

Aim 2. Target the SphKs/S1P/S1PR1 axis with fingolimod/FTY720 for treatment of obesity associated breast cancer to suppress the malicious amplification cascade, and reactivate ER expression in ER-negative breast cancer.

Aim 3. Examine the association of the SphKs/S1P/S1PR1 axis in human breast cancer and prognosis.

The inability to effectively predict, prevent, and treat metastatic breast cancer is a major problem in breast cancer care. This proposal provides evidence that the SphKs/S1P/S1PR1 axis is one of the critical factors that drive breast cancer growth and metastasis and paves the way for development of new adjuvant therapies targeting this axis as a promising strategy for effective treatment of advanced and refractory breast cancer.

3.2. Accomplishments Under These Goals and Significant Results

Aim 1 & Aim 2: Targeting the SphK1/S1P/S1PR1 Axis That Links Obesity, Chronic Inflammation, and Breast Cancer Metastasis

Although obesity with associated inflammation is now recognized as a risk factor for breast cancer and distant metastases, the functional basis for these connections remains poorly understood. In a recently published paper, we show that in breast cancer patients and in animal breast cancer models, obesity is a sufficient cause for increased expression of S1P, which mediates cancer pathogenesis. We have completed our preclinical studies demonstrating that a high-fat diet was sufficient to upregulate expression of SphK1 along with its receptor S1PR1 in syngeneic and spontaneous breast cancer models. This work was published recently in Cancer Research (Nagahashi et al., 2018). We also clearly demonstrated in this study that targeting the SphK1/S1P/S1PR1 axis with FTY720/fingolimod attenuated key proinflammatory cytokines, macrophage infiltration, and tumor progression induced by obesity. S1P produced in lung premetastatic niches by tumor-induced SphK1 increased macrophage recruitment into the lung and induced IL6 and signaling pathways important for lung metastatic colonization. Importantly, FTY720 suppressed IL6, macrophage infiltration, and S1P-mediated signaling pathways in the lung induced by a high-fat diet, and it dramatically reduced formation of metastatic foci. In tumor-bearing mice, we also found that FTY720 similarly reduced obesity-related inflammation, S1P signaling, and pulmonary metastasis, thereby prolonging survival. Our results establish a critical role for circulating S1P produced by tumors and the SphK1/S1P/S1PR1 axis in obesity-related inflammation, formation of lung metastatic niches, and breast cancer metastasis, with potential implications for prevention and treatment (Fig. 1). These findings offer a preclinical proof of concept that signaling by a sphingolipid may be an effective target to prevent obesity-related breast cancer metastasis.

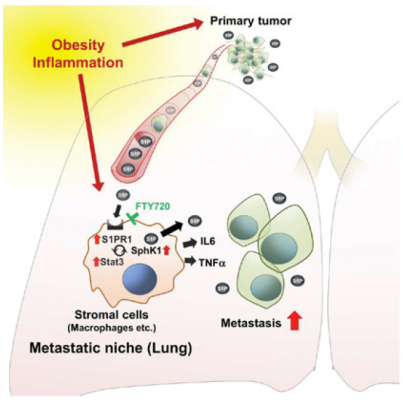

Figure 1. Model illustrating the role of SphK1/S1P/S1PR1 axis in the link between obesity, inflammation, and breast cancer progression and lung metastasis and targeting this axis with FTY720 for treatment.

Aim 3. Examine the Association of the SphKs/S1P/S1PR1 Axis in Human Breast Cancer and Prognosis

3a. ERalpha36 Stimulates SphK1 and Secretion of S1P: Involvement in Tamoxifen Resistance

17β-Estradiol (E2) plays critical roles in breast cancer mainly by binding to its canonical receptor ERα66 and eliciting genomic effects. E2 also triggers rapid, non-genomic responses. We found that E2 activates SphK1, increasing S1P that binds to its receptors leading to signaling important for breast cancer (Geffken and Spiegel, 2018). However, the E2 receptor responsible for SphK1 activation has not yet been identified. In a study we are submitting for publication, we demonstrated in TNBC cells, which lack the canonical ERα66 but express the novel splice variant ERα36, that ERα36 is the receptor responsible for E2-induced activation of SphK1 and formation and secretion of S1P and dihydro-S1P, the ligands for S1PRs. Tamoxifen, the first-line endocrine therapy for breast cancer, is an antagonist of ERα66, but an agonist of ERα36, and like E2, activates SphK1 and markedly increases secretion of S1P (Maczis et al., 2018, submitted).

A major problem with tamoxifen therapy is development of acquired resistance. We found that tamoxifen resistance correlates with increased SphK1 and ERα36 expression in tamoxifen-resistant breast cancer cells, in patient-derived xenografts (PDXs), and in endocrine resistant

breast cancer patients. Immunoblotting of PDXs showed that ERα-positive tumors have low expression of ERα36 compared to ERα-negative tumors that have significantly higher ERα36 expression (Fig. 2A). Similarly, ERα-negative PDXs have significantly higher levels of SphK1 (Fig. 2A). Moreover, mining of the Cancer Genome Atlas (TCGA) breast tumor expression database indicated that TNBC patients have greater SphK1 expression compared to all other breast cancer patients (Fig. 2B). Furthermore, tumors from hormone therapy-resistant patients also have significantly higher SphK1 levels than patients who respond to hormone therapies, such as tamoxifen (Fig. 2C). Our data also indicate that targeting this ERα36 and SphK1 axis may be a therapeutic option to circumvent endocrine resistance and improve patient outcome.

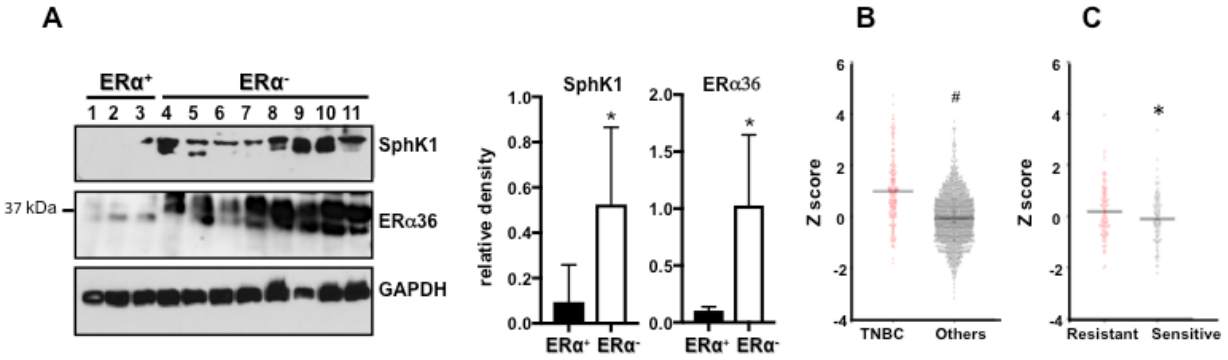

Figure 2. Tamoxifen resistance correlates with increased SphK1 and ERα36 expression in breast cancer patients. (A) Expression levels of SphK1 and ERα36 in the indicated PDXs derived from ERα positive (1-3) and negative (4-11) breast cancer patients were determined by immunoblot analysis and quantified by densitometry. Data are expressed as relative densities normalized to GAPDH. * $p \leq 0.05$. 1. HCI-03; 2. HCI-13; 3. HCI-11; 4 W2; 5. W30; 6. HCI-16; 7. HCI-10; 8. HCI-9; 9. HCI-1; 10. HCI-2; 11. UCD18. (B, C) Breast cancer patient subtypes were from clinical expression information contained within the Cancer Genome Atlas (TCGA) datasets. (**B**) SphK1 expression in TNBC *vs* other ERα-positive breast tumors, # p=0.0001. (**C**) Comparison of SphK1 expression in hormone therapy-sensitive and hormone therapy-resistant tumors. *p=0.04.

3b. ABCC1-Exported S1P Produced by SphK1 Shortens Survival of Mice and Patients with Breast Cancer

Previously, we observed that S1P is exported out of human breast cancer cells by ATP-binding cassette (ABC) transporter ABCC1, but not by ABCB1 (Takabe et al., 2010), both known multidrug resistance proteins that efflux chemotherapeutic agents. However, the pathologic consequences of these events to breast cancer progression and metastasis have not been elucidated. We recently demonstrated that high expression of ABCC1, but not ABCB1, is associated with poor prognosis in breast cancer patients. Overexpression of ABCC1, but not ABCB1, in human MCF7 and murine 4T1 breast cancer cells enhanced S1P secretion, proliferation, and migration of breast cancer cells. Implantation of breast cancer cells overexpressing ABCC1, but not ABCB1, into the mammary fat pad markedly enhanced tumor growth, angiogenesis, and lymphangiogenesis with a concomitant increase in lymph node and lung metastases as well as shorter survival of mice (Yamada et al., 2018). Interestingly, S1P exported via ABCC1 from breast cancer cells upregulated transcription of SphK1, thus promoting and amplifying S1P formation. Importantly, patients with breast cancers that express both activated SphK1 and ABCC1 have significantly shorter disease-free survival (Fig. 3). These findings suggest that export of S1P via ABCC1 functions in a malicious feed-forward manner to amplify the S1P axis involved in breast cancer progression and metastasis, which has important implications for prognosis of breast cancer patients and for potential therapeutic targets. Taken together, our results suggest that multidrug resistant transporter ABCC1 and

activation of SphK1 in breast cancer worsen patient's survival by export of S1P to the tumor microenvironment to enhance key processes involved in cancer progression. Our work further supports the notion that targeting SphK1 should be considered as a useful new therapeutic avenue for breast cancer, particularly TNBC.

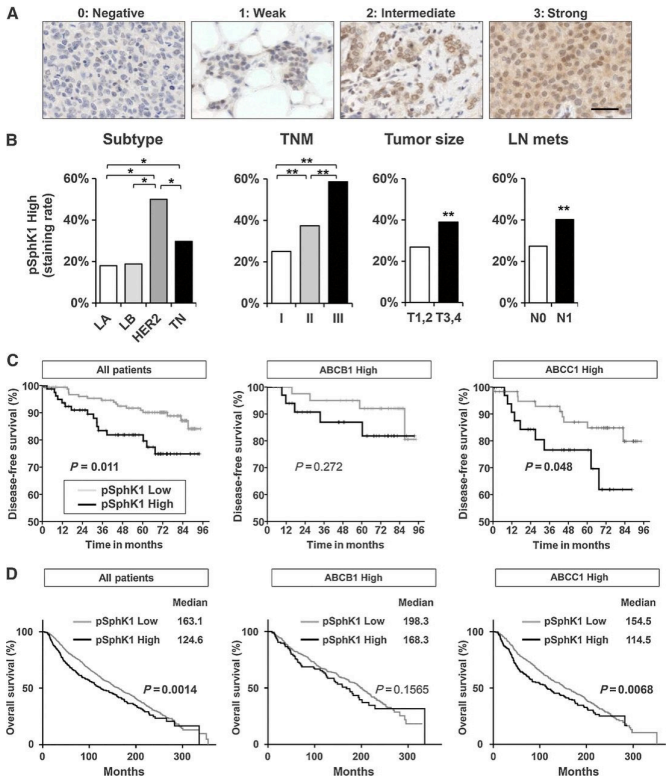

Figure 3. Shorter disease-free survival of patients with breast cancers that express both activated SphK1 and ABCC1. (A) pSphK1 in 275 human breast tumors examined by IHC. **(B)**, Scoring of pSphK1 expression in human breast tumor samples. **(C)** Frequency of high pSphK1 expression in human breast tumors correlated with clinicopathologic factors, tumor size, and lymph node metastasis status and TNM stage. *, P < 0.05. **(D)**, Kaplan–Meier disease-free survival curves according to expression of pSphK1, co-expression of pSphK1 with ABCB1, and coexpression of pSphK1 with ABCC1. **D**, Kaplan–Meier survival analysis of breast cancer patients from the METABRIC database. Data were obtained from patients with clinical and expression information. Median survival is tabulated along with a log-rank P value for significance of high gene expression of SphK1 among all patients, among ABCB1 high patients, or among ABCC1 high patients on patient survival.

Metastasis of breast tumors is greatly decreased by deletion or inhibition of SphK2

We recently made the unexpected observation that breast tumor growth and lung metastasis were greatly suppressed in female SphK2 knockout mice compared to littermate controls. We also found that pulmonary metastatic colonization was greatly reduced when wild type mice were treated with the SphK2 inhibitor SLM6013434. We are now working on this exciting project based on these observations examining the roles of SphK2 and p53 in non-cell autonomous antitumor immunity. We are hoping that by August 2019, we will be able to submit a high impact paper describing these findings.

3.3. Opportunities for Training and Professional Development

This project was not designed to provide training and professional development opportunities. However, we should note that the VCU School of Medicine has developed several new programs to enhance the training and professional development of graduate students and postdoctoral fellows. These vital programs provide these trainees, who are critical to the scientific endeavor, with career and mentoring resources based on the FASEB Individual Development Plan, including web-based tools for an Individual Development Plan, job opportunities in BioCareers, career resources from AAAS, CV/resume writing samples from UCSF Office of Career and Professional Development, and other career development websites. For graduate students these functions reside within the Office of Graduate Education, for Postdoctoral trainees, these reside in the Office of Research and Innovation. While no trainees were included in the original proposal, Melissa Maczis joined my lab two years ago as a PhD student and was supported by the VCU School of Medicine for the first two years. I have been

advising her on career development. Using the "Individual Development Plan" website, she created an Individual Development Plan (IDP) and has been using it to record the immediate and long-term objectives of her research and her career path plan. She has been making outstanding progress toward accomplishing her career goals and based on her accomplishments has successfully applied for and received a predoctoral fellowship from NIH (F31 CA220798).

3.4. How were the results disseminated to communities of interest

I presented several research lectures on this project to the cancer research community at the Massey Cancer Center Retreat and in the regular meetings of the Massey Cancer Center Cancer Cell Signaling Program, that I direct together with Dr. Andrew Larner. I also presented this work to the international scientific community in several Keynote lectures (See below). Melissa Maczis also presented her work in seminars to VCU graduate students.

3.5 The Plan for the Next Reporting Period

Continue as proposed in the original application. As mentioned above, we have already made substantial progress in Aim 1 and completed Aim 2 and have also continued to accomplish Aim 3. As discussed previously, we have uncovered unknown important links between SphK2 to lung metastasis in breast cancer that we are now pursuing.

4. IMPACT

4.1. The impact on the development of the principal discipline of the project

As mentioned in the previous report, our work suggests that a multi-pronged attack with FTY720 is a novel combination approach for effective treatment of conventional hormonal therapy-resistant breast cancer and triple-negative breast cancer. We previously found that the active phosphorylated form of FTY720 is a potent histone deacetylase inhibitor that reactivates ERalpha expression and enhances hormonal therapy for breast cancer (Hait et al., 2015). FTY720 has several advantages over available HDAC inhibitors as potential treatments for breast cancer patients: it is an orally bio-available pro-drug; it has already been approved for human use; it regulates expression of only a limited number of genes compared to other HDAC inhibitors; it has good pharmacokinetics and a long half life; it suppresses several survival and proliferative pathways; and it is much less toxic, accumulates in tumor tissues, and both the phosphorylated and unphosphorylated forms target important pathways in breast cancer. We also found that FTY720, which targets the SphK1/S1P/S1PR1 axis, prevents the amplification cascade and mitigates obesity-promoted metastatic niche formation and breast cancer metastasis (Yamada et al., 2018). Hence, we hope that our studies will pave the way for exploration of new clinical trials using FTY720 as a prototype of new adjuvant treatment strategies for hormonal resistant breast cancer. This might be particularly relevant in view of the increase in obesity that is now endemic and in de novo and acquired resistance to hormonal therapy. In an unrelated project, we found that treatment of mice with FTY720 targeted S1PR1 and blocked and reversed neuropathic pain induced by bortezomib (Stockstill et al., 2018). The development of chemotherapy-induced painful peripheral neuropathy is a major dose-limiting side effect of many chemotherapeutics, including bortezomib. As FTY720 also shows promising anticancer potential and is FDA approved, rapid clinical translation of our findings is anticipated. More recently, our work also suggests that targeting other components of the S1P axis, such as SphK2, other kinase involved in formation of S1P, might be especially beneficial for metastatic breast cancer.

4.2. The Impact on Other Disciplines

Although this work may not have a direct impact on other disciplines it might contribute to them, particularly in the treatment of cognitive impairment. HDAC inhibitors have shown promise as a treatment to combat the cognitive decline associated with aging and neurodegenerative disease, as well as to ameliorate the symptoms of depression and posttraumatic stress disorder, among others. Due to its unique features described above and its high brain penetration, FTY720 might be more effective than other HDAC inhibitors as an adjuvant therapy for erasing aversive memories (Hait et al., 2014). This might also be relevant to suppression of cognitive impairment and neuropathic pain associated with chemotherapy. Moreover, our recent work shows that targeting the S1P/S1PR1 axis by treatment with FTY720 greatly reduces cancer-induced bone pain and neuroinflammation (Grenald et al., 2017) and supports potential fast-track clinical application of the FDA-approved drug, FTY720, as a therapeutic avenue for preventing "chemo brain", i.e. chemotherapy-related cognitive impairment or cognitive dysfunction.

4.3. The Impact on Technology Transfer

Nothing to report

4.4. The impact on Society Beyond Science and Technology

Nothing to report

5. CHANGES/PROBLEMS

There are no significant changes in the project or its direction. We are pursuing our interesting results on the role of SphK2 in breast cancer growth and particularly in pulmonary metastasis. However, Melissa Maczis received her own fellowship from NIH (F31CA220798). Therefore, her salary will no longer be covered by the DOD. We have recruited an outstanding postdoctoral fellow to help completion of this project. We anticipate finishing this project by August 2019.

6. PRODUCTS

Publications

Geffken K, **Spiegel S**. Sphingosine kinase 1 in breast cancer. Adv Biol Regul. 2018 Jan;67:59-65. doi: 10.1016/j.jbior.2017.10.005. PMC5807162

Nagahashi M, Yamada A, Katsuta E, Aoyagi T, Huang WC, Terracina KP, Hait NC, Allegood JC, Tsuchida J, Yuza K, Nakajima M, Abe M, Sakimura K, Milstien S, Wakai T, **Spiegel S**, Takabe K. Targeting the SphK1/S1P/S1PR1 Axis That Links Obesity, Chronic Inflammation, and Breast Cancer Metastasis. Cancer Res. 2018 Apr 1;78(7):1713-1725. PMC in progress.

Yamada A, Nagahashi M, Aoyagi T, Huang WC, Lima S, Hait NC, Maiti A, Kida K, Terracina KP, Miyazaki H, Ishikawa T, Endo I, Waters MR, Qi Q, Yan L, Milstien S, **Spiegel S**, Takabe K. ABCC1-Exported Sphingosine-1-phosphate, Produced by Sphingosine Kinase 1, Shortens Survival of Mice and Patients with Breast Cancer. Mol Cancer Res. 2018 Jun;16(6):1059-1070. PMC in progress.

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Abstracts

Maczis MA, Maceyka M, Waters MR, Singh M, Turner TH, Alzubi M, Harrell JC, Milstien S, **Spiegel S**. Key Role of Sphingosine-1-phosphate in De novo and acquired tamoxifen resistant breast cancer. Massey Cancer Research Retreat, June 8, 2018

Maczis MA Lynch KR, Santo SL, Milstien S, **Spiegel S**. Importance of Sphingosine kinase 2 and

spinster 2 in breast cancer progression and metastasis. Massey Cancer Research Retreat, June 8, 2018

Maczis MA, Lynch KR, Santo WL, Milstien S, **Spiegel S**. The Sphingosine-1-Phosphate Gradient regulates Breast cancer Metastasis. 34th Daniel T. Watts Research Poster Symposium, VCU, October 26, 2017

Maczis MA, Milstien S, **Spiegel S**. Role of sphingosine-1-phosphate in non-genomic effects of estrogen in triple negative breast cancer. 34th Daniel T. Watts Research Poster Symposium, VCU, October 26, 2017

Lynch KR, Santo WL, Milstien S, **Spiegel S.** The Sphingosine-1-Phosphate Gradient Regulates Breast Cancer Metastasis. Maczis MA, FASEB Lysophospholipid and Related Mediators-from Bench to Clinic, New Orleans, 2017

Maczis MA, Milstien S, **Spiegel S**. Role of sphingosine-1-phosphate in non-genomic effects of estrogen in triple negative breast cancer. FASEB Lysophospholipid and Related Mediators-from Bench to Clinic, New Orleans, 2017

Maczis MA, Milstien S, **Spiegel S**. Non-genomic effects of estrogen Stimulated Sphingosine-1-Phosphate Signaling in triple negative breast cancer Expressing only Estrogen receptor alpha 36. Massey Cancer Research Retreat, June 16, 2017

Presentations

1. New aspects of sphingosine-1-phosphate in inflammation and cancer, 2017 FASEB Conference on Lysophospholipid and Related Mediators: from Bench to Clinic, New Orleans, LA, August 20-25, 2017
2. New aspects of sphingosine-1-phosphate in inflammation and cancer, Keynote Lecture, 12th Sphingolipid Club Meeting, Trabia, Sicily, Italy, September 6-10, 2017
3. S1P gradient and Spns2 transporter in lymphocyte trafficking and breast cancer metastasis, The FEBS Congress 2017, Jerusalem, Israel, September 10-14, 2017
4. S1P in breast cancer metastasis, Fifty-Eighth International Symposium on Biological Regulation and Enzyme Activity in Normal and Neoplastic Tissues University of Bologna, Italy October 2-3, 2017
5. Sphingosine-1-Phosphate In Breast Cancer Metastasis, 3rd Meeting of the GRK Sphingolipids in health and diseases, Essen, Germany, December 6-8, 2017
6. Sphingosine Kinase 1 in Endocytic Membrane Trafficking and p53-Dependent Autophagic Cell Death, Gordon Research Conference on Glyco and Sphingolipid Biology, Galveston, Texas, February 11 - 16, 2018

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals who have worked on the project

Name: Sarah Spiegel
Project Role: PI – No change

Name: Sheldon Milstien
Project Role: Co-Investigator – No change

Name: Kazuaki Takabe
Project Role: Co-Investigator – Left VCU June 2016, now at Roswell Park, Clinical Chief of Breast Surgery and Breast Disease Site Leader. We will continue our long-standing collaboration but his salary will be covered by his new institution.

