Upregulation of the S1P₃ receptor in metastatic breast cancer cells increases migration and invasion by induction of PGE₂ and EP₂/EP₄ activation

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Abbreviations

Aza-C, 5'-aza-2'-deoxycytidine; COX-2, cyclooxygenase-2; cPLA₂, cytosolic phospholipase A₂; DMEM, Dulbecco's modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; EP, prostaglandin E₂ receptor; ERK1/2, extracellular signal-regulated protein kinase; GPCR, G protein-coupled receptor; HBSS, Hanks buffered salt solution; IL, interleukin; mPGES, microsomal prostaglandin E₂ synthase; NFκB, nuclear factor kappa-light-chain enhancer of activated B cells; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase polymerase chain reaction; PGE₂, prostaglandin E₂; S1P, sphingosine 1-phosphate; S1P₁₋₅, sphingosine 1-phosphate receptors; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; [Ca²⁺]_i, intracellular free Ca²⁺ concentration; STAT3, signal transducer and activator of transcription 3.

Abstract

Breast cancer is one of the most common and devastating malignancies among women worldwide. Recent evidence suggests that malignant progression is also driven by processes involving the sphingolipid molecule sphingosine 1-phosphate (S1P) and its binding to cognate receptor subtypes on the cell surface. To investigate the effect of this interaction on the metastatic phenotype, we used the breast cancer cell line MDA-MB-231 and the sublines 4175 and 1833 derived from lung and bone metastases in nude mice, respectively. In both metastatic cell lines expression of the S1P₃ receptor was strongly upregulated compared to the parental cells and correlated with higher S1P-induced intracellular calcium ($[Ca^{2+}]_i$), higher cyclooxygenase (COX)-2 and microsomal prostaglandin (PG) E₂ synthase expression, and consequently with increased PGE₂ synthesis. PGE₂ synthesis was decreased by antagonists and siRNA against S1P₃ and S1P₂. Moreover, in parental MDA-MB-231 cells overexpression of S1P₃ by cDNA transfection also increased PGE₂ synthesis, but only after treatment with the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine, indicating reversible silencing of the COX-2 promoter. Functionally, the metastatic sublines showed enhanced migration and Matrigel invasion in adapted Boyden chamber assays, which further increased by S1P stimulation. This response was abrogated by either S1P₃ antagonism, COX-2 inhibition or prostaglandin E₂ receptor 2 (EP₂) and 4 (EP₄) antagonism, but not by S1P₂ antagonism. Our data demonstrate that in breast cancer cells overexpression of S1P₃ and its activation by S1P has pro-inflammatory and pro-metastatic potential by inducing COX-2 expression and PGE₂ signaling via EP₂ and EP₄.

Keywords: breast cancer metastasis, sphingosine 1-phosphate, sphingosine 1-phosphate receptor 3, cyclooxygenase, prostaglandin E₂; prostaglandin E₂ receptor

1. Introduction

Breast cancer is the most common and devastating malignancy among women behind lung cancer and its metastasis is a major cause of poor prognosis [1,2]. The proliferative, migratory and invasive properties of metastatic