Name: Melissa Maczis

Project Role: Graduate Student
Will continue to work on this project. However, since she is an outstanding student, she received a fellowship from NIH and her salary starting July 1, 2017 will not be covered by the current DOD grant.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? No changes from the last reporting period.

What other organizations were involved as partners?
Nothing to Report

8. REFERENCES

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9. APPENDICES

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Sphingosine kinase 1 in breast cancer

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ABSTRACT

Breast cancer affects 1 out of 8 women in the US and is the second highest cause of death from cancer for women, leading to considerable research examining the causes, progression, and treatment of breast cancer. Over the last two decades, sphingosine-1-phosphate (S1P), a potent sphingolipid metabolite, has been implicated in many processes important for breast cancer including growth, progression, transformation and metastasis, and is the focus of this review. In particular, one of the kinases that produces S1P, sphingosine kinase 1 (SphK1), has come under increasing scrutiny as it is commonly upregulated in breast cancer cells and has been linked with poorer prognosis and progression, possibly leading to resistance to certain anti-cancer therapies. In this review, we will also discuss preclinical studies of both estrogen receptor (ER) positive as well as triple-negative breast cancer mouse models with inhibitors of SphK1 and other compounds that target the S1P axis and have shown good promise in reducing tumor growth and metastasis. It is hoped that in the future this will lead to development of novel combination approaches for effective treatment of both conventional hormonal therapy-resistant breast cancer and triple-negative breast cancer.

1. Introduction

Breast cancer affects nearly 1 out of every 8 women over their lifetime and is the second leading cancer cause of death for women behind lung cancer in the US. Fortunately, over the last 30 years, breast cancer death rates have been dropping due to increased awareness of the disease, advances in detection, and better treatments. A large factor in these better treatments has been development of hormonal therapies to directly target specific receptors in the cancer cells such as estrogen (ER) and progesterone receptors (PR) that are present in roughly 70% of breast cancers. ER positive tumors in particular can be treated with estrogen antagonists such as tamoxifen to great effect with less side effects than traditional chemotherapy. The human epidermal growth factor receptor 2 (HER2), that is upregulated in 10–15% of breast cancers tumors can also be treated with a monoclonal antibody. However, there are still 15–20% of tumors that are ER/PR/HER2 negative, termed triple negative breast cancer (TNBC), which are usually more aggressive and metastatic with significantly worse prognosis. Therefore, current cancer research is also focused on deeper understanding of novel signaling pathways that can contribute to breast cancer growth and metastasis. In the last 20 years, it has become apparent that the bioactive sphingolipid metabolite, sphingosine-1-phosphate (S1P), regulates processes important for breast cancer including inflammation that can drive tumorigenesis, angiogenesis, which provides cancer cells with nutrients and oxygen, cell growth and

Abbreviations: BCSCs, breast cancer stem cells; ER, estrogen receptor; EGFR, epidermal growth factor receptor; ERK, extracellular signal regulated kinase; E2, 17β-estradiol; HDAC, histone deacetylase; HER2, human epidermal growth factor receptor 2; MAPK, mitogen activated protein kinase; PKC, protein kinase C; RTK, receptor tyrosine kinase; SphK, sphingosine kinase; S1P, sphingosine-1-phosphate; S1PR, S1P receptor; TNBC, triple-negative breast cancer

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survival, as well as migration and invasion important for metastasis (Espaillat et al., 2017; Maczis et al., 2016; Nagahashi et al., 2014; Newton et al., 2015; Pyne et al., 2014, 2016; Pyne and Pyne, 2010). In this review, we will summarize current research findings on S1P in breast cancer and examine the roles of the S1P/sphingosine kinase 1 (SphK1) axis in breast cancer signaling, prognosis, progression and as a possible target for future treatments, especially for TNBC and tumors that show resistance to typical first line treatments.

2. Formation of sphingosine-1-phosphate

Sphingolipids are important membrane constituents of all eukaryotic cells that also generate bioactive metabolites, such as S1P. The formation of S1P from sphingosine, produced by degradation of sphingolipids, begins with the activation of one of two enzymes, SphK1 or SphK2, resulting in the former case in its translocation from the cytosolic compartment to the plasma membrane where its substrate sphingosine resides (Hannun and Obeid, 2008). Numerous growth factors such as EGF, hormones, such as estradiol (E2), and pro-inflammatory cytokines such as IL-1 and IL-6 activate SphK1 (Gao et al., 2015; Maceyka et al., 2012; Maceyka and Spiegel, 2014; Maczis et al., 2016). In many cases, it has been shown that this is due to stimulation of extracellular signal-regulated kinases 1/2 (ERK1/2) that in turn phosphorylate SphK1 on Ser225 allowing for its specific targeting to the plasma membrane (Pitson et al., 2003). This is in contrast to SphK2 that also resides in intracellular compartments, including the nucleus, and produces S1P there (Hait et al., 2009). As with other potent mediators, S1P is rapidly turned over either by dephosphorylation back to sphingosine by phosphatases or irreversibly cleaved by S1P lyase to ethanolamine phosphate and hexadecenal (Aguilar and Saba, 2012; Hannun and Obeid, 2008; Maceyka and Spiegel, 2014).

3. Sphingosine-1-phosphate signaling in breast cancer

Following activation of SphK1 and restricted formation of S1P, the majority of the effects mediated by S1P occur after its export from the cell by the specific transporter called spinster 2 (Spns2) or by ATP-binding cassette transporters ABCA1, ABCC1, and ABCG2. S1P then can bind to one of five specific G protein-coupled cell surface S1P receptors (S1PR1-5) in an autocrine/paracrine manner, termed “inside-out” signaling. This leads to stimulation of downstream signaling mediated by overlapping G-proteins (Maceyka et al., 2012; Maczis et al., 2016; Takabe et al., 2008) (Fig. 1). A complete description of all of the interconnected signaling pathways that are activated by S1P is beyond the scope of this review, and this area has been extensively reviewed (Kihara et al., 2014; Pyne et al., 2016). Therefore, we will mainly focus on S1PR1 and S1PR3, two receptors that have been linked to breast cancer progression.

Intriguingly, S1PR1 has been linked to persistent activation of signal transducer and activator of transcription 3 (STAT3). STAT3

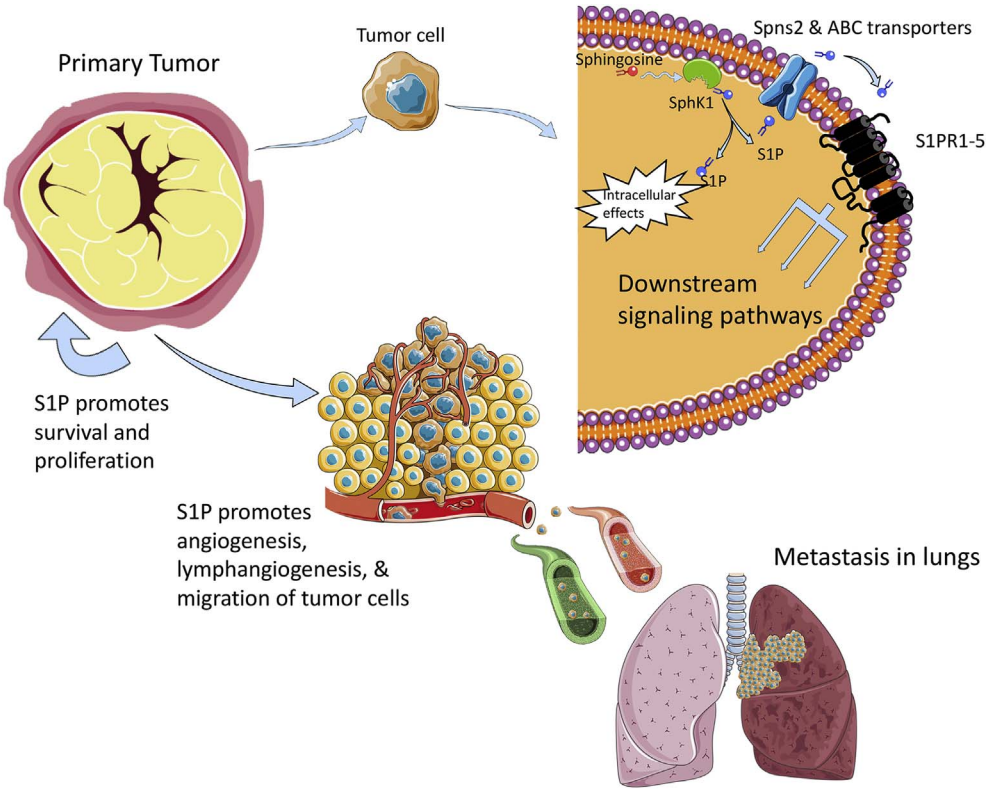

Fig. 1. Role of the SphK1/S1P axis in breast tumor progression and metastasis.

has been shown to be involved in many aspects of tumor growth and metastasis by activating a wide range of pathways promoting proliferation, survival, inflammation, invasion, and angiogenesis (Yu et al., 2014). STAT3 also enhances transcription of S1PR1 and activation of S1PR1 by S1P reciprocally activates STAT3 (Alshaker et al., 2014, 2015; Espaillat et al., 2017; Lee et al., 2010; Liang et al., 2013; Nagahashi et al., 2014). In breast cancer in particular, persistent STAT3 activation seems to be mainly due to upregulation of the pro-inflammatory cytokine IL-6 and S1PR1 (Alshaker et al., 2014, 2015; Lee et al., 2010). Moreover, IL-6 can activate SphK1 leading to a strong feed-forward mechanism promoting cancer cell progression (Lee et al., 2010). This signaling pathway is further complicated in ER negative breast cancer cells. as the adipokine leptin, a product of adipocytes, has also been shown to upregulate STAT3 and SphK1. SphK1 in turn induces production of IL-6, which then activates STAT3 (Alshaker et al., 2014, 2015). Pharmacological and molecular approaches further demonstrated that leptin-induced SphK1 activity and expression are mediated by activation of ERK1/2 and Src family kinase pathways, but not by the major pathways downstream of the leptin receptor, janus kinase 2 (JAK2) (Alshaker et al., 2015). As obesity is a risk factor for breast cancer and related to poorer prognosis, these studies could have implications for ER-negative breast cancer.

Binding of S1P to S1PR1 has also been shown to activate various receptor tyrosine kinases (RTKs) important for angiogenesis and proliferation such as VEGFR, EGFR, and PDGFR. This can result in “criss-cross” pathway activations as the growth factors that activate these RTKs can also activate SphK1. For example, EGF activation of SphK1 plays an important role in the migration of breast cancer cells towards EGF along with increased cell growth (Sarkar et al., 2005). S1P also potentiates the EGFR signaling pathway by insulin-like growth factor binding protein 3 (IGFBP-3), a growth promoter associated with poorer prognosis, suggesting that inhibition of both EGFR and SphK1 could have beneficial therapeutic effects in TNBC (Martin et al., 2014). Moreover, VEGF-mediated activation of SphK1 plays an essential role in regulating angiogenesis and lymphangiogenesis (Anelli et al., 2010; Nagahashi et al., 2012).

As for S1PR3, its activation via S1P was linked to the activation of the Notch signaling pathway along with p38MAPK in breast cancer stem cells (BCSCs) leading to proliferation and tumorigenicity (Hirata et al., 2014). BCSCs can also be activated by carcinogens, such as benzyl butyl phthalate, which has been shown to increase SphK1 expression leading to S1PR3 activation, implying that S1PR3 is a determinant of pollutant-driven breast cancer metastasis (Wang et al., 2016).

Most of S1PR3's cancer promoting and pro-survival effects can be attributed to sustained activation of ERK1/2 and AKT/PI3K pathways, key regulators of cell cycle progression, survival, and proliferation mechanisms in breast cancer cells (Datta et al., 2014; Wang et al., 2016; Watson et al., 2010). In triple-negative MDA-MB-231 breast cancer cells, early and sustained phosphorylation of both ERK1/2 and AKT/PI3K was inhibited by a SphK1 inhibitor while only sustained activation was inhibited by pertussis toxin, a potent G protein inhibitor, suggesting that S1PRs are crucial only for sustained activation (Datta et al., 2014). Aside from activating its own downstream signaling cascade, the AKT/PI3K pathway is involved in crosstalk with several other pathways, including RAS/RAF/MEK and ER, further strengthening the interconnecting pro-survival and progression pathways (Maiti et al., 2017). Another study in TNBC cells substantiated a link between sphingosine, SphK1, and the protein kinase C (PKC) serine/threonine kinase family, important regulators of cell proliferation and survival (Kotelevets et al., 2012). This study also showed that targeting SphK1 in triple-negative MDA-MB-231 breast cancer cells decreased proliferation and survival by compromising PKC activity and cytokinesis (Kotelevets et al., 2012). While the exact mechanisms of these pathways have not been elucidated, they support the significance of SphK1 as a target for cancer therapy. A recent study with MDA-MB-231 cells looked at how S1P signaling affected adhesion and invasion via the tumor cell microenvironment. It was reported that extracellular matrix rigidity-dependent S1P secretion regulates metastatic cancer cell invasion and adhesion (Ko et al., 2016). These results suggest that alterations in the mechanical environment of the extracellular matrix surrounding the tumor cells actively regulate secretion of S1P, which in turn, may contribute to cancer progression. In summary, many of the pathways modulated by the SphK1/S1P/S1PR axis in breast cancer cells are overlapping, promoting their growth, survival, proliferation, and metastasis (Fig. 1).

In addition to the very well-known functions of S1P as a ligand for S1PRs, recent studies suggest that S1P also has important intracellular actions (Maceyka et al., 2012). Especially relevant is the observation that SphK2 is present in the nucleus of many breast cancer cell lines (Hait et al., 2009; Igarashi et al., 2003; Sankala et al., 2007) where it produces S1P that inhibits class I histone deacetylases (HDACs) (Hait et al., 2009). Thus, it was suggested that HDACs are direct intracellular targets of S1P and link nuclear sphingolipid metabolism and S1P to epigenetic regulation of expression of specific genes (Hait et al., 2009). Recently, we found that FTY720 is also phosphorylated in breast cancer cells by nuclear SphK2 and accumulates there. Moreover, like S1P, nuclear FTY720-P is also a potent inhibitor of class I HDACs. Furthermore, we observed that high fat diet increased triple-negative spontaneous breast tumors and HDAC activity in MMTV-PyMT transgenic mice that was suppressed by oral administration of FTY720. Interestingly, this treatment not only inhibited HDACs, it also reversed high fat diet-induced loss of ER and PR in advanced carcinoma (Hait et al., 2015). Furthermore, treatment with FTY720 also re-expressed ER and increased therapeutic sensitivity of TNBC syngeneic breast tumors to tamoxifen in vivo more potently than a known HDAC inhibitor. This work suggests that in combination, FTY720 could be an effective treatment of both conventional hormonal therapy-resistant breast cancer and triple-negative breast cancer (Hait et al., 2015).

4. Sphingosine kinase 1 and estrogen receptor signaling

Nearly 80% of breast cancers are ER positive, meaning they are dependent on estrogens such as 17β-estradiol (E2) to signal growth, proliferation and metastasis. E2 normally binds to ER in the cytoplasm and after dimerization, translocates to the nucleus. In the nucleus, the ER dimers bind to estrogen response elements and act as transcription factors to activate or repress gene transcription (Klinge, 2001). E2 can also induce rapid, non-genomic cellular changes through membrane ERs that are still ill defined, including the

splice variant ER36 and the G protein-coupled receptor GPR30 (Wang and Yin, 2015; Zhou et al., 2016). These membrane ERs have been shown to activate SphK1, producing S1P and activate signaling pathways downstream of S1PRs leading to increased cell growth, higher microvessel density in tumors, and enhanced resistance to anti-cancer drugs in response to hormonal therapies (Maczis et al., 2016; Sukocheva et al., 2006, 2013; Takabe et al., 2010). GPR30 was suggested to activate SphK1 as its downregulation by anti-sense oligonucleotides inhibited E2-mediated activation of SphK1 in MCF-7 breast cancer cells (Sukocheva et al., 2006). However, the identity of the responsible receptor has not yet been conclusively established. E2-mediated formation of S1P led to rapid release of S1P from breast cancer cells via the ABCC1 and the ABCG2 transporters (Takabe et al., 2010) and “inside out” signaling by S1P (Maczis et al., 2016; Sukocheva et al., 2006, 2013). Furthermore, inhibiting these transporters blocked E2-induced activation of ERK1/2 (Takabe et al., 2010). It was convincingly demonstrated that activation of S1PR3 by S1P transactivated EGFR through a pathway mediated by Src and matrix metalloproteases. This switch from E2/ER-mediated growth to SphK1/EGFR activation has also been thought to contribute to resistance to hormonal therapies such as tamoxifen (Maczis et al., 2016; Sukocheva and Wadham, 2014; Sukocheva et al., 2006).

SphK1 activity has also been linked to the effects of several microRNAs that are regulated by ER. miR-515-5P, a tumor suppresser, was shown to reduce SphK1 activity and loss of miR-515-5P resulted in increased oncogenic SphK1 activity. In addition, E2 treatment downregulated miR-515-5P levels, and miR-515-5p is downregulated in ER-positive compared to ER-negative breast cancers (Pinho et al., 2013).

5. Sphingosine kinase 1 and breast cancer prognosis

Over the last few years, new evidence from several studies has illuminated the multi-factorial role of the SphK1/S1P axis in breast cancer and its link with worse prognosis and overall outcomes (Maczis et al., 2016; Ruckhaberle et al., 2008). It also usually corresponds with upregulation of associated S1PRs and chemotherapeutic resistance (Gao et al., 2015). In one study, 62.5% of tumors analyzed (20 out of 32) had at least a 2-fold increase in SphK1 mRNA expression compared to surrounding normal breast tissue (Datta et al., 2014). Furthermore, ER negative tumors had higher SphK1 levels than ER-positive tumors and the deadlier, triple-negative tumors had the highest levels of SphK1 expression of all tumor types examined. Overall, the analysis revealed an inverse correlation between SphK1 levels and survival of breast cancer patients. One of the possible causes investigated in this study was resistance to doxorubicin and docetaxel-based chemotherapies, mainstays for treatment of ER positive breast cancer, and it was found that non-responders to treatment had significantly higher SphK1 mRNA levels. This infers that SphK1 does not just promote progression and growth of tumors but also impacts survival through its effects on drug resistance (Datta et al., 2014). Patients with high levels of cytoplasmic Sphk1 compared to low SphK1 had a nearly 8-years shorter mean time to recurrence on tamoxifen (12.61 years with low SphK1 and 4.65 years with high SphK1 expression). Further investigations examined expression of S1PR1 and S1PR3 in particular and it was noted that patients with high membrane S1PR1 had a roughly 3 years shorter mean time to recurrence on tamoxifen and just over 8 years shorter disease-specific survival. It has been speculated that these differences in recurrence and survival could be due to E2 activation of SphK1 leading to the activation of the ERK1/2 pathways downstream of S1PR3 (Watson et al., 2010). Similar observations were made in another study (Ohotski et al., 2013).

Interestingly, S1P levels in breast cancer patients with lymph node metastasis that correlate with poor prognosis were significantly higher than those with negative lymph nodes, consistent with the notion that S1P plays an important role in angiogenesis, lymphangiogenesis, and metastasis (Tsuchida et al., 2016). Another interesting finding was that SphK1 levels determined by immunohistochemistry in deadlier TNBC tumors were lower, in contrast with some earlier studies. However, the S1P levels were higher, possibly suggesting the tumor microenvironment is responsible for the increase in S1P, not the tumor itself. This agreed with their observation of higher levels of S1P in patients with increased white blood cells, and suggested that since TNBCs are more immunogenic and immune cells express SphK1 and secrete S1P, they could increase S1P levels in the microenvironment (Tsuchida et al., 2016).

In sum, high levels of SphK1 expression and resulting high levels of S1P are most likely related to poorer prognosis for most patients. This could be due to the ability of the SphK1/S1P axis to promote cancer cell growth, proliferation, survival, and drug resistance. Thus, decreasing SphK1 expression and activity and S1P production could represent a new approach to improve prognosis of breast cancer.

6. Sphingosine kinase 1 in animal models of breast cancer progression and metastasis

Most of the data on SphK1 and its relationship to breast cancer in humans have come from analysis of tumor samples combined with patient follow-up data. An increasing number of studies have used mouse models to examine the role of the SphK1/S1P axis in breast cancer progression. The first observation was that breast cancer cells stably overexpressing SphK1 formed more and larger tumors in mice than vector transfectants with higher microvessel density in their periphery (Nava et al., 2002). Similar results were obtained by orthotopically implanting 4T1-luc2 murine breast cancer cells into the mammary fat pads of immune competent female mice (Nagahashi et al., 2012). The 4T1-luc2 tumors are rapidly growing and metastasize first to the lymph nodes and then the lungs, reminiscent to human breast cancer progression. Interestingly, circulating levels of S1P in tumor bearing mice were also significantly increased. Treatment of these mice with the specific SphK1 inhibitor SKI-1 decreased plasma S1P levels concomitantly with significant reductions in tumor volume, weight, and mitotic activity as well as lymph node and lung metastsis (Nagahashi et al., 2012). Moreover, cancer stem cells overexpressing SphK1 had increased ability to develop tumors in nude mice. Tumorigenicity of these cancer stem cells was inhibited by S1PR3 knockdown or a S1PR3 antagonist indicating that S1P promotes expansion of cancer stem

cells via S1PR3 by a ligand-independent Notch activation (Hirata et al., 2014).

Growth of tumors to beyond a certain size requires the formation of new blood vessels, termed angiogenesis, to continue to feed the rapidly growing and dividing cells (Nagahashi et al., 2012). To further spread throughout the body, the tumor cells usually extravasate and travel through the lymph system while also promoting formation of new lymph vessels through lymphangiogenesis. Both tumor size and metastasis are crucial in determining the staging and prognosis of a cancer (Nagahashi et al., 2016). There are also many cellular factors that contribute to angiogenesis and lymphangiogenesis, and perhaps others still to be discovered. However, it is becoming clear that S1P plays an important role in these processes. The angiogenic and lymphangiogenic actions of S1P are likely mediated via activation of S1PR1 on endothelial cells (Anelli et al., 2010; Nagahashi et al., 2012). As discussed above, S1P is commonly elevated in cancer tissues and in the circulation and also in lymph interstitial fluid from human breast cancer tumors (Nagahashi et al., 2016).

7. SphK1/S1P/S1P receptor axis as a therapeutic target for breast cancer

With such strong connections between SphK1/S1P/S1PR axis and the growth and progression of breast cancer cells, SphK1 and S1PR offer new and novel targets for possible future treatment avenues aimed at treating breast cancer, especially TNBC. Several preclinical studies have used mouse breast cancer models to investigate the effects of SphK1 inhibiters or S1PR modulators on tumor growth. A combination of the non-specific SphK inhibitor SKI-II with gefitinib, an EGFR inhibitor, significantly inhibited growth of xenograft MDA-MB-468 TNBC tumors whereas neither SKI-II or gefitinib alone had any effects (Martin et al., 2014). Another SphK1 inhibitor, SKI-5C, also significantly reduced growth of tumors from another TNBC cell line, MDA-MB-231, in xenografted SCID mice (Datta et al., 2014). Using an improved syngeneic breast cancer cell implantation method that mimics human breast cancer biology better than conventional xenograft subcutaneous implantation, treatment with the specific SphK1 inhibitor SK1-I suppressed tumor growth of murine 4T1 breast cancer cells and S1P levels and reduced metastases to lymph nodes and lungs (Nagahashi et al., 2012).

Lastly, one of the most promising possible future avenues for breast cancer treatments that target the S1P axis is Fingolimod (FTY720), a sphingosine analog pro-drug currently used to treat multiple sclerosis that has long been known to have beneficial effects in many preclinical breast cancer models (Azuma et al., 2002; Deng et al., 2012; Hait et al., 2015; Rincon et al., 2015). FTY720 effects are not limited only to suppressing the development and progression of breast tumors on its own but also is an effective adjuvant therapy. Treatment with FTY720 potentiated the anti-cancer effects of doxorubicin in MDA-MB-231 xenograft tumors and particularly in MDA-MB-231 cells that acquired resistance to doxorubicin (Rincon et al., 2015). FTY720 has been shown to synergize with the effect of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) reducing tumor volume and inducing apoptosis in xenograft breast cancer models without affecting normal cells (Woo et al., 2015). FTY720 has several anti-cancer targets that contribute to its multi-potent effectiveness. When phosphorylated by SphK2, FTY720-P is a S1P mimetic that acts as a functional antagonist of S1PR1, reducing the persistent activation of STAT3 (Deng et al., 2012) and thus diminishes accumulation of regulatory T cells in tumors (Priceman et al., 2014).

We found that FTY720 is phosphorylated by nuclear SphK2 in breast cancer cells. FTY720-P accumulates in the nucleus and potently inhibits class I histone deacetylases (HDACs) leading to increased histone acetylations and expression of a restricted set of genes independently of its known effects on S1PRs. We also observed that feeding a high-fat diet accelerated formation of tumors and increased triple-negative spontaneous breast tumors in MMTV-PyMT transgenic mice and that oral treatment with FTY720 inhibited development and aggressiveness of spontaneous breast tumors in these mice, reduced HDAC activity and dramatically reversed high-fat diet-induced loss of ER and PR in advanced carcinoma. Like other HDAC inhibitors, treatment of ER-negative breast cancer cells with FTY720 reactivated expression of silenced ER and sensitized them to tamoxifen. Furthermore, treatment with FTY720 also re-expressed ER and increased therapeutic sensitivity of ER-negative syngeneic breast tumors to tamoxifen in vivo more strongly than a pan HDAC inhibitor.

Unphosphorylated FTY720 also has anti-cancer actions. It inhibits SphK1 by binding to an allosteric site that exerts auto-inhibition on the catalytic site. It also induces proteasomal degradation of SphK1 and thus inhibits actions of S1P (Lim et al., 2011). Moreover, part of the effectiveness of FTY720 in tumor suppression can be attributed to its ability to activate the tumor suppressor PP2A (Perrotti and Neviani, 2013; Saddoughi et al., 2013), which is commonly inhibited in breast cancer and is crucial for maintaining tumor cell properties (Rincon et al., 2015).

Overall these studies show that FTY720 is a multi-faceted drug with the potential to work as an effective anti-cancer drug by itself and also as an adjuvant to hormonal therapies, traditional chemotherapies, and even radiation therapies to treat not only ER-positive tumors but also the more difficult TNBCs and tumors that develop resistance to chemotherapeutic agents. As FTY720 is already an FDA approved drug for treating humans, it is hoped that it can re-purposed for use as a cancer treatment.

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Competing interests statement

The authors declare no competing financial interests.

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Tumor Biology and Immunology

Cancer Research

Targeting the SphK1/S1P/S1PR1 Axis That Links Obesity, Chronic Inflammation, and Breast Cancer Metastasis

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Abstract

Although obesity with associated inflammation is now recognized as a risk factor for breast cancer and distant metastases, the functional basis for these connections remain poorly understood. Here, we show that in breast cancer patients and in animal breast cancer models, obesity is a sufficient cause for increased expression of the bioactive sphingolipid mediator sphingosine-1-phosphate (S1P), which mediates cancer pathogenesis. A high-fat diet was sufficient to upregulate expression of sphingosine kinase 1 (SphK1), the enzyme that produces S1P, along with its receptor S1PR1 in syngeneic and spontaneous breast tumors. Targeting the SphK1/S1P/S1PR1 axis with FTY720/fingolimod attenuated key proinflammatory cytokines, macrophage infiltration, and tumor progression induced by obesity. S1P produced in the lung premetastatic niche by tumor-induced SphK1 increased macrophage recruitment into the lung and induced IL6 and signaling pathways important for lung metastatic colonization. Conversely, FTY720 suppressed IL6, macrophage infiltration, and S1P-mediated signaling pathways in the lung induced by a high-fat diet, and it dramatically reduced formation of metastatic foci. In tumor-bearing mice, FTY720 similarly reduced obesity-related inflammation, S1P signaling, and pulmonary metastasis, thereby prolonging survival. Taken together, our results establish a critical role for circulating S1P produced by tumors and the SphK1/S1P/S1PR1 axis in obesity-related inflammation, formation of lung metastatic niches, and breast cancer metastasis, with potential implications for prevention and treatment.

Significance: These findings offer a preclinical proof of concept that signaling by a sphingolipid may be an effective target to prevent obesity-related breast cancer metastasis. *Cancer Res; 78(7); 1713–25.* *©2018 AACR.*

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Introduction

Obesity has drastically increased to become one of the leading health concerns in the United States (1), and is now recognized as a risk factor for breast cancer incidence, progression, recurrence, and prognosis (2, 3). Epidemiologic and clinical studies indicate that obesity increases breast cancer risk by approximately 40% in postmenopausal women and is associated with endocrine therapy resistance (2, 4). Obese breast cancer patients are more likely to be diagnosed with larger, higher-grade tumors, have an increased incidence of distant metastases, and elevated risks of recurrence and death (3, 5). However, the mechanisms by which obesity increases breast cancer incidence and worsens prognosis remain ill defined.

High body weight has also been associated with larger and more aggressive tumors in animal models of breast cancer (6–10). Obesity in both humans and rodents is characterized by increased production of insulin and growth factors, low-grade chronic inflammation, and secretion of proinflammatory cytokines that regulate breast cancer development and progression (11–13). Animal models that recapitulate human cancers, such as syngeneic or transgenic murine models with intact immune functions, have provided important clues to the critical roles of the cytokines TNFα and IL6, and have highlighted the key roles played by the master transcription factors NFκB and STAT3 in the link between chronic inflammation and

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breast cancer (14–16). Infiltration of macrophages (12) that produce these cytokines into the tumor microenvironment is now recognized as an important enabler of cancer progression, and tumor-associated macrophages (TAM) correlate with increased angiogenesis, metastasis, and decreased survival of breast cancer patients (17). Macrophages have also been shown to be recruited to premetastatic niches, specialized microenvironments in distant organs primed by factors secreted from cancer cells that promote metastatic progression (18–20), but the underlying mechanisms guiding their assembly are largely unknown.

There is growing evidence that sphingosine-1-phosphate (S1P), a pleiotropic bioactive sphingolipid metabolite enriched both in blood and lymphatic fluid is involved in inflammation, obesity, and breast cancer (21). S1P generated by activation of sphingosine kinase 1 (SphK1) is exported out of cells and signals through specific S1P receptors to regulate numerous cellular processes important for breast cancer, including cell growth, survival, invasion, immune cell trafficking, vascular integrity, angiogenesis, and cytokine and chemokine production (22–25). Previous clinical studies have shown that SphK1 is overexpressed in breast cancer and its expression is associated with poor patient outcomes (26, 27). Because it has been suggested that S1P levels are elevated in plasma of obese humans and rodents (28), in this work we explored the role of S1P in obesity promoted breast cancer in patients and in animal models. We uncovered that the SphK1/S1P/S1PR1 axis is a critical factor linking obesity, low-grade chronic inflammation, and breast cancer, identified S1P as an important new factor in metastatic niche formation and demonstrated that targeting the SphK1/S1P/S1PR1 axis is an effective treatment for metastatic breast cancer exacerbated by obesity.

Materials and Methods

Cell culture

A C57Bl/6 mouse mammary fat pad–derived adenocarcinoma cell line E0771 was obtained from CH3 BioSystems. A BALB/c mouse mammary fat pad–derived adenocarcinoma cell line 4T1-luc2 that has been engineered to express luciferase was obtained from PerkinElmer. E0771 cells were cultured in DMEM with 10% FBS. 4T1-luc2 cells were cultured in RPMI medium 1640 with 10% FBS. SphK1 was overexpressed by transfection with Lipofectamine Plus (Invitrogen) as described (29). Transfection efficiency was determined by quantitative PCR (qPCR) and Western blot analysis. All these cell lines were used within 10 passages after reception in the current experiments, and have been routinely tested for mycoplasma contamination using the PCR Mycoplasma Detection Kit (ABM) and the last mycoplasma test was performed in August 2017. Mycoplasma-free cell lines were used in all of our experiments.

Patient samples

Blood was taken prior to operation from 19 breast cancer patients who did not have any complications, and underwent surgery at Niigata University Medical and Dental Hospital. Serum was separated by centrifugation, and preserved at −180°C. All the patients were Japanese, and obesity was defined as body mass index (BMI) 25 kg/m^2 among that population. Collection and use of all specimens in this study were approved by the Institutional Review Board of Niigata University. Written informed consent was obtained from all participants and the studies were conducted in accordance with the Declaration of Helsinki.

Animal models

All animal studies were conducted in the Animal Research Core Facility at VCU School of Medicine in accordance with the institutional guidelines. Animals were bred and maintained in a pathogen-free environment and all procedures were approved by the VCU Institutional Animal Care and Use Committee (IACUC) that is accredited by Association for Assessment and Accreditation of Laboratory Animal Care.

Female C57Bl/6 mice and BALB/c mice were obtained from Jackson Labs. Mice were fed with either normal diet (ND) or high-fat diet (HFD; TD.88137, Harlan Labs) containing cholesterol (0.2%), total fat (21% by weight; 42% kcal from fat), saturated fatty acids (>60% of total fatty acids), sucrose (34% by weight), protein (17.3% by weight), and carbohydrate (48.5% by weight) for 12 weeks prior to implantation of cancer cells. E0771 breast cancer cells (5×10^4 cells in 10-μL Matrigel) were surgically implanted in the upper mammary fat pad under direct visualization as described previously (23). Tumor size was measured with calipers every 2 days and total tumor volume was estimated by the cylinder formula. Tumor-bearing mice were randomized 2 days after implantation prior to treatment with saline or FTY720. FTY720 (Cayman Chemicals) was administered by gavage at a dose of 1 mg/kg/day in PBS. Mice were sacrificed by exsanguination, blood was collected, tumors excised, weighed, fixed in formalin, and embedded in paraffin or frozen in liquid nitrogen. For survival studies, mice were euthanized according to a morbidity scale approved by IACUC.

For tumor-conditioned media (TCM) treatments, mice were injected intraperitoneally with TCM (300 μL) from E0771, HeLa, or 4T1-luc2 cells overexpressing *Sphk1* or from vector-transfected cells for 5 days prior to tail vein injections of E0771 cells or 4T1 cells (1×10^5/100 μL/mouse), respectively, as described previously (19, 20, 30). Metastatic lesions in the lung were determined histologically, by examining hematoxyin and eosin (H&E)–stained sections.

For the spontaneously developed breast cancer model, male MMTV-PyMT mice on a FVB/N background (Jackson Laboratories) were randomly bred with normal FVB/N females to obtain females heterozygous for the PyMT oncogene. Female heterozygous mice developed palpable mammary tumors as early as 5 weeks of age. Tumor sizes were measured every 3 days by caliper and total tumor volume was estimated by the cylinder formula (31). HFD or ND feeding and FTY720 administration was started at weaning.

Bioluminescent quantification of tumor burden

D-Luciferin (0.2-mL of 15 mg/mL stock, PerkinElmer) was injected intraperitoneally into mice previously implanted with 4T1-luc2 cells, and Living Image Software (Xenogen) was used to quantify the photons/second emitted by the cells as described previously (23).

Interstitial fluid collection

Interstitial fluid (IF) from breast and breast tumors was collected as described previously (32, 33). Briefly, tissue was excised and placed in preweighed tubes on ice. Tubes were reweighed to

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determine tissue weight and the tissue was sectioned several times with scissors. Samples were then transferred into inserts capped with nylon mesh, and placed into preweighed centrifuge tubes. The tubes were centrifuged at 100 × *g* for 10 minutes at 4°C and the IF accumulated in the bottom. The volume of IF was quantified by weight. PBS containing phosphatase inhibitors (100 μL) was added to the IF and the tubes were centrifuged at 1,000 × *g* for 10 minutes at 4°C to remove any contaminating cells.

Quantification of S1P and dihydro-S1P by mass spectrometry

Lipids were extracted from blood, tissues, and interstitial fluid, and S1P and dihydro-S1P quantified by liquid chromatography, electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS, 4000 QTRAP, AB Sciex) as described previously (23, 34, 35).

Histopathologic analysis

Tissue slices (5 μm) were stained with H&E for morphologic analysis. Paraffin-embedded slides were deparaffinated, and antigen unmasking was carried out by microwave heating in citrate buffer for 20 minutes. Slides were incubated with 3% H_2O_2 and then with goat or horse serum (DAKO) for 30 minutes at room temperature. After washing with PBS, slides were incubated at 4°C overnight with the following primary antibodies with indicated dilutions: IL6 (1:200, Abcam), SphK1 (1:100, Abcam), S1PR1 (1:100, Santa Cruz Biotechnology), Ki-67 (1:25, Dako). Biotinylated secondary antibodies (1:200) were added and incubated at room temperature for 20 minutes. After 5 minutes with streptavidin–HRP, sections were stained with DAB substrate and counterstained with hematoxylin. Slides were examined with a Zeiss Axioimager A1 (Jena) and images captured with an AxioCam MRc camera.

Immunofluorescence analysis

Tumors were also frozen, and embedded in optimal cutting medium (OCT 4583; Sakura Finetek) for immunofluorescence analysis. Sections were fixed in 4% paraformaldehyde, blocked with horse serum containing 2.5% of fraction V for 1 hour, and then stained with primary antibodies at 4°C overnight: anti–IL6 (1:200, Abcam), or anti-F4/80 (1:200, AbD Serotec). After two washes with PBS, sections were stained with Alexa488- and Alexa594-conjugated secondary antibodies (1:500, Invitrogen) for 30 minutes. Nuclei were counterstained with Hoechst 33432 (Invitrogen) for 5 minutes. Slides were mounted and examined with a LSM710 laser-scanning confocal microscope (Zeiss).

Immunoblotting

Frozen tissue samples were homogenized and sonicated in 300 μL of buffer containing 50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 2 mmol/L sodium orthovanadate, 4 mmol/L sodium pyrophosphate, 100 mmol/L NaF, 1:500 protease inhibitor mixture (Sigma). Equal amounts of proteins were separated by SDS-PAGE, transblotted to nitrocellulose, and immunopositive bands visualized by ECL (36).

Real-time PCR

Total RNA was isolated from tissues and cells using TRIzol (Life Technologies) and reverse transcribed with the High Capacity cDNA Reverse Transcription Kit. Premixed primer-probe sets and TaqMan Universal PCR Master Mix (Applied Biosystems) were employed to examine mRNA levels. cDNAs were diluted 10-fold (for the target genes) or 100-fold (for GAPDH) and amplified using the ABI7900HT cycler. *Gapdh* mRNA was used as an internal control to normalize mRNA expression.

Statistical analysis

Statistical analysis was performed using unpaired two-tailed Student *t* test for comparison of two groups and ANOVA followed by *post hoc* tests for multiple comparisons (GraphPad Prism). $P < 0.05$ was considered significant. Experiments were repeated at least three times in triplicate with consistent results. *In vivo* experiments were repeated three times and each experimental group consisted of at least six mice.

Results

Obesity increases S1P levels in breast cancer patients and in a syngeneic breast cancer model

Because obesity is now recognized as an independent prognostic factor for breast cancer patients (37, 38), and we have shown that S1P levels are elevated in breast tumors (23), it was of interest to examine the effects of obesity on S1P levels in breast cancer patients that did not yet receive therapy. S1P levels in serum from obese breast cancer patients were significantly higher than those from nonobese patients (Fig. 1A). Likewise, serum levels of dihydro-S1P, which also binds to all S1P receptors, were significantly higher in the obese patients (Fig. 1A).

Next, HFD induced obesity in C57Bl/6 mice, a common model because of its similarities to metabolic changes in obese humans, was used to investigate the mechanisms underlying obesity-promoted breast cancer progression. To this end, E0771 mouse breast cancer cells were implanted into mammary fat pad of syngeneic C57Bl/6 mice fed with HFD or ND for 12 weeks (Fig. 1B). As expected, mice fed with HFD developed significantly larger tumors within 30 days than those on ND (Fig. 1C). HFD significantly increased S1P levels in both normal mammary fat pad and breast tumors (Fig. D). HFD also significantly increased S1P levels in the tumor IF, which is a component of the tumor microenvironment and bathes cancer cells in the tumor (Fig. 1D). This is in agreement with our previous finding that tumor generated S1P is secreted into tumor IF (33), and that S1P levels are higher in human breast cancer and its tumor IF compared with those of normal breast tissue (33, 39). Similar to patients, levels of S1P in serum from nontumor-bearing mice and tumor-bearing mice fed with HFD were also significantly increased compared with mice on ND, and the difference between mice fed with ND and those with HFD were larger in the tumor bearing mice than nontumor-bearing mice (Fig. 1D). Moreover, S1P levels in the lung were also increased in the mice fed with HFD regardless of tumor existence (Fig. 1D). Consistent with elevation of S1P in tumors, expression of SphK1, but not SphK2, and S1PR1, albeit to a much lesser extent, was increased in the tumors from mice fed HFD (Fig. 1E).

The SphK1/S1P/S1PR1 axis connects obesity, chronic inflammation, and breast cancer progression

To investigate the involvement of the S1P/S1PR1 axis in tumor progression in obese animals, we utilized the prodrug FTY720/fingolimod that is phosphorylated *in vivo* to its active form FTY720-P, an S1P mimetic that acts as a functional antagonist of S1PR1 by inducing its internalization and

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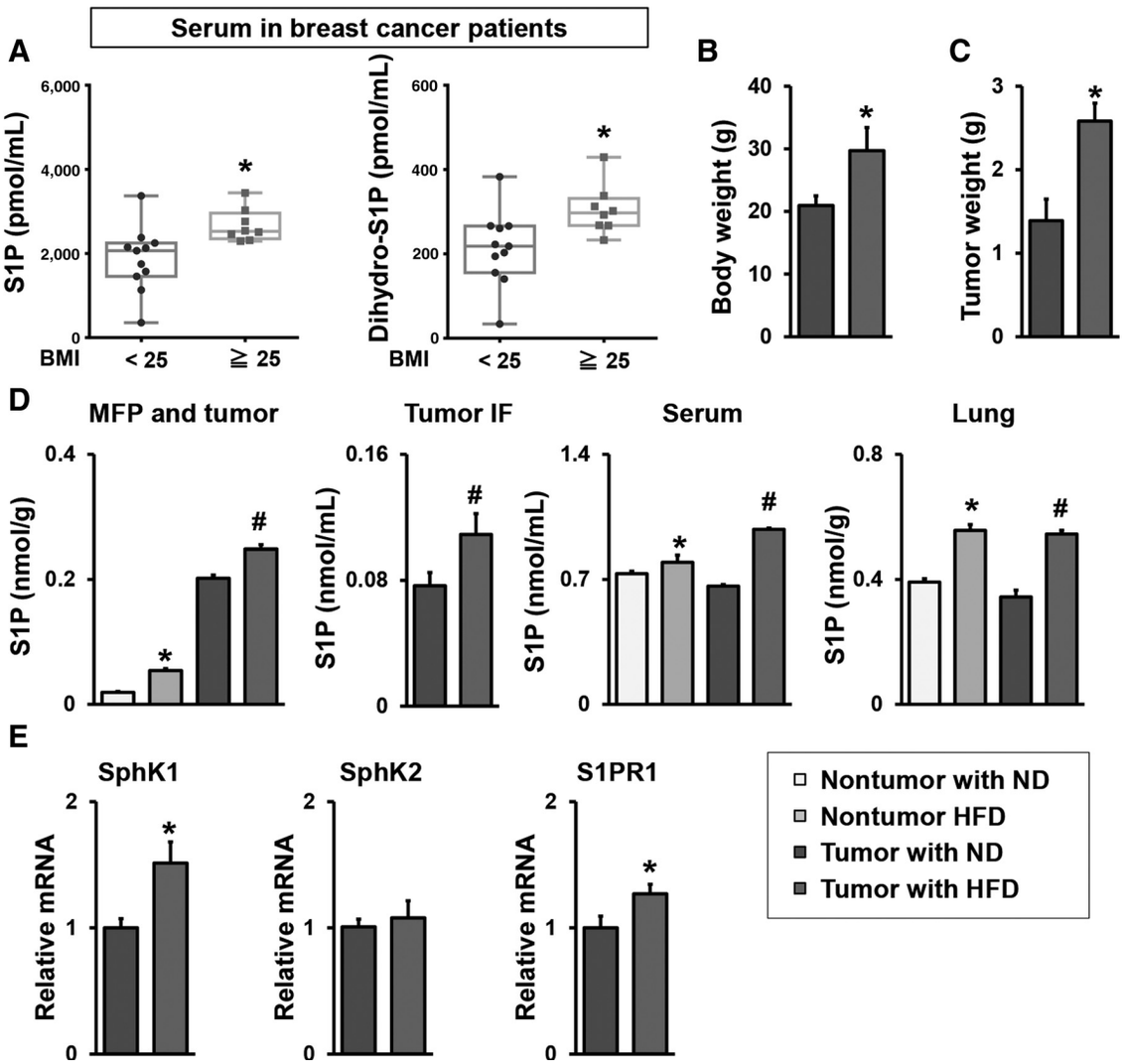

Figure 1.

Obesity increased circulating levels of S1P in humans and mice with breast cancer. **A,** S1P and dihydro-S1P levels in serum from preoperative breast cancer patients with BMI < 25 kg/m^2 ($n = 11$), or BMI 25 kg/m^2 ($n = 8$) were measured by LC-ESI-MS/MS. The S1P and dihydro-S1P levels are shown in the box plot. The central rectangle spans the first quartile to the third quartile. A segment inside the rectangle shows the median and "whiskers" above and below the box shows the value of the minimum and maximum. All data points are also shown as dots. *, $P < 0.05$. **B–E,** The SphK1/S1P/S1PR1 axis in HFD promoted breast cancer progression. **B,** Prior to implantation of E0771 cells into the chest mammary fat pad under direct vision, C57Bl/6 mice were fed ND or HFD for 12 weeks and body weight was measured. **C,** Tumors were harvested 30 days after the implantation, and the tumor weight was measured. Data are means ± SEM. *, $P < 0.05$. **D,** Levels of S1P in mammary fat pad (MFP) and breast tumors, tumor IF, serum without or with breast tumors, and lung without or with breast tumors from mice fed with ND or HFD were measured by LC-ESI-MS/MS. **E,** Expression of *Sphk1*, *Sphk2*, and *S1pr1* in breast tumors was determined by qPCR and normalized to *Gapdh* mRNA. Data are expressed as means ± SEM. *, $P < 0.05$.

degradation (40). When E0771 tumors in syngeneic mice on a HFD reached 5 mm in diameter, mice were treated orally daily with FTY720 (1 mg/kg) or saline. FTY720 significantly suppressed tumor progression determined by decreases of primary tumor volumes and tumor weights in the obese mice (Fig. 2A and B). FTY720 administration, however, did not significantly affect weight gain of HFD-fed mice (Fig. 2C), indicating that FTY720 did not affect diet intake. Treatment with FTY720 also significantly suppressed HFD-induced elevation of S1P in serum, tumors, and IF (Fig. 2D).

The proinflammatory cytokines, IL6 and TNFα, produced by tumor-infiltrating stromal cells, such as TAMs, are known to have important roles in obesity-related cancer progression (12). Indeed, expression of these cytokines was increased in the tumors of HFD-fed mice, compared with those fed ND, which was suppressed by FTY720 treatment (Fig. 2E). Moreover, as expected, HFD increased recruitment of TAMs, as revealed by immunofluorescence with anti-F4/80, (Fig. 2F). Of note, FTY720 dramatically decreased infiltration of TAMs in tumors of HFD-fed mice (Fig. 2F).

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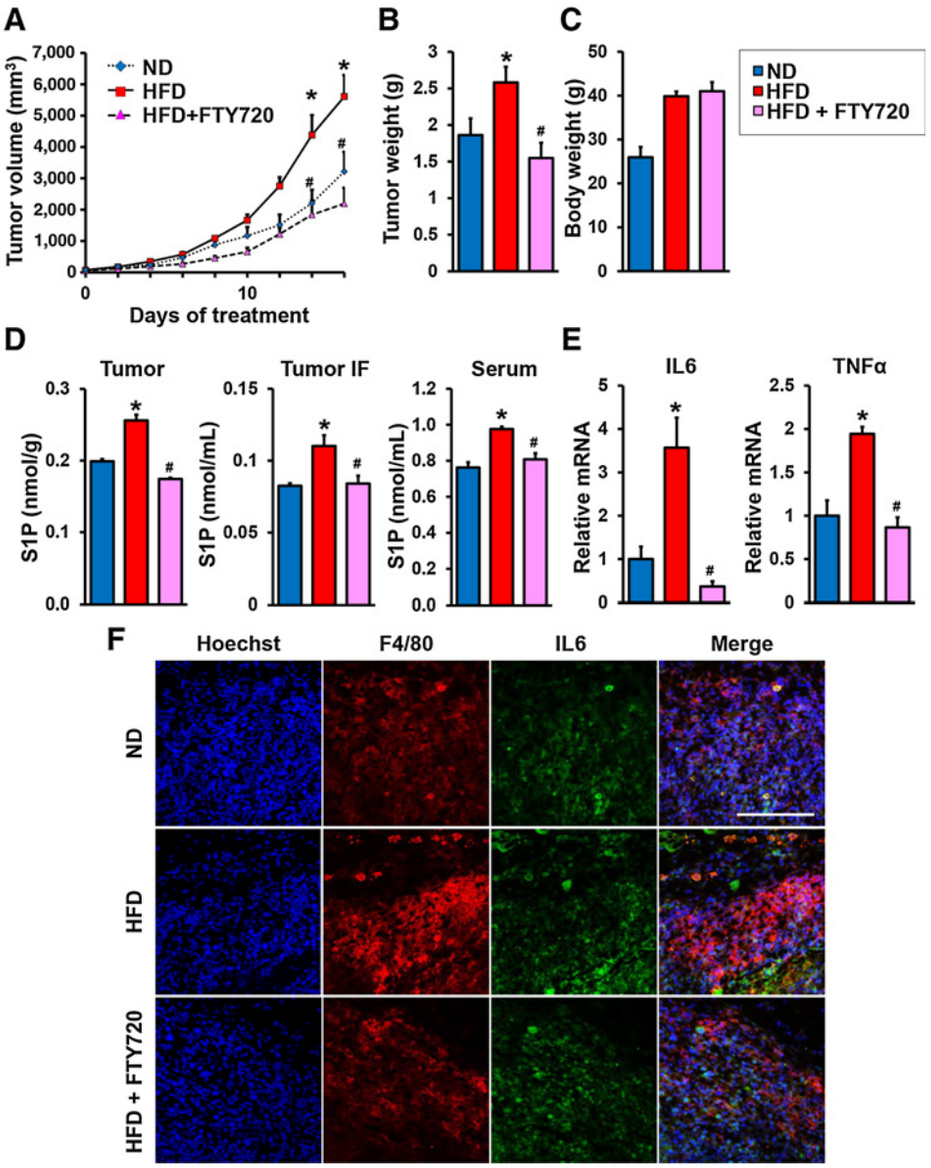

Figure 2.

The SphK1/S1P/S1PR1 axis connects obesity, chronic inflammation, and breast cancer progression. **A–E,** Mice were treated as above and when tumors reached 5 mm in diameter, HFD-fed mice were treated by gavage daily with PBS or FTY720 (1 mg/kg). **A,** Tumor volumes were measured on the indicated days. **B–E,** After treatment for 18 days, tumors were harvested and tumor and body weights determined (**B** and **C**). **D,** Levels of S1P in breast tumors, tumor IF, and serum were measured by LC-ESI-MS/MS. **E,** *Tnfα* and *IL6* mRNA levels in tumors were determined by qPCR and normalized to *Gapdh* mRNA. Data are expressed as means ± SEM. *, $P < 0.05$ versus ND; #, $P < 0.05$ versus HFD. **F,** Immunofluorescence analysis of tumors stained for IL6 (green), F4/80 (red), and Hoechst (blue). Scale bar, 100 μm.

Targeting the SphK1/S1P/S1PR1 axis with FTY720 attenuates obesity-induced tumor progression and inflammation

An obesogenic HFD has been shown to enhance primary tumorigenesis and metastasis in MMTV-PyMT transgenic mice, which spontaneously develop breast cancer accompanied by recruitment of TAMs and increased tissue inflammation (9, 10). Therefore, we also sought to examine the role of the SphK1/S1P/S1PR1 axis in the link between inflammation and obesity-promoted breast cancer progression in this mouse model that closely mimics progression of the human disease (41). Consistent with previous reports (7–10, 31), HFD increased tumor incidence, multiplicity, and size with a significant increase in proliferation determined by Ki67 staining (Fig. 3A and B). HFD feeding, which increased circulating S1P levels (Fig. 3C), also enhanced mRNA expression of SphK1, but not SphK2, and S1PR1 in tumors, corresponding with increased protein levels determined by IHC (Fig. 3D and E). Because we and others have shown that enhanced S1PR1 expression reciprocally activates STAT3, leading to its persistent activation and upregulation of IL6 expression (36, 42), we also studied the effects of interfering with S1P formation and S1PR1 function and this feed-forward amplification loop with FTY720. Daily administration of FTY720 to HFD-fed MMTV-PyMT transgenic mice not only decreased HFD-induced S1PR1 expression, but also, in agreement with the notion that it is an inhibitor of SphK1 and induces its proteasomal degradation (43, 44), FTY720 almost completely abrogated the increase in SphK1 protein in the tumors (Fig. 3D and E). Concomitantly, FTY720 administration prevented increased levels of circulating S1P (Fig. 3C), prevented activation of Stat3, and increases in IL6 expression, and reduced tumor development of HFD-fed MMTV-PyMT mice (Fig. 3).

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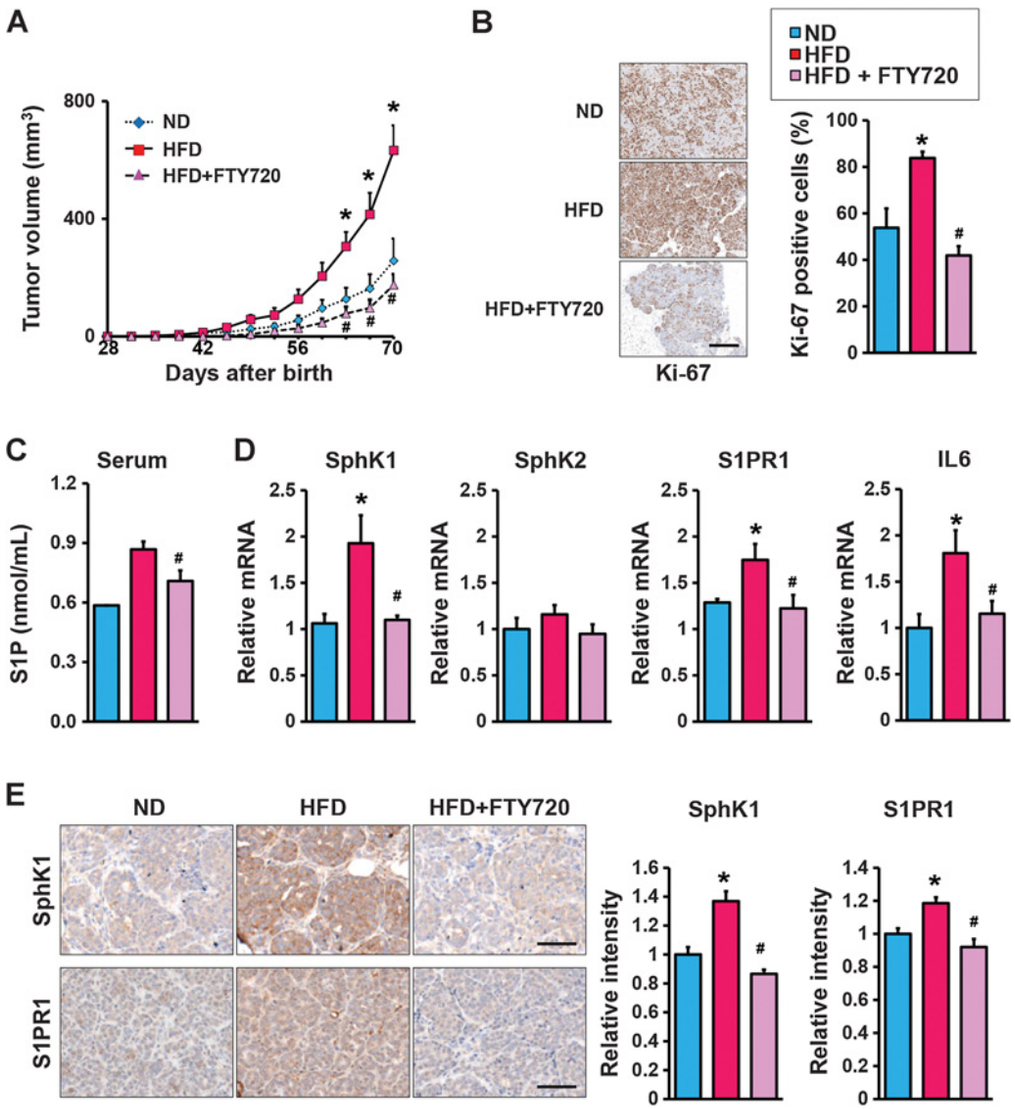

Figure 3.

Targeting the SphK1/S1P/S1PR1 axis with FTY720 mitigates HFD-induced inflammation and tumorigenesis in MMTV-PyMT transgenic mice. **A,** Beginning at 3 weeks of age, MMTV-PyMT transgenic mice were fed ND or HFD and treated daily by gavage with PBS or FTY720 (1 mg/kg). **A,** Spontaneous tumor sizes were determined on the indicated days. **B,** Tumor sections were stained with Ki67 antibody and percent Ki67–positive cells determined. Scale bar, 50 μm. **C,** Levels of S1P in serum were measured by LC-ESI-MS/MS. **D,** Expression of *Sphk1, Sphk2, S1pr1,* and *IL6* in breast tumors was determined by qPCR and normalized to *Gapdh* mRNA. **E,** Breast tumors were immunostained with anti-SphK1 or anti-S1PR1. Scale bar, 50 μm. Relative intensity of the immunostaining was quantified. Data are expressed as means ± SEM. *, $P < 0.05$ versus ND; #, $P < 0.05$ versus HFD.

S1P produced by tumor SphK1 primes distant premetastatic sites

Our results show that S1P is secreted from the primary tumor into the tumor IF that drains into systemic circulation via lymphatic flow. Taken together with a recent study suggesting that S1P transported out of lymph endothelial cells by Spns2 can regulate metastatic colonization (45), it was intriguing to examine whether increased levels of circulating S1P can also promote formation of "premetastatic niches" in distant sites, such as the lung, that assist circulating cancer cells to form metastatic lesions at that location. To this end, prior to tail vein injections of E0771 breast cancer cells (Fig. 4A), mice were treated for 5 days with TCM from control or SphK1-overexpressing E0771 cells that contained increased levels of S1P (Fig. 4A; Supplementary Fig. S1).

Seven days after tumor challenge when there were no significant metastases, H&E staining showed extensive infiltration of inflammatory cells into the lungs of mice receiving TCM containing high levels of S1P (TCM-*Sphk1*) compared with those receiving control TCM (Fig. 4B). Histologic analysis also revealed extensive clusters of macrophages and greater IL6 staining (Fig. 4C). *Sphk1, S1pr1,* and *IL6* mRNA levels were also all significantly higher in those lungs (Fig. 4D). Furthermore, treating mice with TCM

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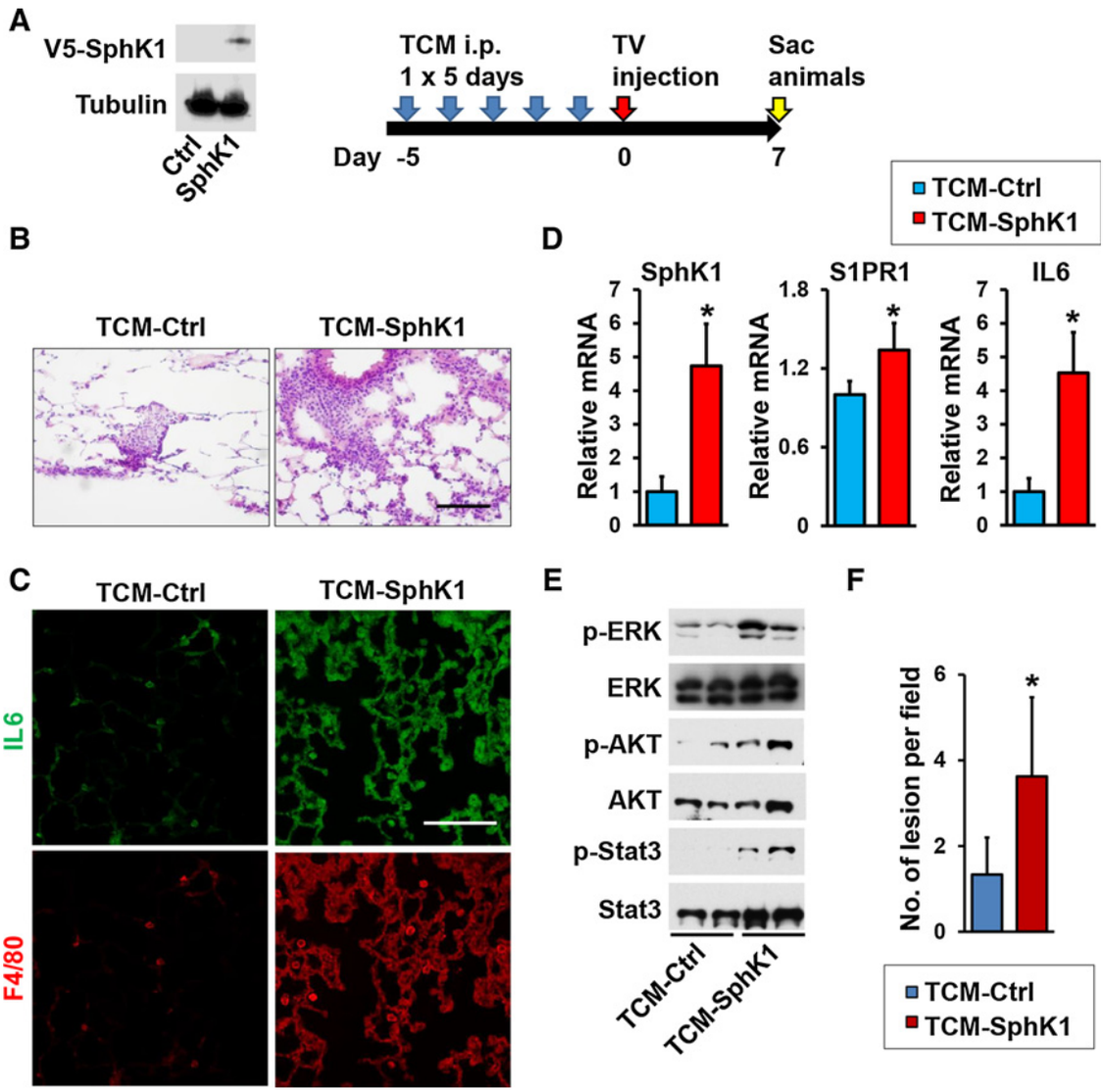

Figure 4.

S1P in tumor conditioned medium increased macrophage recruitment and induced factors and signaling pathways important for lung premetastatic niches. **A,** Equal amounts lysates of E0771 cells with SphK1 overexpression or control (Ctrl) analyzed by immunoblotting with the indicated antibodies (left). Schematic overview of regimen for examination of premetastatic niche formation in the lung utilizing TCM (right). **B–E,** Mice were treated with TCM from *Sphk1*-overexpressing E0771 cells (TCM-*Sphk1*) or control E0771 cells (TCM-Ctrl) for 5 days, followed by systemic tumor challenge. Lungs were harvested 7 days later. **B,** H&E staining of lung sections. Scale bar, 100 μm. **C,** Immunofluorescence analysis of lung sections stained for IL6 (green), F4/80 (red), and Hoechst (blue). Scale bar, 100 μm. **D,** Expression of *Sphk1, S1pr1*, and *IL6* mRNA in lungs determined by qPCR and normalized to levels of *Gapdh* mRNA. Data are means ± SEM. *, $P < 0.05$. **E,** Equal amounts of lung lysates analyzed by immunoblotting with the indicated antibodies. **F,** Quantitation of metastatic lesions in the mouse model with TV injection of 4T1-luc2. Data are means ± SD. *, $P < 0.05$ versus TCM-Ctrl.

generated from SphK1-expressing tumor cells, but not TCM derived from control tumor cells, activated ERK, Akt, and STAT3 (Fig. 4E), known survival signaling pathways downstream of S1P/S1PR1, that have been implicated in premetastatic niche formation (19, 36, 42). We confirmed our findings by repeating experiments utilizing tail vein injections of another breast cancer cell line of 4T1-luc2 after 5-day TCM treatment. As expected, histologic analysis revealed that there were increased metastatic foci in the lung in the mice treated with TCM-*Sphk1* compared with those treated with control TCM (Fig. 4F).

FTY720 inhibits HFD-induced inflammation and lung-seeding ability for breast cancer cells

Because low-grade inflammation is induced by cancer, and obesity may exacerbate that inflammation, obesity has been suggested to increase cancer cell colonization and promote pulmonary breast cancer metastasis (6, 9, 10, 46). We next examined the involvement of the SphK1/S1P/S1PR1 axis in lung seeding ability for breast cancer cells. We performed tail vein injections of 4T1-luc2 cells after 5-day TCM-*Sphk1* treatment, and treated with clinically relevant doses of FTY720

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(Fig. 5A), and found that FTY720 significantly decreased the tumor burden detected by IVIS imaging (Fig. 5B). To examine the effect of HFD, mice were fed HFD or ND for 12 weeks prior to treatment with TCM-*Sphk1* and subsequent intravenous injection of E0771 cells (Fig. 5C). The lungs of HFD-fed mice were heavier than those of mice fed ND, suggesting the presence of increased cancer cell–seeding lesions (Fig. 5D). Indeed, histologic analyses showed significantly increased numbers of cancer cell–seeding foci in the lungs of the HFD-fed mice (Fig. 5E). Moreover, HFD-induced obesity also increased recruitment of macrophages (Fig. 5F) that have been shown to mediate inflammatory responses, and increase expression of IL6 (Fig. 5F), which facilitate tumor cell recruitment, extravasation, and colonization into the niche (42, 46). S1P-stimulated signaling including pERK, pAKT, and pStat3, was also enhanced in lungs of mice fed HFD compared with ND (Fig. 5G). Importantly, treatment of mice fed HFD with clinically relevant doses of FTY720 dramatically suppressed HFD-induced formation of cancer cell–seeding foci (Fig. 5D and E), IL6, and macrophage infiltration (Fig. 5F), as well as S1P-mediated signaling pathways (Fig. 5G). Finally, we repeated experiments utilizing tail vein injections of 4T1-luc2 cells after 5-day TCM-*Sphk1* treatment into HFD-fed mice, and treated with FTY720 (Fig. 5H). We found significant decreases of tumor burden in the mice treated with FTY720 compared with control mice (Fig. 5H).

FTY720 suppresses pulmonary metastasis in MMTV-PyMT transgenic and E0771 syngeneic orthotopic HFD-fed mice

In the metastatic lung colonization assay, cancer cells are injected intravenously and travel directly to the lung rather than from the primary tumor. Accordingly, we next tested the effect of FTY720 on obesity-related cancer metastasis of spontaneous and syngeneic breast tumors where cancer cells metastasize from the breast to the lung, more closely mimicking the pathology of the human disease. HFD enhanced pulmonary metastasis in MMTV-PyMT mice (Fig. 6A), consistent with previous reports (47). FTY720 administration drastically reduced lung metastases (Fig. 6A). Likewise, increased metastasis of breast cancer cells to the lung from E0771 orthotopic breast tumors was observed in mice fed HFD compared with those fed ND (Fig. 6B), with expression of SphK1 and S1PR1 significantly elevated in those lungs (Fig. 6C). Treatment of HFD-fed mice with FTY720 significantly reduced metastasis as well as expression of SphK1 and S1PR1 (Fig. 6B and C), indicating that elevated SphK1/S1P/S1PR1 correlates with HFD increased lung metastasis.

FTY720 suppresses obesity-related inflammation and S1P signaling and prolongs survival in tumor-bearing mice

The key inflammatory cytokine IL6, which is known to enhance metastatic potentials of tumor cells (46), was elevated in lungs from HFD-fed mice (Fig. 7A) concomitantly with increased S1P levels (Fig. 7B) and activation of key signaling pathways (Fig. 7C). FTY720 suppressed elevation of the proinflammatory cytokines, S1P levels, and stimulation of signaling pathways downstream of S1PR1 as demonstrated by reduction in phosphorylation of ERK, AKT, Stat3, and p65 (Fig. 7A–C). To determine whether FTY720 treatment also affects the survival of mice developing breast cancer lung metastases, we carried out a long-term study of syngeneic mice orthotopically implanted with E0771 cells. In agreement with previous studies in other animal models of breast cancer (48), Kaplan–Meier survival analysis revealed that HFD significantly worsened the survival of these mice compared with those fed ND (Fig. 7D). Daily administration of FTY720 significantly prolonged the survival of HFD-fed mice (Fig. 7D).

Discussion

Obesity-induced chronic inflammation has decisive roles in the pathogenesis of breast cancer and distal recurrence; however, the underlying mechanisms linking obesity and chronic inflammation to an increased risk of breast cancer and metastasis are poorly understood. In the current study, we identified a novel mechanism involving the SphK1/S1P/S1PR1 axis in a malevolent feed-forward amplification loop that connects obesity, inflammation, and breast cancer progression and metastasis. Targeting this axis with FTY720, which reduced expression of SphK1 and S1PR1 and S1P levels, significantly suppressed breast cancer metastasis and prolonged survival in obese HFD-fed MMTV-PyMT transgenic and E0771 syngeneic orthotopic breast cancer mice. In agreement with the critical role of the SphK1/S1P/S1PR1 axis in persistent activation of NFκB and STAT3 and production of proinflammatory cytokines IL6 and TNFα (19, 36, 42), we found that in animals bearing breast tumors, increased S1P by HFD is essential for the production of IL6, the multifunctional NFκB-regulated cytokine, as well as activation of STAT3 and the upregulation of its target gene *S1pr1* (36). Administration of FTY720 interfered with the SphK1/S1P/S1PR1 axis and prevented STAT3 activation along with decreasing these proinflammatory cytokines and macrophage recruitment, resulting in suppression of obesity-promoted chronic inflammation and breast cancer progression and metastasis.

We have revealed that HFD increases S1P levels in the circulation of not only tumor-bearing animals, but also of nontumor-bearing animals (Fig. 1). Furthermore, S1P levels in the normal mammary fat pad and lung tissue were also increased with HFD without tumor. These findings indicate that obesity itself increase the levels of S1P in the body. Indeed, it has been reported that S1P levels are positively associated with obesity in humans. Plasma levels of S1P were reported to be higher in obese patients than those in nonobese and lean individuals (49). In addition, it was demonstrated that levels of plasma S1P directly correlate with BMI and total body fat percentage (28, 50). Taken together, these findings indicate that the S1P levels in the blood and local organs are increased with obesity most likely in addition to increased S1P secretion from enlarged tumor.

Our data show that HFD-induced obesity increased levels of S1P not only in the primary tumor itself but also in tumor interstitial fluid—a component of the tumor microenvironment, in the systemic circulation, and in distant sites such as the lungs. Similarly, serum S1P levels from obese breast cancer patients are higher than those in normal weight patients. The "seed-and-soil hypothesis," first proposed by Paget, spawned the idea that primary tumors secrete factors that contribute to the development of premetastatic niches, characterized by an abundance of bone marrow–derived cells and stromal cells (30, 51). It has also been shown that even before tumor cells arrive premetastatic niches in distant organs are formed to create a favorable microenvironment for disseminating tumor cell colonization (52). Our data suggest that S1P is one of these

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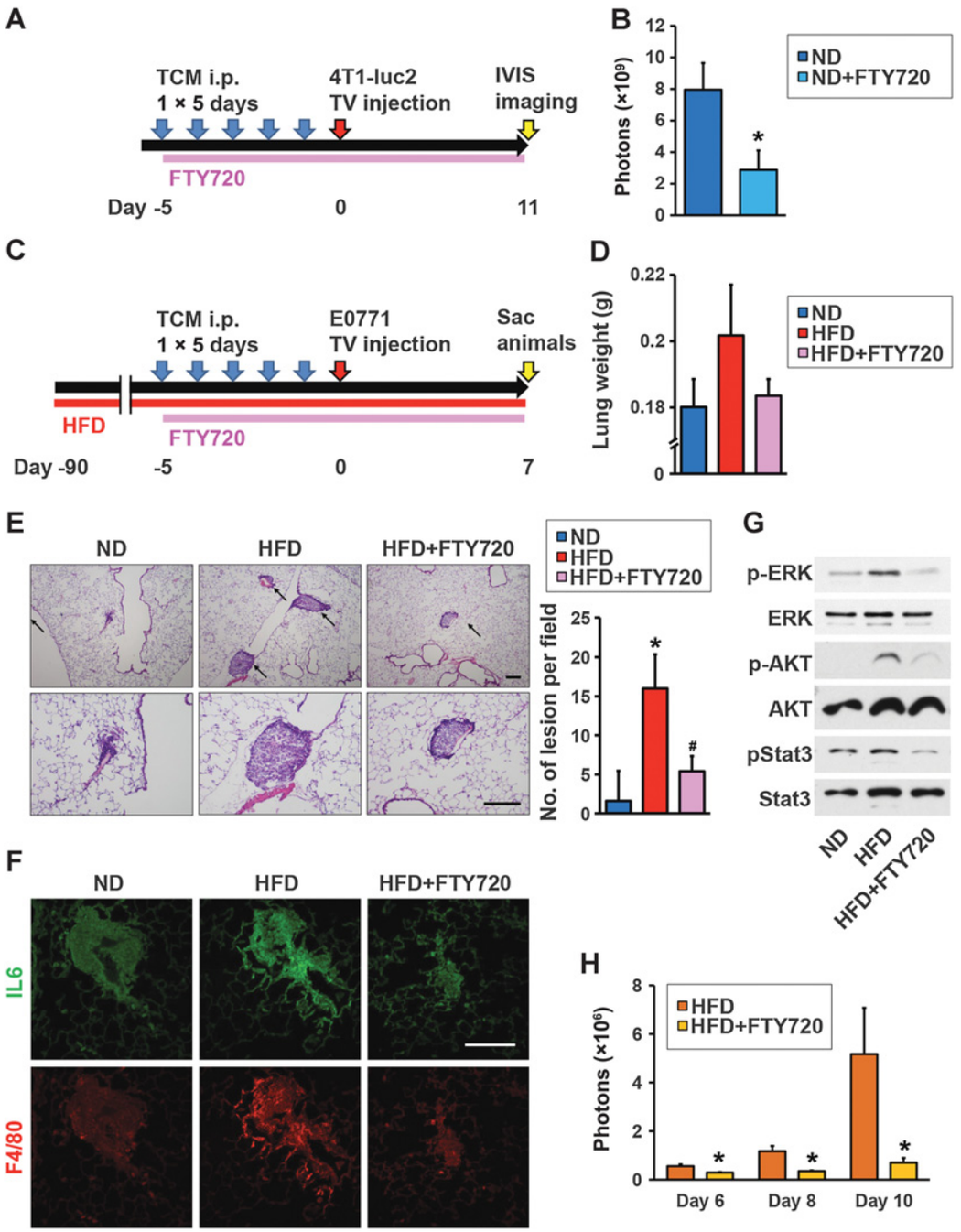

Figure 5.

FTY720 suppressed HFD-induced inflammation and lung-seeding ability of breast cancer cells. **A,** Schematic overview of treatment regimen for lung colonization. Mice were fed ND and treated with TCM from *Sphk1*-overexpressing 4T1-luc2 cells (TCM-*Sphk1*) for 5 days and also daily treated orally with PBS or with FTY720 (1 mg/kg) as indicated. At day 6, 4T1 cells were injected intravenously and tumor burden was quantified by *in vivo* bioluminescence on the indicated days. **B,** Tumor burden of mice with tail vein injection of 4T1-luc2 cells treated without or with FTY720 quantified by *in vivo* bioluminescence on day 11. Data are means ± SEM. *, $P < 0.05$ versus ND. **C,** Schematic overview of treatment regimen for lung colonization. Mice were fed ND or HFD for 12 weeks and treated with TCM from *Sphk1*-overexpressing E0771 cells (TCM-*Sphk1*) for 5 days and also daily treated orally with PBS or with FTY720 (1 mg/kg) as indicated. At day 6, E0771 cells were injected intravenously and 7 days later, lungs were harvested. **D,** Lung weights. **E,** H&E staining of lung sections. Top and bottom panels show lower (×40) and higher (×100) magnifications, respectively. Arrows, cancer cell–seeding lesions. Scale bar, 200 μm. Quantitation of cancer cell–seeding lesions. Data are means ± SEM. *, $P < 0.05$ versus ND; #, $P < 0.05$ versus HFD. **F,** Immunofluorescence analysis of lung sections stained for IL6 (green) and F4/80 (red). Scale bar, 100 μm. **G,** Equal amounts of lung lysates analyzed by immunoblotting, with the indicated antibodies. **H,** Tumor burden of HFD-fed mice with tail vein injection of 4T1-luc2 cells treated without or with FTY720 quantified by *in vivo* bioluminescence on the indicated days. Data are means ± SEM. *, $P < 0.05$ versus HFD, respectively.

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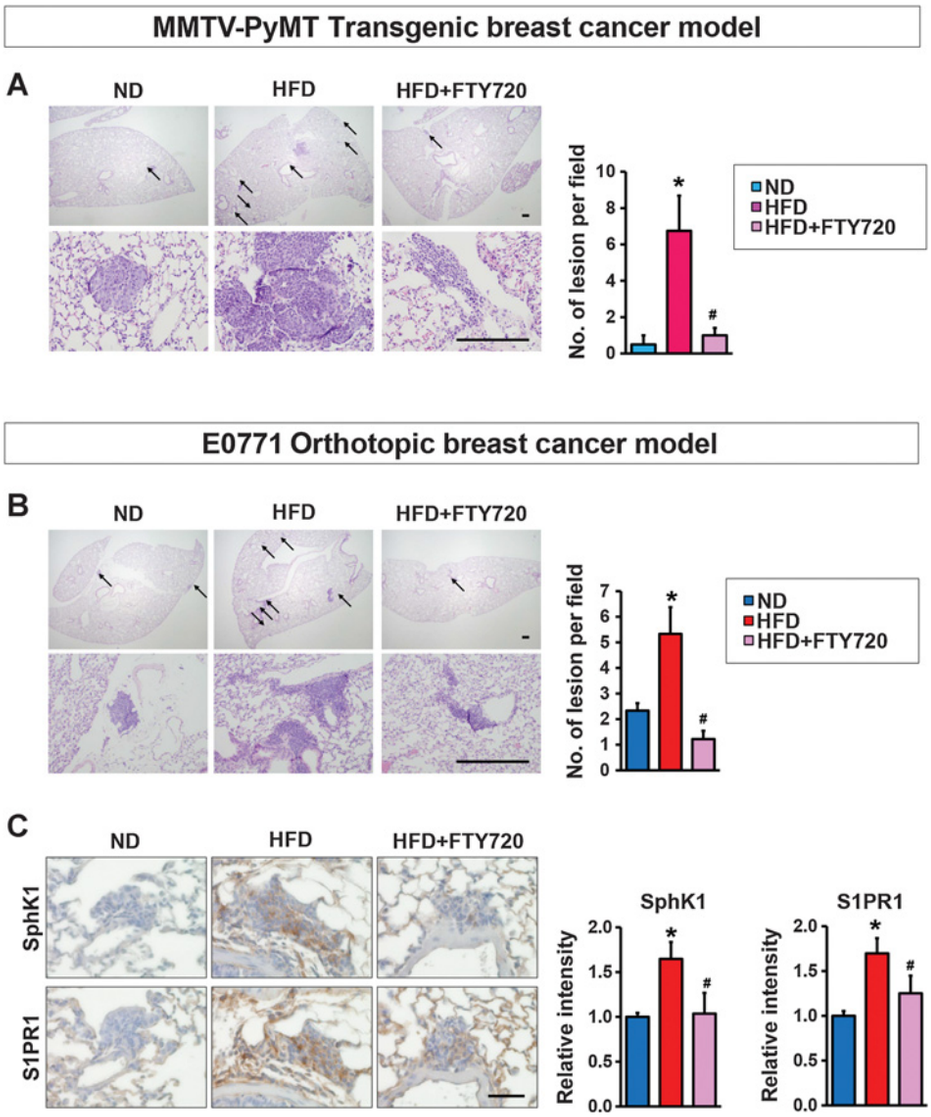

Figure 6.

FTY720 suppresses lung metastasis in HFD-fed MMTV-PyMT transgenic and E0771 syngeneic orthotopic breast cancer mice. **A,** MMTV-PyMT transgenic mice were fed with ND or HFD and treated daily by gavage with PBS or FTY720 (1 mg/kg) as indicated. Lungs were harvested when mice were 10 weeks old. **C,** H&E staining of lung sections. Top and bottom panels show lower (×20) and higher (×200) magnifications, respectively. Arrows, metastatic lesions. Scale bar, 100 μm. Quantitation of metastatic lesions. Data are means ± SEM. *, $P < 0.05$ versus ND; #, $P < 0.05$ versus HFD. **B** and **C,** C57Bl/6 mice were fed with ND or HFD for 12 weeks, before E0771 cells were implanted into the chest mammary fat pad under direct vision. When tumor sizes reached 5 mm in diameter, mice fed HFD were randomized into two groups and treated by gavage with PBS or FTY720 (1 mg/kg/day). Lungs were examined 18 days later. **B,** H&E staining of lung sections. Top and bottom panels show lower (×20) and higher (×200) magnifications, respectively. Arrows, metastatic lesions. Scale bar, 100 μm. Quantitation of metastatic lesions. Data are means ± SEM. *, $P < 0.05$ versus ND; #, $P < 0.05$ versus HFD. **C,** Lung sections were immunostained with anti-SphK1 or anti-S1PR1. Scale bar, 50 μm. Relative intensity of immunostaining was quantified. Data are means ± SEM. *, $P < 0.05$ versus PBS; #, $P < 0.05$ versus HFD.

factors secreted by tumor cells due to upregulation of SphK1. We found that TCM from SphK1-overexpressing breast cancer cells, which contains high levels of S1P, promoted metastatic niche formation and lung metastasis. S1PR1 has previously been identified as a key component for persistent activation of STAT3 in both primary tumors and in various cell types including myeloid cells in distant organs, leading to premetastatic niche formation (19, 42). Although in the past it was not clear how S1PR1 was activated, our results suggest that S1P secretion from tumor cells is the primary driver of S1PR1 activation to influence the microenvironment of distant organs such as lung by promoting recruitment of macrophages known to promote tumor cell extravasation, seeding, and persistent growth, and enabling metastasis (Fig. 7E). Consistent with this key role for S1P, a genome-wide screening of 800 mutant mice using an *in vivo* assay for the discovery of new microenvironmental regulators of metastatic colonization identified the S1P transporter Spns2 that regulates levels of S1P in lymph and blood as a critical new player (45).

We also found that in HFD-induced obese animals, SphK1 and S1PR1 expression is increased in metastatic lesions, along with higher levels of proinflammatory cytokines, IL6 and TNFα. Taken together with previous findings, this indicates that upregulation of SphK1, formation of S1P, and subsequent activation of S1PR1 leads to persistent activation of survival signaling and STAT3 in a malicious feed-forward amplification loop critical for breast cancer proliferation, survival, crosstalk with the microenvironment and metastasis.

FTY720, which targets the SphK1/S1P/S1PR1 axis, prevents the amplification cascade and mitigates obesity-promoted metastatic niche formation and breast cancer metastasis. We found that FTY720 decreased S1P levels as well as SphK1 and S1PR1 expression in the breast tumors. It has been reported that FTY720 inhibits SphK1 activity and promotes its proteosomal degradation (53). It has also been reported that FTY720 induces functional antagonism by the rapid polyubiquitylation, endocytosis, and proteasomal degradation of S1PR1 (54). This mechanism may also contribute to FTY720-induced cancer

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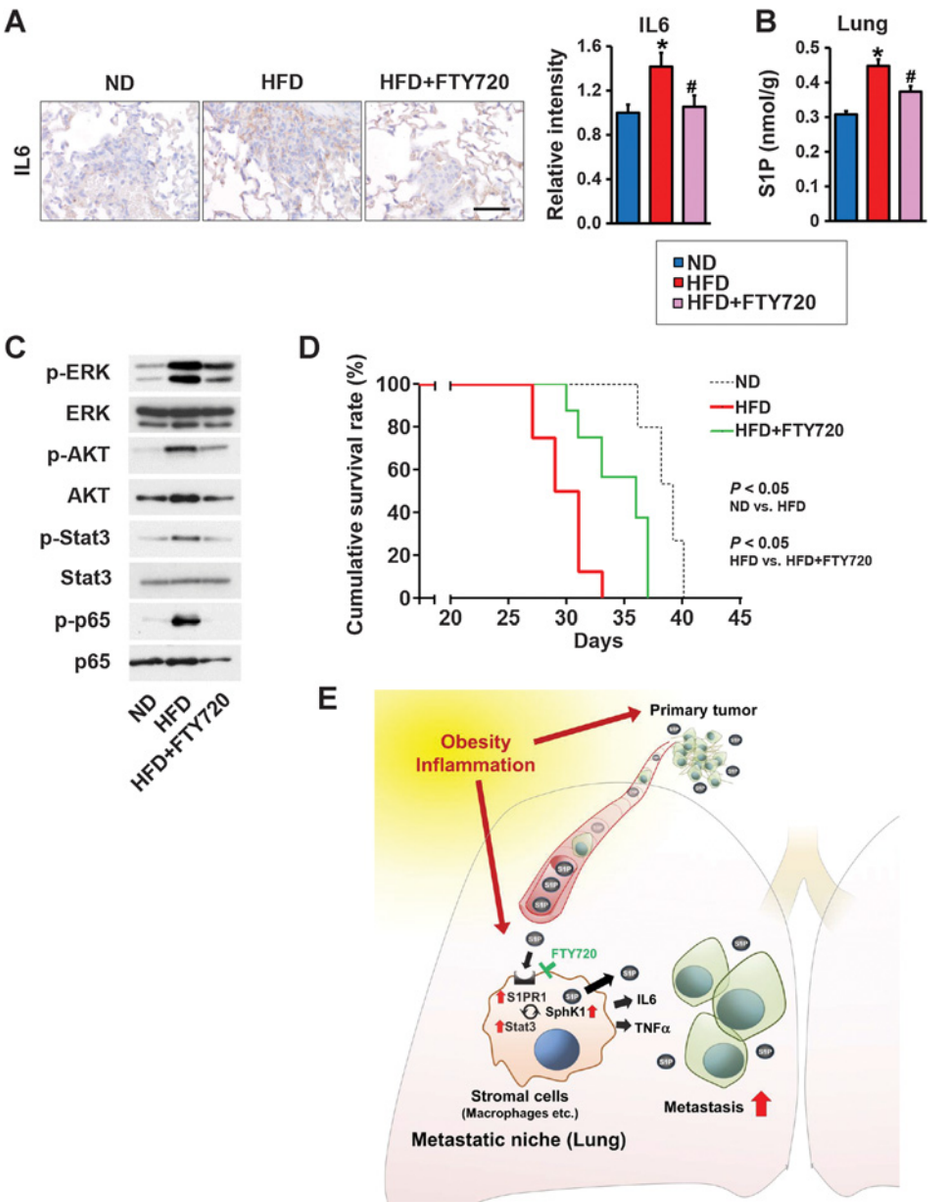

Figure 7.

FTY720 mitigates obesity-related lung inflammation and S1P signaling, and prolongs survival of breast cancer–bearing mice. **A–D,** C57Bl/6 mice were fed ND or HFD for 12 weeks. Tumor-bearing mice fed HFD were randomized into two groups 2 days after implantation and then treated by gavage with PBS or FTY720 (1 mg/kg/day) and 18 days later, lungs were examined. **A,** Lung sections were immunostained with anti-IL6. Scale bar, 50 μm. Relative intensity of the immunostaining was quantified. **B,** Levels of S1P in lungs were measured by LC-ESI-MS/MS. Data are means ± SEM. *, $P < 0.05$ versus. ND; #, $P < 0.05$ versus HFD. **C,** Equal amounts of lung lysates were analyzed by Western blotting with the indicated antibodies. **D,** Kaplan–Meier cumulative survival curves for mice fed with ND, HFD, or HFD plus FTY720 treatment. Data from days after E0771 implantation are shown. **E,** Scheme illustrating the role of SphK1/S1P/S1PR1 axis in the link between obesity, inflammation, and breast cancer progression and lung metastasis and targeting this axis with FTY720 for treatment. See text for more details.

cell–selective apoptosis (55) and its inhibition of tumor vascularization and angiogenesis (56). Taken together, these direct effects of FTY720 on sphingolipid metabolism may explain the mechanisms through which FTY720 targets the SphK1/S1P/S1PR1 axis.

It is possible that the reduction of primary tumor size may be at least partly responsible for the decrease of metastatic tumor burden. Although our results of survival data (Fig. 7) showed a benefit from FTY720, the effect was not as dramatic as the results shown in Figs. 4–6. In this case, response to therapy was not linear to the length of survival. While the reduction in disease burden substantiates an important role for the SphK1/S1P/S1PR1 axis, there remained a significant disease burden in treated mice, implying limited efficacy as a single modality therapy. Clinically, it is well known that therapies that reduce metastatic burden often do not result in longer survival in a variety of settings of human patients (57).

Our objective is to elucidate the role of the SphK1/S1P/S1PR1 axis in cancer associated with inflammation and to show the effects of FTY720 in that setting. FTY720 acts by multiple means in addition to its effects on obesity-related inflammation. In agreement with previous reports, we also found that FTY720 inhibited tumor growth in mice treated with ND. In this research, our discovery is that FTY720 is effective on cancer with obesity-associated inflammation. Future development of more specific drugs that target the SphK1/S1P/S1PR1 axis will further aid in elucidating its importance in control of pulmonary metastatic burden and the usefulness of targeting this axis therapeutically for obesity-promoted metastatic breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Nagahashi, K. Takabe

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Nagahashi, A. Yamada, E. Katsuta, T. Aoyagi, W.-C. Huang, K.P. Terracina, N.C. Hait, J.C. Allegood, J. Tsuchida, K. Yuza, M. Nakajima, M. Abe

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Nagahashi, T. Aoyagi, W.-C. Huang, J.C. Allegood, S. Spiegel, K. Takabe

Writing, review, and/or revision of the manuscript: M. Nagahashi, K.P. Terracina, S. Milstien, S. Spiegel, K. Takabe

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Nagahashi, T. Aoyagi, W.-C. Huang, T. Wakai, K. Takabe

Study supervision: M. Nagahashi, K. Sakimura, T. Wakai, S. Spiegel, K. Takabe

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Tumor Microenvironment

Molecular Cancer Research

ABCC1-Exported Sphingosine-1-phosphate, Produced by Sphingosine Kinase 1, Shortens Survival of Mice and Patients with Breast Cancer

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Abstract

Sphingosine-1-phosphate (S1P), a bioactive sphingolipid mediator, has been implicated in regulation of many processes important for breast cancer progression. Previously, we observed that S1P is exported out of human breast cancer cells by ATP-binding cassette (ABC) transporter ABCC1, but not by ABCB1, both known multidrug resistance proteins that efflux chemotherapeutic agents. However, the pathologic consequences of these events to breast cancer progression and metastasis have not been elucidated. Here, it is demonstrated that high expression of ABCC1, but not ABCB1, is associated with poor prognosis in breast cancer patients. Overexpression of ABCC1, but not ABCB1, in human MCF7 and murine 4T1 breast cancer cells enhanced S1P secretion, proliferation, and migration of breast cancer cells. Implantation of breast cancer cells overexpressing ABCC1, but not ABCB1, into the mammary fat pad markedly enhanced tumor growth, angiogenesis, and lymphangiogenesis with a concomitant increase in lymph node and lung metastases as well as shorter survival of mice. Interestingly, S1P exported via ABCC1 from breast cancer cells upregulated transcription of sphingosine kinase 1 (SPHK1), thus promoting more S1P formation. Finally, patients with breast cancers that express both activated SPHK1 and ABCC1 have significantly shorter disease-free survival. These findings suggest that export of S1P via ABCC1 functions in a malicious feed-forward manner to amplify the S1P axis involved in breast cancer progression and metastasis, which has important implications for prognosis of breast cancer patients and for potential therapeutic targets.

Implication: Multidrug resistant transporter ABCC1 and activation of SPHK1 in breast cancer worsen patient's survival by export of S1P to the tumor microenvironment to enhance key processes involved in cancer progression. *Mol Cancer Res; 16(6); 1059–70. ©2018 AACR.*

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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Introduction

Despite the recent improvement of 5-year survival due to advances in chemotherapy and targeted therapy, close to 40,000 women in the United States continue to succumb to breast cancer every year (1). ATP-binding cassette (ABC) transporters are transmembrane proteins that transport various molecules across cellular membranes including chemotherapeutic agents, functioning as a xenobiotic protective mechanism (2). Some of the ABC transporters, such as ABCB1 (multidrug resistance protein 1: MDR1) and ABCC1 (multidrug resistance associated protein1: MRP1), were originally identified as "multi-drug resistant genes and proteins." Indeed, ABCB1 was demonstrated to export doxorubicin, one of the most frequently used chemotherapeutics for breast cancer that led to development of ABCB1 inhibitors to fight drug resistance (3, 4). Disappointingly, all 12 clinical trials that examined ABCB1-targeted therapy failed to improve survival (5). This suggested to us that other ABC transporters may also drive drug resistance not only because they export drugs, but because they export molecules that biologically aggravate cancer progression and that targeting these transporters may not be sufficient to improve survival.

The bioactive sphingolipid mediator sphingosine-1-phosphate (S1P) is a key regulatory molecule in cancer that promotes cell proliferation, migration, invasion, angiogenesis, and

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lymphangiogenesis (6–8). S1P is generated intracellularly by two sphingosine kinases, SphK1 and SphK2, and is exported out of the cells, where it regulates many functions by binding to and signaling through a family of five G protein–coupled receptors (S1PR1–5) in an autocrine, paracrine, and/or endocrine manner, which is known as "inside-out" signaling (9). We previously demonstrated that SphK1, but not SphK2, produces S1P that is exported from MCF7 breast cancer cells stimulated by estradiol (10). We also demonstrated that expression of SphK1 is upregulated in human breast cancers (11) and the level of activated, phosphorylated SphK1 correlates with lymphatic metastasis (12), in agreement with reports by others (13, 14).

It has previously been proposed that ABC transporters such as ABCC1 may function not only as an export mechanism for drugs but also exacerbate cancer progression. However, no candidates for this potential mechanism have yet been uncovered (3, 15). High expression of ABCC1 has also been associated with poor prognosis in several types of human cancers, including breast cancer (5). Based upon our finding that ABCC1 exports S1P (10), it was tempting to suggest that S1P export via ABCC1 contributes to aggressive breast cancers with poor prognosis. Here, we show that export of S1P via ABCC1 functions in a feed-forward manner to amplify the S1P axis involved in breast cancer progression and contributes to shortened survival of mice and humans with breast cancer

Materials and Methods

Cell culture

MCF7 human mammary adenocarcinoma cells were obtained from ATCC; a murine mammary adenocarcinoma cell line that overexpresses luciferase 4T1-luc2 was obtained from Perkin Elmer. Human umbilical vein endothelial cells (HUVEC) and human lymphatic endothelial cells (HLEC) were obtained from Angio-Proteomie. Cells were purchased in 2010 to 2012. After purchase, cell lines were expanded and frozen after one to three passages. Cells were expanded and stored according to the manufacturer's instructions. MCF7 and 4T1-luc2 cells were used for no longer than 10 passages, whereas HUVEC and HLEC cells were used for no longer than 3 passages. All cell cultures were routinely tested to rule out mycoplasma infection using Mycoplasma Detection Kit (ABM). MCF7 was cultured in modified IMEM without phenol red supplemented with 10% FBS, 0.22% dextrose, and 2 mmol/L glutamine. 4T1-luc2 was cultured in RPMI 1640 medium with 10% FBS. HUVECs and HLECs were maintained in endothelial cell medium supplemented with 5% FBS and endothelial cell growth supplement (ScienCell Research Laboratories).

Full length *Homo sapiens ABCB1* (NM_000927.4) and *ABCC1* (NM_004996.3) were subcloned into pcDNA3.1 in frame with a C-terminal V5-His tag (Invitrogen) using PCR with the following primers: ABCB1-forward 5′-TAA TAT GGA TCC ATG GAT CTT GAA GGG GAC CG-3′; ABCB1-reverse 5′-TAA TAT GGA TCC ATG GAT CTT GAA GGG GAC CG-3′; ABCC1-forward 5′-TAA TAT TCT AGA TTC TGG CGC TTT GTT CCA GC-3′; ABCC1-reverse 5′-TAA TAA TCT AGA TTC ACC AAG CCG GCG TCT TTG G-3′. Lipofectamine (Invitrogen) and Lipofectamine Plus reagents (Invitrogen) were used to transfect MCF7 and 4T1-luc cells and Geneticin (G418) at 0.8 or 0.1 g/L, respectively, was used to select stably transfected clones.

In vitro assays

Cell proliferation was determined with a WST-8 Kit. Motility was determined by wound healing assays (16). *In vitro* angiogenesis and lymphangiogenesis were determined by tube formation assays as described previously (17, 18). qPCR and western blotting were carried out essentially as described previously (10). Lipids were extracted and sphingolipids quantified by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS; refs. 10, 19). Cells were fixed for 5 minutes in 4% paraformaldehyde in PBS and blocked by horse serum, and immunocytochemistry was performed using the following primary antibodies: anti-ABCB1 (C219, Abcam), anti-ABCC1 (MRPr1, Monosan), and anti-SphK1 phospho-Ser225 (ECM Biosciences). The specificities of anti-ABCC1 and anti-SphK1 antibodies and anti–phospho-SphK1 specific antibody, phospho-Ser225, were previously confirmed using siRNA knockdown (10, 20). After incubation with biotinylated secondary antibodies, antigens were visualized with 3,30-diaminobenzidine (Dako), and cells were counterstained with hematoxylin.

Animal studies

All procedures were approved by the VCU Institutional Animal Care and Use Committee that is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. For mice xenograft experiments, 10- to 18 week-old female BALB/c nu/nu mice (Harlan Laboratories) were ovariectomized via the dorsal approach, and 0.72 mg 17-β-estradiol pellets (Innovative Research) were implanted subcutaneously as described previously (21). MCF7 cells stably expressing ABCB1 (B1), ABCC1 (C1), or vector (V) were orthotopically implanted into the right upper mammary fat pads of nude mice as described previously (18, 22, 23). For syngeneic mice experiments, 4T1-luc2 cells stably expressing ABCB1, ABCC1, or vector were implanted in the same manner in 8- to 12-week-old female BALB/c mice (Harlan Laboratories). Tumor volumes in mm^3 were determined by measurements of length and width using calipers every 2 to 3 days. Bioluminescence was used to determine total tumor burden as well as metastases in *ex vivo* in axillary lymph nodes and lungs and was measured and quantified utilizing Xenogen IVIS 200 and Living Image software (Caliper Life Sciences; refs. 24, 25). Tumor interstitial fluid was collected as described previously (26).

For FACS, tumors were minced and digested, and cell suspensions were processed (18). Alexa 488–conjugated LYVE-1 (eBioscience); PE-conjugated podoplanin, PerCP-Cy5.5–conjugated CD45, APC-conjugated CD31, Alexa 700–conjugated TER-119 (BioLegend), or appropriate matched fluorochrome-labeled isotype control mAbs were used for staining. Cells were analyzed by FACS using BD FACSCanto II and BD FACSAria II (BD Biosciences), and data were assessed with BD FACSDiva Software version 6.1.3 (BD Biosciences). The remaining tumor sections were fixed with 10% of neutral-buffered formalin for histopathologic analysis.

Patient samples

This study was approved by the Institutional Review Board of Yokohama City University (Yokohama, Japan), and the patients provided informed consents before inclusion in the study. The study followed the Declaration of Helsinki and good clinical practice guidelines. Tissues were obtained from 275 patients with stage 1 to 3 breast cancers treated in Yokohama City University Medical Center, Japan, between 2006 and 2008. The clinical

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characteristics are presented in Supplementary Table S1. Tissue microarrays were constructed as described previously (27). Because SphK1 is activated by phosphorylation in breast cancer cells, we examined the activation status of SphK1 in breast cancer patients by immunostaining of a tissue microarray from breast cancer patients with a phospho- SphK1–specific antibody. Specificity of the anti-phosphorylated SphK1 antibody was confirmed by immunocytochemistry of MCF7 human breast cancer cells. In agreement with previous studies (10, 28), pSphK1 staining was increased after stimulation of MCF7 cells with estradiol and was absent when SphK1 was downregulated by a specific siRNA (data not shown).

Histopathologic analyses of mouse and human tumor samples

Five-micron sections of mouse and human tumors were stained with anti-Ki67 (DAKO), anti-SphK1 (Abcam), anti-SphK1 phospho-Ser225 (ECM Biosciences), anti-CD31 (BD), anti–LYVE-1 (Abcam), or anti-CK8 (Abcam). Sections were examined with a BX-41 light microscope (Olympus) or TCS-SP2 AOBS Confocal Laser Scanning Microscope (Leica), and microvessel density was determined as described previously (29). ABCB1 and ABCC1 staining in the breast tumors was assessed according to the intensity and population of staining. We scored each sample by 0 to 3: 0 = negative, there is no staining in the tumor cells; 1 = weak, more than 10 % of tumor cells stained with weak intensity; 2 = moderate, more than 30% of tumor cells stained with intermediate intensity or less than 30% of tumor cells stained with strong intensity; 3 = strong, more than 30% of tumor cells stained with strong intensity. Negative: 0 and weak: 1 are considered as low expression, whereas moderate: 2 and strong: 3 are considered as high expression as described previously (27).

Interstitial fluid collection and quantification of sphingolipids by mass spectrometry

Cells, culture medium, and interstitial fluid from breast tumors were collected as described previously (26). Lipids were extracted from these samples, and sphingolipids were quantified by LC-ESI-MS/MS (4000 QTRAP, AB Sciex) as described previously (26).

METABRIC data acquisition and preprocessing and survival analysis

Level 3 *z*-score normalized gene expression data were downloaded from the METABRIC breast cancer study using CBioPortal. All of 2,509 patients with both overall survival data and U133 microarray data from Curtis and colleagues and Pereira and colleagues studies were considered (30, 31). For single gene survival analyses based on expression of SphK1, patients were classified as having high or low expression of the given gene using a gene-specific *z*-score threshold. Patients were labeled as "high" if the expression of the interrogated gene was above the threshold and "low" if below the threshold. To determine the SphK1-specific threshold, *z*-scores of 0, ±0.5, ±1, and ±2 were investigated. For each cutoff, a survival curve was generated. The cutoff that generated the lowest log-rank *P* value was chosen as the SphK1 cutoff. For dual ABCC1 or ABCB1 transporter and SphK1 survival analyses, an inclusive *z*-score threshold of above and below 0 for each gene was used to maximize the size of the study sample. Two patient groups were classified by their patterns of expression of the pair of genes. For all analyses, Kaplan–Meier analysis was performed using GraphPad prism software. Statistical analysis was performed using the Mantel–Cox log-rank test and considered significant at $\alpha = 0.05$ significance level.

Statistical analysis

In vitro and *in vivo* experiments were repeated at least three times and consistent results are presented. Results were analyzed for statistical significance with the Student *t* test for unpaired samples. Correlations among the clinicopathologic parameters and each transporter or activated SphK1 were evaluated by the Pearson v2 test, the Fisher exact test, and the Mann–Whitney test. Patient outcomes were assessed by disease-free survival, and distributions were estimated by the Kaplan–Meier method using SPSS 19.0 (SPSS Inc.) Differences were compared using the log-rank test. $P < 0.05$ was considered statistically significant.

Results

High expression of ABCC1, but not ABCB1, is associated with poor prognosis in breast cancer patients

To further solidify the association of ABC transporter expression with breast cancer patient prognosis, we extended our previous study, which had a limited number of patients and a short duration (27), utilizing a tumor tissue microarray from a larger cohort of patients, and we analyzed expression of ABC transporters by IHC. Patient and tumor characteristics are summarized in Supplementary Tables S1 and S2. ABCB1 was highly expressed in 32.4% (89/275), whereas ABCC1 was highly expressed in 38.9% (107/275) of the tumors analyzed. Representative images of ABCB1 and ABCC1 stained tissues are shown in Fig. 1A. Patients with high ABCC1 expression had significantly shorter disease-free survival compared with patients with low ABCC1-expressing tumors with the follow-up period up to 8 years ($P = 0.004$; Fig. 1B). In contrast, expression levels of ABCB1 were not associated with disease-free survival despite the extended number of patients and follow-up period (Fig. 1B).

Overexpression of ABCC1, but not ABCB1, enhances S1P secretion, proliferation, and migration of breast cancer cells and promotes angiogenesis and lymphangiogenesis *in vitro*

To investigate the role of ABC transporters in cancer progression, we generated MCF7 human breast cancer cells and 4T1-luc2 murine breast cancer cells stably overexpressing vector, ABCB1, and ABCC1. Expression of ABCB1 and ABCC1 was confirmed at the mRNA and protein levels by qPCR and Western blotting, respectively, and IHC confirmed that these ABC transporters are expressed on the plasma membrane (Fig. 2A; Supplementary Fig. S1).

Consistent with our previous reports demonstrating that S1P is exported via ABCC1 but not ABCB1 using siRNA (10), and specific inhibitors (20), overexpression of ABCC1, but not ABCB1, significantly increased S1P secretion from human MCF7 and murine 4T1 breast cancer cells, as measured by LC-ESI-MS/MS (Fig. 2B and C). In addition, ABCC1 overexpression increased not only S1P, but also dihydro-S1P (DHS1P; Supplementary Fig. S2A). Moreover, as expected, intracellular S1P was decreased, whereas sphingosine (Sph) was increased (Supplementary Fig. S2B). Although intracellular ceramides and sphingomyelins levels were slightly decreased (Supplementary Figs. S2C, S3A, and S3B), there were no significant changes in intracellular monohexosylceramides (Supplementary Figs. S2C and S3C). Our results imply

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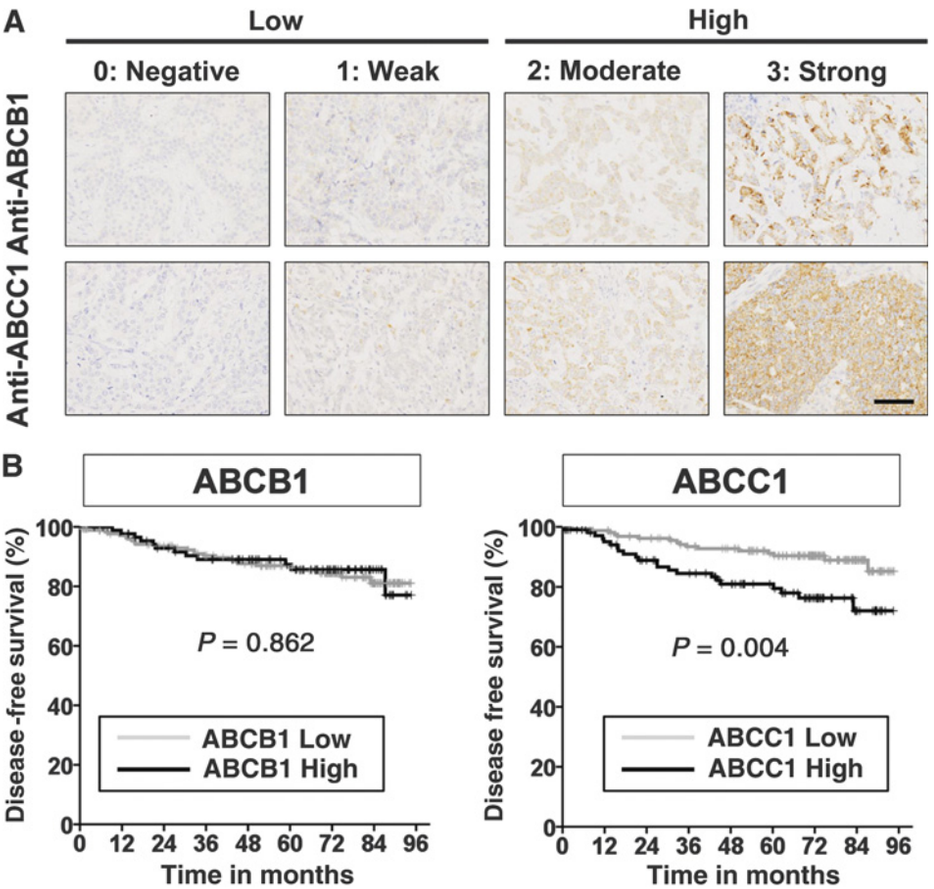

Figure 1.
Expression of ABCC1 but not ABCB1 correlates with poor prognosis in human breast cancer patients. Tissue microarrays containing 275 breast tumor tissues were stained with anti-ABCB1 or anti-ABCC1 antibodies. **A,** Expression of ABCB1 and ABCC1 was scored as 0, negative; 1, weak; 2, moderate; or 3, strong. Scores 0 and 1 are considered as low expression, whereas scores 2 and 3 are considered as high expression. Representative images are shown under high magnification for ABCB1 staining (top) and ABCC1 staining (bottom). **B,** Kaplan–Meier disease-free survival curves according to expression of ABCB1 and ABCC1. *P* values were calculated by the log-rank test. Scale bar, 50 μm.

that overexpression of ABCC1 that increases secretion of S1P leads to increased degradation of ceramide to sphingosine to compensate for the loss of intracellular S1P.

We next examined several biological processes important for cancer progression known to be regulated by S1P (7, 8). Overexpression of ABCC1 but not ABCB1 significantly enhanced cell proliferation (Fig. 2D). Moreover, MK571, an ABCC1 inhibitor, not only prevented secretion of S1P, but also suppressed the growth-stimulating effect of ABCC1 overexpression (Fig. 2D). In agreement with previous reports showing that directly adding S1P enhances breast cancer cell proliferation and migration (17, 32), cells expressing ABCC1 also showed significantly enhanced migration in scratch assays that was reduced by treatment with MK571. In contrast, MK571 had no significant effects on migration of vector or ABCB1 transfected cells (Fig. 2E and F). These results suggest that overexpression of ABCC1 increases the export S1P that enhances proliferation and migration of breast cancer cells.

Because S1P is a potent angiogenic and lymphangiogenic factor (18), we next examined whether S1P secreted from cells overexpressing ABC transporters could affect angiogenesis or lymphangiogenesis. Conditioned media from breast cancer cells expressing ABCC1, but not ABCB1 cells, promoted both angiogenesis and lymphangiogenesis of HUVECs and HLECs, respectively (Fig. 2G and H). However, conditioned medium from MCF7 cells expressing ABCC1 that were treated with MK571, an inhibitor of ABCC1 or with SK1-I, a specific inhibitor of SphK1, lost its ability to stimulate *in vitro* angiogenesis and lymphangiogenesis of endothelial and lymph endothelial cells, respectively (Fig. 2G and H). Collectively, these results suggest that S1P produced by SphK1 and secreted from breast cancer cells via ABCC1 transporter could affect not only the cancer cells themselves but also the microenvironment.

Overexpression of ABCC1, but not ABCB1, markedly enhances tumorigenesis in MCF7 xenografts

We next examined the role of ABCC1 and secreted S1P in breast cancer progression *in vivo* by comparing tumors produced by MCF7 cells stably overexpressing ABCC1 or ABCB1 implanted into ovariectomized athymic nude mice in the presence of estradiol pellets. MCF7 cells were utilized because they readily secrete S1P in response to estradiol (10). Tumors from MCF7 cells overexpressing ABCC1 grew significantly faster and were much larger than tumors from mice implanted with MCF7 cells overexpressing vector or ABCB1 (Fig. 3A). Morphologically, MCF7/C1 tumors appeared more poorly differentiated by H&E staining (Fig. 3B). In agreement, these tumors also had significantly higher mitotic activity than tumors from MCF7 cells overexpressing vector or ABCB1 by Ki67 staining (Fig. 3B and C). Likewise, mice implanted with MCF7 cells overexpressing ABCC1 had significantly higher blood vessel densities detected by CD31

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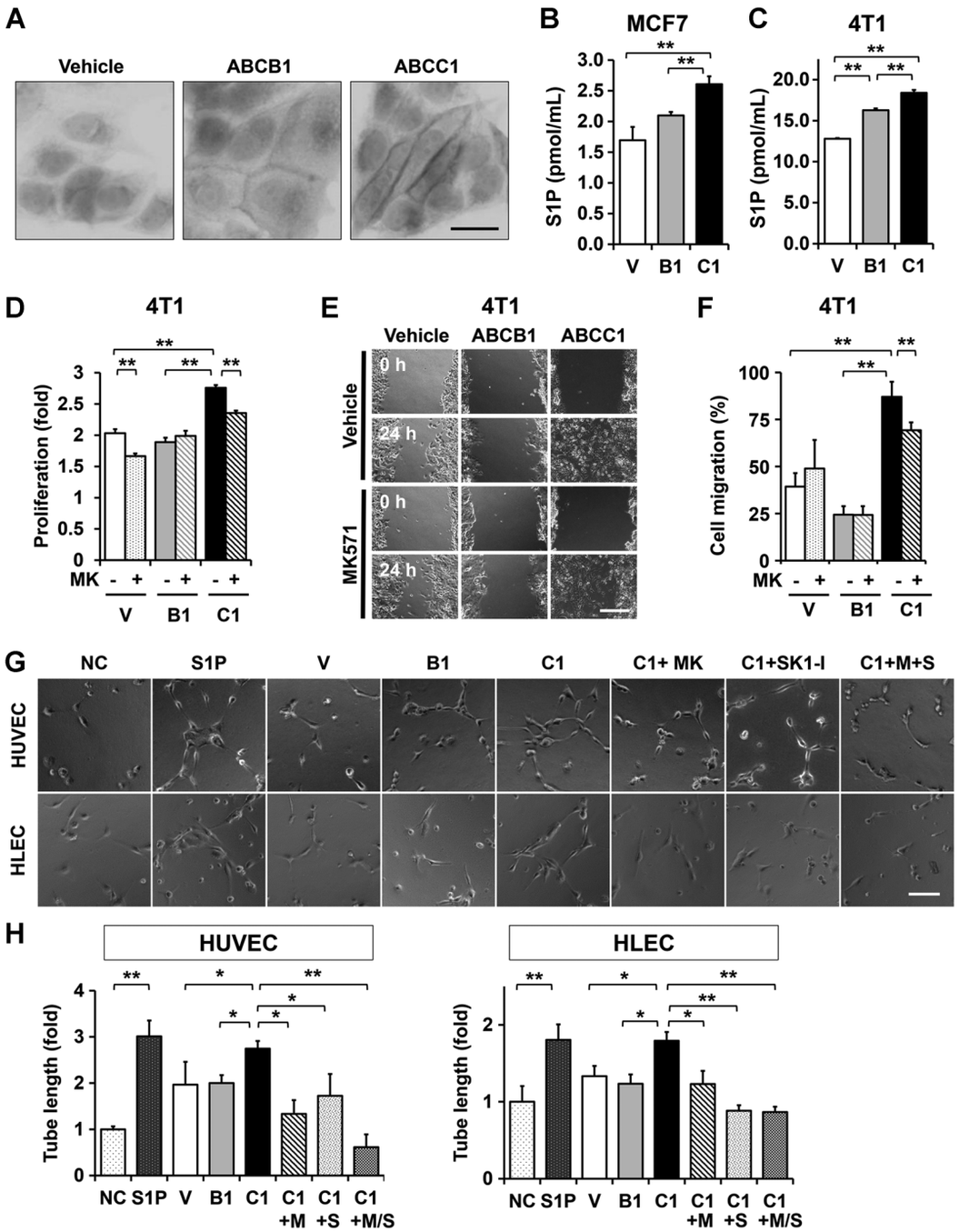

Figure 2.

Overexpression of ABCC1, but not ABCB1, enhances proliferation and cell migration of breast cancer cells and promotes S1P secretion–mediated angiogenesis and lymphangiogenesis of endothelial cells. **A–E,** MCF7 or 4T1 breast cancer cells were transfected with vector (V), ABCB1 (B1), or ABCC1 (C1) as indicated. **A,** IHC reveals that expressed ABCB1 and ABCC are localized to the plasma membrane. **B** and **C,** S1P secreted from the indicated breast cancer cells was determined by LC-ESI-MS/MS. **D,** Proliferation of cells treated with vehicle or 20 μmol/L MK571 for 48 hours was determined by WST8 assay. Data are expressed as fold increase compared with 0 time. **E** and **F,** Monolayers of the indicated 4T1 cells treated with vehicle or 20 μmol/L MK57 were wounded and migration of cells into the wounded area was measured 24 hours later. **E,** Representative photographs of wounded areas are shown. **F,** Cell migration was determined as percent of initial wounded area and expressed as means ± SD of 6 determinations. **G** and **H,** HUVECs and HLECs were cultured on reduced growth factor basement membrane matrix-coated 48-well plates and incubated for 6 hours without or with S1P (1 μmol/L) or conditioned medium from MCF cells overexpressing ABCC1 (C1) were pretreated with vehicle (NC, nontreated control) or 20 μmol/L MK571 without or with 10 μmol/L SK1-I for 12 hours and conditioned medium prepared. +M, treated with MK571; +S, treated with SK1-I. Six random fields per condition were photographed (**G**) and total tube length determined (**H**). Scale bars, 20 μm (**A**), 200 μm (**E**), and 100 μm (**G**). *, $P < 0.05$; **, $P < 0.01$ determined by Student t test. Data, means ± SD.

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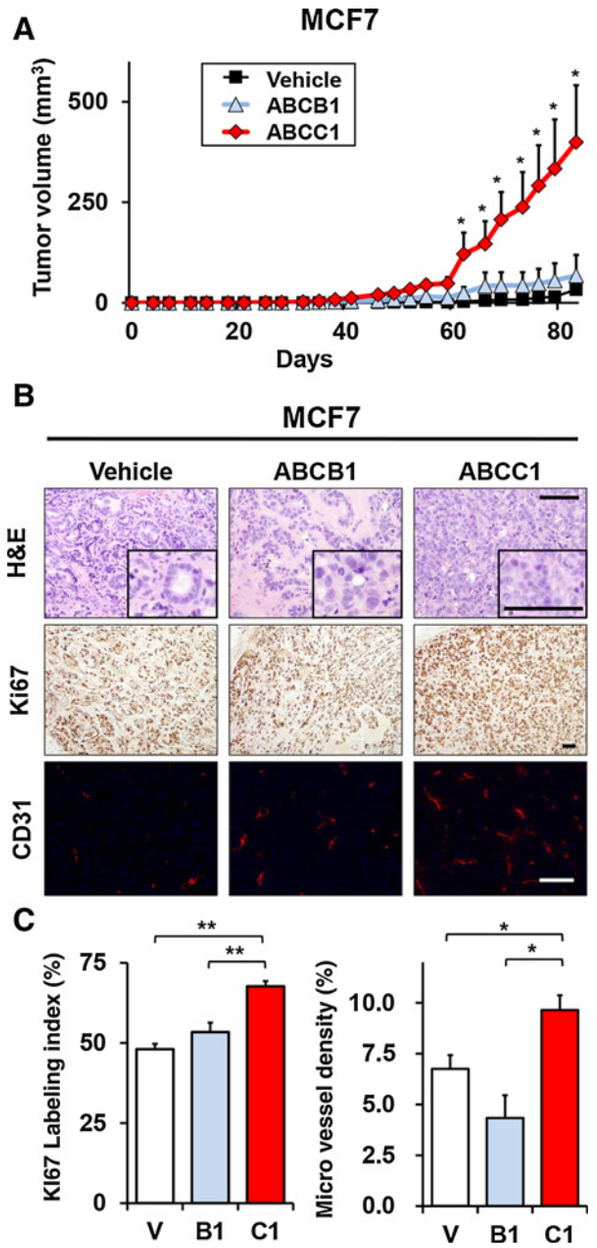

Figure 3.

Overexpression of ABCC1, but not ABCB1, enhances MCF7 tumor growth in a mouse xenograft model. **A,** BALB/c nude mice were ovariectomized and estrogen pellets implanted under anesthesia. Tumors were established by surgical implantation of MCF7 cells stably overexpressing vector, ABCB1, or ABCC1 into chest mammary fat pads. Tumor size was measured at the indicated times ($n = 5$ mice/group). **B,** Representative images of H&E and Ki67 staining and confocal immunofluorescent images of stained blood vessels with anti-CD31 (red) and nuclei costained with Hoechst (blue) in tumor sections 83 days after implantation. **C,** Percentage of Ki67-positive cells and microvessel density were determined. Scale bar, 100 μm. *, $P < 0.05$; **, $P < 0.01$ determined by Student t test. Data, means ± SEM.

immunofluorescence (Fig. 3B and C). These results suggest that overexpression of ABCC1 enhances breast cancer progression and angiogenesis.

Overexpression of ABCC1, but not ABCB1, enhances tumor growth, angiogenesis, and lymphangiogenesis and contributes to poor survival of mice bearing 4T1 syngeneic tumors

Because it is well established that S1P plays critical roles in immune responses and affects the tumor microenvironment, we next examined the role of ABCC1 in an immunocompetent syngeneic breast cancer model. *In vivo* bioluminescence revealed that tumors from 4T1-luc2 cells overexpressing ABCC1 orthotopically implanted in BALB/C mice grew significantly faster and to a much greater size than 4T1-luc2 tumors overexpressing vector or ABCB1 (Fig. 4A). Similar to the xenograft model, tumors of 4T1 cells overexpressing ABCC1 also had high mitotic activity measured by Ki67 staining (Fig. 4B). These tumors also had increased angiogenesis and lymphangiogenesis compared with tumors overexpressing ABCB1 or empty vector, as quantified by microvessel density of blood vessels (MVD) and lymphatic vessels (MLD) determined by immunofluorescence staining for CD31 and Lyve1, respectively (Fig. 4C). These results were further confirmed by flow cytometry of cells from mammary site tumors that quantified blood endothelial cells (BEC) and lymphatic endothelial cells (LEC) using CD31, a marker for both BECs and LECs, and gp38 (podoplanin), a specific marker for LECs (Fig. 4D). Given the significant increase in both angiogenesis and lymphangiogenesis, we then determined lymph node and lung metastasis and survival of the mice. As shown in Fig. 4E, mice implanted with 4T1 cells overexpressing ABCC1 had significantly more metastases not only in lymph nodes but also in the lung, both measured by *ex vivo* bioluminescence, than in mice implanted with 4T1 cells overexpressing ABCB1. Similarly, the numbers of metastatic lesions were significantly greater in the mice bearing ABCC1-expressing tumors, determined by immunofluorescence and H&E staining (Fig. 4F). Furthermore, these mice had significantly shorter survival compared with mice bearing ABCB1-expressing tumors (23 ± 3 days compared with 30 ± 2 days; Fig. 4G). Together, these results suggest that tumors overexpressing ABCC1 are much more aggressive with worse survival, possibly due to enhanced S1P secretion.

S1P exported via ABCC1 upregulates transcription of SphK1 and enhances its own production of S1P

Previous studies demonstrated that expression of SphK1 is elevated (33, 34) and correlates with poor survival in human breast cancer patients (13) and in mice breast cancer models (18). Therefore, it was of interest to determine expression of SphK1 in breast cancer cells and tumors overexpressing ABC transporters. *SphK1* mRNA levels were significantly increased in both MCF7 and 4T1 cells overexpressing ABCC1 (Fig. 5A; Supplementary Fig. S4). Similarly, IHC revealed increased activated SphK1 determined with a phospho-SphK1–specific antibody (Fig. 5B). Upregulation of *SphK1* mRNA in cells overexpressing ABCC1 was suppressed by MK571, an ABCC1 inhibitor, but had no effects on SphK1 levels in cells overexpressing ABCB1 or vector (Fig. 5C). As these results suggest that S1P secreted through ABCC1 leads to upregulation of SphK1, the kinase that produces it, we next examined whether exogenous S1P can upregulate SphK1 in naïve breast cancer cells. Indeed, *SphK1* mRNA was increased by treatment of 4T1 cells with S1P in a time- and dose-dependent manner (Fig. 5D), supporting the notion that S1P exported by ABCC1 can act in a positive feedback manner to amplify its own production. In agreement with these *in vitro* data, IHC revealed that tumors overexpressing ABCC1 had higher levels of SphK1 and activated

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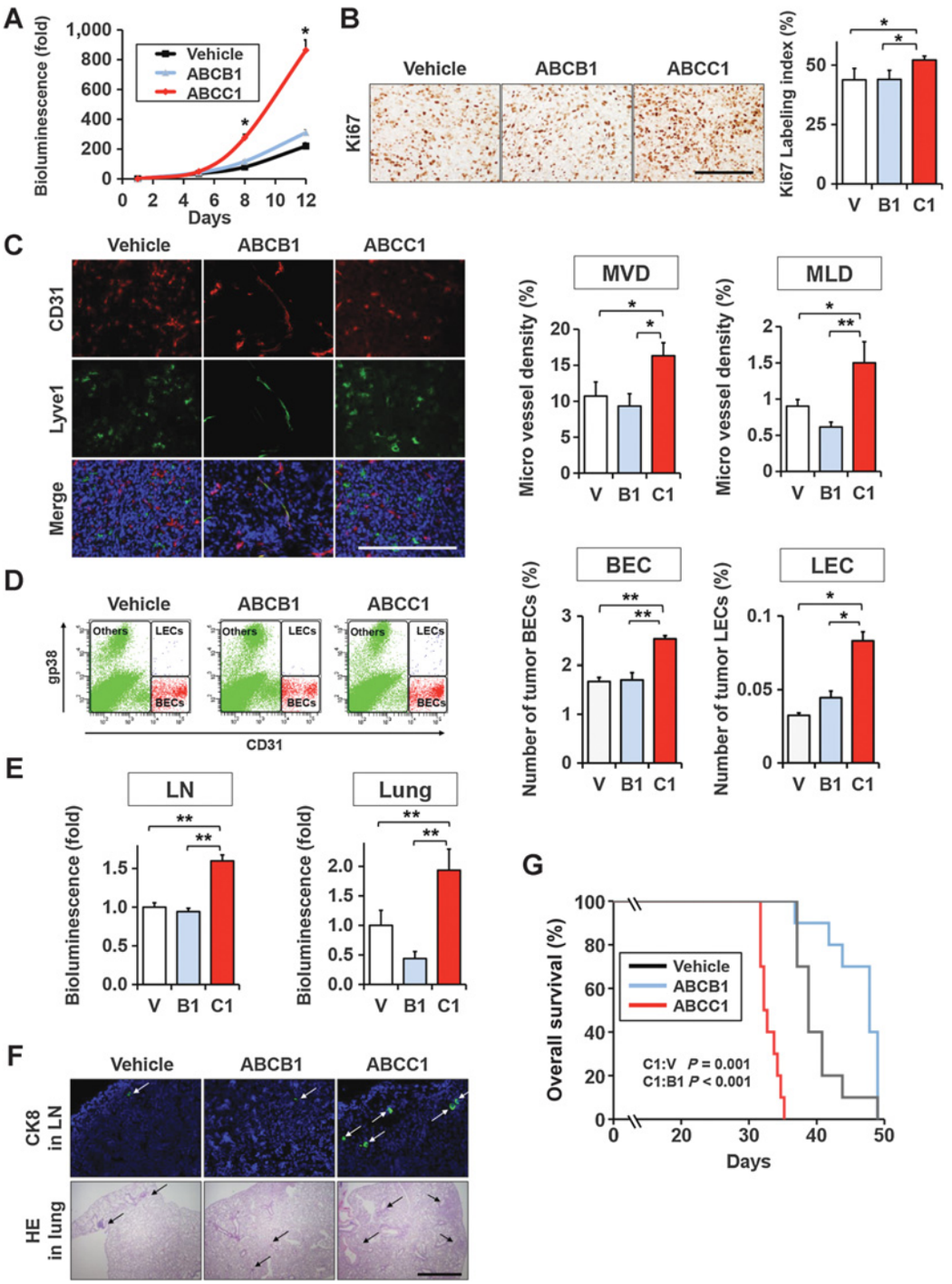

Figure 4.

Overexpression of ABCC1, but not ABCB1, enhances 4T1 tumor growth and decreases survival in a syngeneic mouse model. **A–F,** 4T1-luc2 cells transfected with vector, ABCB1, or ABCC1 were implanted into mammary fat pads of BALB/c mice under direct vision (23). **A,** Tumor burden was determined by *in vivo* bioluminescence ($n = 10$ mice/group). **B,** Representative images of Ki67 staining of tumor sections are shown and the percentage of Ki67 positive cells within tumors was enumerated. ($n = 5$) **C,** Confocal immunofluorescent images of tumors stained for blood vessels (anti-CD31, red), lymphatic vessels, (anti-lyve1, green), and nuclei (Hoechst, blue). Microvessel density and lymphatic vessel density were determined. **D,** Tumors were minced, digested with collagenase, and BECs and LECs were quantified by FACS. Representative panels of FACS analysis are shown. **E,** Regional lymph node metastases and lung metastases were determined by *ex vivo* bioluminescence 12 days after implantation. **F,** Confocal immune fluorescent images of lymph nodes stained for adenocarcinoma (anti-CK8, green, white arrows) and nuclei (Hoechst, blue) and H&E-stained lung sections show metastases (black arrow). **G,** Kaplan–Meier survival curves of mice bearing 4T1/V, 4T1/B1, and 4T1/C1 tumors. Days were counted after cancer cell implantation. *P* values were calculated by log-rank test. Scale bar, 100 μm (**B** and **C**); 2 μm (**F**). *, $P < 0.05$; **, $P < 0.01$ based on Student *t* test. Data, means ± SEM.

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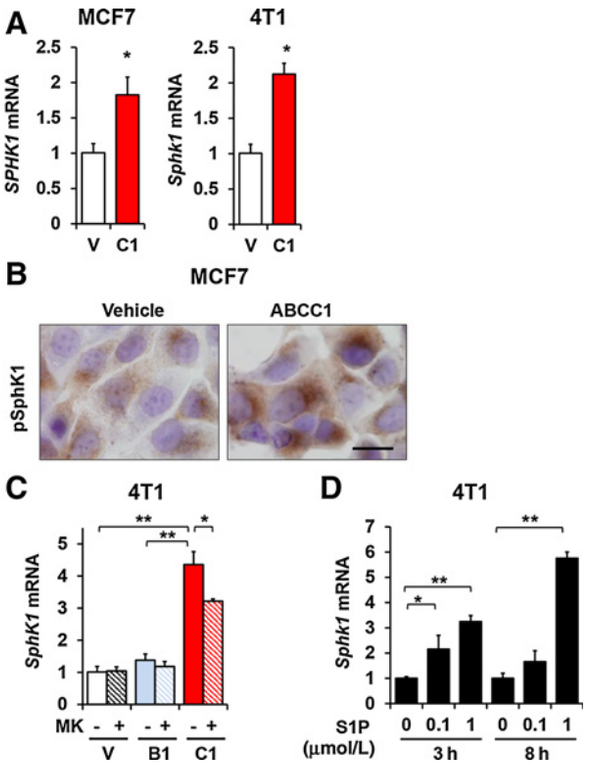

Figure 5.

S1P exported via ABCC1 upregulates expression of SphK1. **A,** SphK1 expression in MCF7 and 4T1 cells transfected with vector or ABCC1 was determined by QPCR and normalized to GAPDH. Data, means ± SEM. **B,** Activation of SphK1 in transfected MCF7 cells was determined by immunocytochemistry with pSphK1 antibody. Scale bar, 20 μm. **C,** 4T1 cells transfected as indicated were treated with vehicle or 20 μmol/L MK571 for 24 hours in serum-free medium. Cells were then stimulated in medium containing 10% serum for 3 hours. *SphK1* mRNA levels were determined by qPCR and normalized to *Gapdh*. Data, means ± SD. **D,** Naïve 4T1 cells were starved for 24 hours and treated with 100 nmol/L or 1 μmol/L S1P in 0.4% fatty acid free BSA for 3 or 8 hours as indicated. SphK1 and GAPDH mRNA was determined by qPCR.

SphK1 than those expressing ABCB1 or vector (Fig. 6A–D). Likewise, *SphK*1 mRNA expression was higher in tumors overexpressing ABCC1, as demonstrated by qPCR (Fig. 6E). Consistent with increased SphK1 expression, S1P levels in tumors and in tumor interstitial fluid were significantly higher in tumors overexpressing ABCC1 than tumors overexpressing ABCB1 or vector (Fig. 6F).

Patients with breast cancers that express both activated SphK1 and ABCC1 have shorter disease-free survival

Because expression of ABCC1 in murine breast tumors upregulates SphK1 and decreases survival, we investigated whether increased expression of SphK1 and ABCC1 in human breast tumors could be a prognosis indicator. To this end, expression of ABCC1 and activated SphK1 in human breast cancer tissues was determined by IHC of human breast tumor microarrays and ER, PgR, and HER2 status was determined by an expert pathologist and tumors divided into 4 subtypes of breast cancer: Luminal [estrogen receptor positive (ER^+), $HER2^-$]; Luminal-HER2 (ER^+, $HER2^+$); HER2 (ER^-, $HER2^+$); and triple-negative breast cancer (TNBC: ER^-, $HER2^-$; Supplementary Table S1). Scoring of pSphK1 expression in human breast tumor samples was shown in Fig. 7A. The frequency of strong pSphK1 expression was higher in HER2 overexpressing or TNBC, the more aggressive breast cancer subtypes. pSphK1 was more prevalent and increased in a larger tumors (higher T stage) and in tumors from patients with lymph node metastases (higher TNM stage; Fig. 7B; Supplementary Table S3). Finally, we correlated clinical outcomes with expression of pSphK1 and ABCC1. Importantly, patients with breast tumors that had higher expression of pSphK1 had worse disease-free survival compared with those with weaker pSphK1 levels ($P = 0.011$; Fig. 7C). Strikingly, in patient tumors with high expression of both pSphK1 and ABCC1, disease-free survival was significantly decreased (Fig. 7C). In contrast, there was no significant difference in survival of those expressing high levels of ABCB1 and pSphK1 (Fig. 7C). In agreement with previous studies showing that expression of SphK1 is elevated in patients with breast cancer and correlates with poor prognosis (13), mining of METABRIC breast tumor expression database showed that SphK1 expression significantly correlates with worse survival prognosis (median survival of 124 months with high SphK1 expression compared with 163 months for patients with low SphK1 expression, $P = 0.0014$; Fig. 7D). Furthermore, those with high levels of both SphK1 and ABCC1 had much worse prognosis with median survival of 114 months ($P < 0.0068$, Fig. 7D). Such correlations were not observed with ABCB1 expression (Fig. 7D).

Together, these findings support the notion that breast cancer patients with tumors that have high ABCC1 expression have poorer survival, at least in part, due to enhanced expression of SphK1 and secretion of S1P into the tumor microenvironment.

Discussion

Among the many known ABC transporters, ABCB1 and ABCC1 are two multidrug resistant proteins that are upregulated in breast cancer in response to chemotherapy and contribute to chemoresistance (2). ABCB1 is the most well studied as it effluxes the commonly used anticancer drugs, anthracyclines and taxanes. However, clinical trials with agents targeting ABCB1 have all failed (35). Recent reports suggest that the functions of ABC transporters are not limited merely to the efflux of drugs as they also transport other types of molecules including lipids (36). Lipid-derived signaling molecules, such as leukotrienes and prostaglandins, conjugated organic anions, and S1P, have also been identified as substrates of multitasking ABCC1 transporter (37). Among them, the bioactive sphingolipid mediator S1P is now recognized as a critical regulator of many physiologic processes important for breast cancer progression (38). S1P is generated inside cancer cells by SphK1, and then exported outside of the cell into the tumor microenvironment where it can bind to five G protein–coupled receptors whose downstream signaling is responsible for most of the action of S1P. This "inside-out" signaling by S1P plays a pivotal role in cancer cells and in the tumor microenvironment by regulating inflammatory cells recruitment and stimulating angiogenesis and lymphangiogenesis (39). SphK1 levels are upregulated in many malignant tumors, including lung (40), kidney (33), colon (41), breast (13, 18), prostate (42), stomach (43), liver (44), brain (45), in non-Hodgkin lymphoma (46), and chronic myeloid leukemia (47), and has been reported to be associated with poor prognosis in several types of cancer, including esophageal (48), bladder

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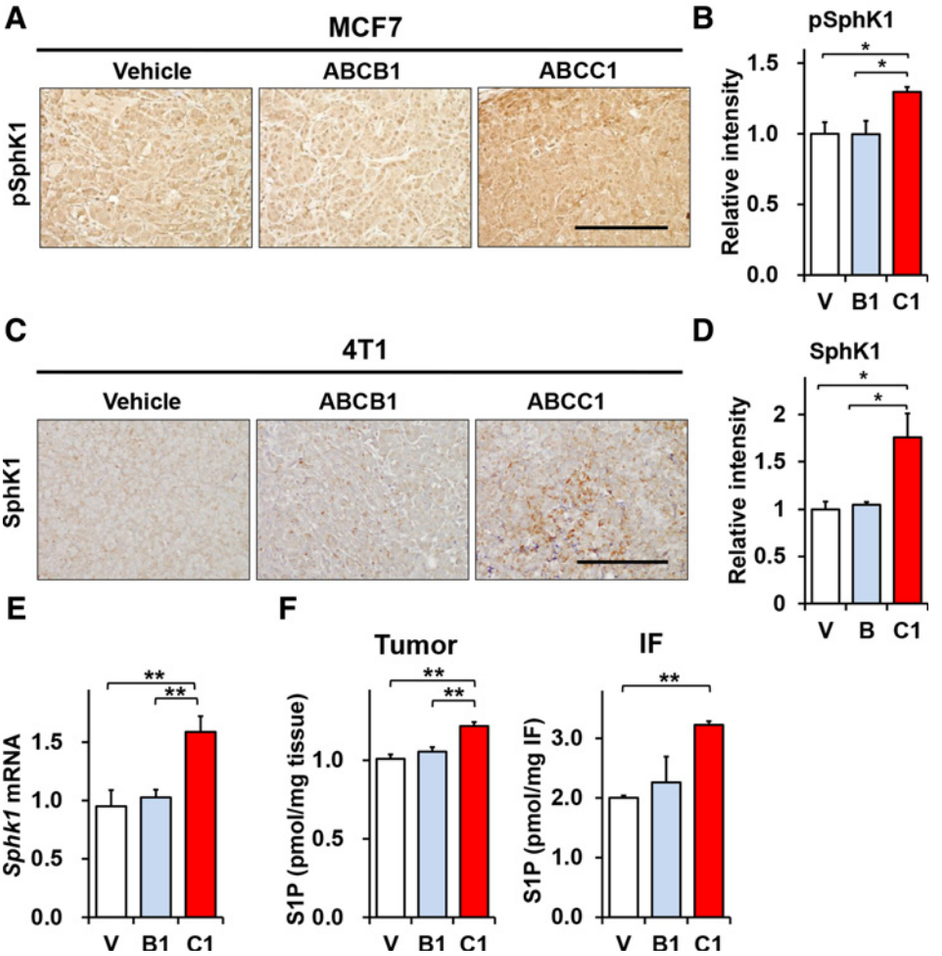

Figure 6.
Overexpression of ABCC1 in breast tumors enhances expression of SphK1 and increases S1P in tumor interstitial fluid. **A** and **B,** MCF7 cells transfected with vector, ABCB1, or ABCC1 were implanted into chest mammary fat pads of ovariectomized BALB/c nude mice. pSphK1 in tumors determined by IHC on day 32. **C–E,** 4T1-luc2 cells transfected with vector, ABCB1, or ABCC1 were implanted into mammary fat pads of BALB/c mice and tumors analyzed on day 12. SphK1 expression in 4T1 tumors was examined by staining with anti-SphK1 antibody. **B** and **D,** Relative intensity of immunostaining was quantified by NIH ImageJ. Scale bar, 200 μm. **E,** *Sphk1* mRNA level in tumors were determined by qPCR and normalized to *Gapdh*. **F,** S1P levels in tumors ($n = 10$) and in tumor interstitial fluid ($n = 5$) were determined by LC-ESI-MS/MS. Data, means ± SEM. *, $P < 0.05$; **, $P < 0.01$.

(49), prostate (50), and breast (13, 51). In agreement, we found that expression of SphK1 correlates with breast cancer staging. We also demonstrated for the first time that activated SphK1, determined with a phospho-SphK1–specific antibody, which is a direct reflection of S1P production, is associated with poor survival in human breast cancer. Interestingly, patients whose breast tumor had high expression of both activated SphK1 and ABCC1 had significantly shorter disease-free survival, whereas no associations were observed with expression of ABCB1. These findings are consistent with the view that increased production of S1P by higher levels of pSphK1 and its increased efflux of S1P by ABCC1 combine to shorten survival.

It is now recognized that tumors display another level of complexity by regulating the tumor microenvironment (52). We demonstrated previously that S1P is increased in breast tumors and in their interstitial fluid that fills the space of the tumor microenvironment (26) and that S1P can enhance breast cancer–induced angiogenesis and lymphangiogenesis (18, 53). In the current study, we have shown that S1P levels in tumor and interstitial fluid are higher in breast tumors that overexpress ABCC1. Furthermore, export of S1P by ABCC1 not only affected the cancer cells themselves and markedly enhanced tumor growth, it also influenced the tumor microenvironment, increasing angiogenesis, and lymphangiogenesis. It is thus not surprising that these rapidly growing tumors aggressively metastasized to lymph nodes or distant organs and shortened survival of mice bearing these tumors. In sharp contrast, overexpression of ABCB1 could result in a slightly increased level of S1P around breast cancer cells that have very high endogenous SphK1, but the effects of small increases in S1P levels on physiologic functions, such as tumor progression and metastasis, would not be expected to be significant. Our results suggest that export of S1P by ABCC1 is as important as production of S1P by SphK1 in cancer progression and in the tumor microenvironment.

Another unexpected and important finding in this study was that S1P exported via ABCC1 from breast cancer cells upregulated their expression of SphK1, leading to further increased production of S1P that in turn acts on the tumor cells themselves and on the tumor microenvironment. Hence, exported S1P acts in a malicious feed-forward amplification loop to amplify the S1P axis that drives tumorigenesis and metastasis. Although not examined in the current study, the results of many previous studies suggest that these effects of S1P are mediated by binding to S1P receptors present on the cancer cells or on cells in the microenvironment, including endothelial and lymphendothelial cells (8). Similar to the shorter survival of mice bearing tumors overexpressing ABCC1 (that upregulates SphK1), patients with tumors that overexpress both SphK1 and ABCC1 have poor prognosis. Taken together, our

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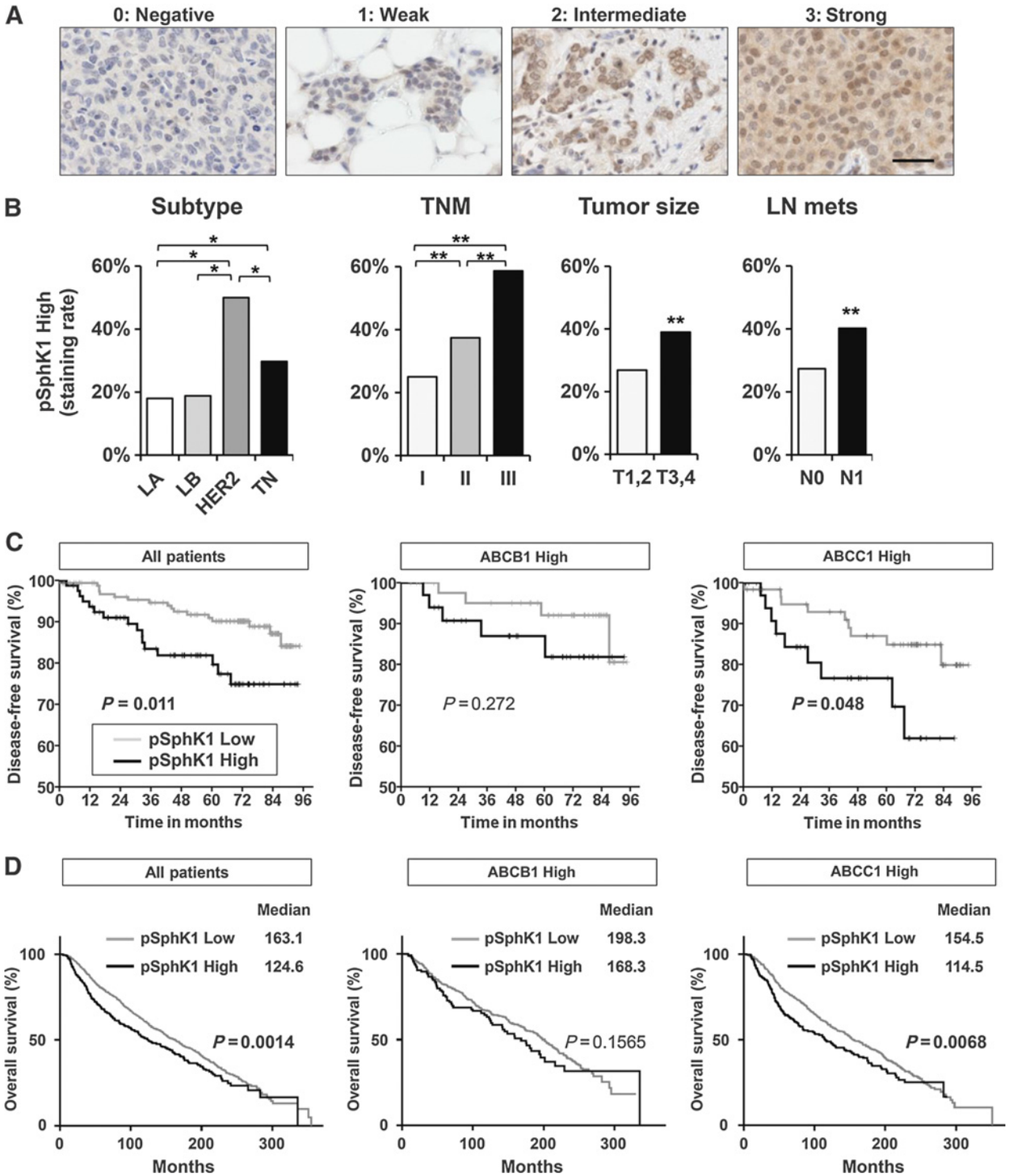

Figure 7.

Patients with breast cancers that express both activated SphK1 and ABCC1 have shorter disease-free survival. pSphK1 in 275 human breast tumors examined by IHC. **A,** Scoring of pSphK1 expression in human breast tumor samples. **B,** Frequency of high pSphK1 expression in human breast tumors correlated with clinicopathologic factors, tumor size, and lymph node metastasis status and TNM stage. *, $P < 0.05$; **, $P < 0.01$. **C,** Kaplan–Meier disease-free survival curves according to expression of pSphK1, co-expression of pSphK1 with ABCB1, and coexpression of pSphK1 with ABCC1. *P* values were calculated by the log-rank test. **D,** Kaplan–Meier survival analysis of breast cancer patients from the METABRIC database. Data were obtained from patients with clinical and expression information. Median survival is tabulated along with a log-rank *P* value representing the significance of high gene expression of SphK1 among all patients, among ABCB1 high patients, or among ABCC1 high patients on patient survival.

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results suggest that combined therapies that target SphK1 (S1P production) and ABCC1 (S1P secretion) should be more beneficial than targeting each of them alone or then inhibitors of ABCB1. Our study might also explain the failure of previous clinical trials targeting ABCB1. We suggest that ABCC1, not ABCB1, contributes to worse prognosis through the export of the potent lipid mediator, S1P, independently of its effects on efflux chemotherapeutic drugs. This represents a new concept of action of ABCC1 and a significant advancement in understanding of the role of ABC transporters in breast cancer, with the potential for applicability in other malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Yamada, S. Spiegel, K. Takabe
Development of methodology: W.-C. Huang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Yamada, M. Nagahashi, T. Aoyagi, W.-C. Huang, S. Lima, N.C. Hait, A. Maiti, K. Kida, K.P. Terracina, H. Miyazaki, T. Ishikawa
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Yamada, W.-C. Huang, M.R. Waters, Q. Qi, L. Yan, S. Spiegel, K. Takabe
Writing, review, and/or revision of the manuscript: A. Yamada, K.P. Terracina, S. Milstien, S. Spiegel, K. Takabe
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Ishikawa, I. Endo, K. Takabe
Study supervision: K. Takabe

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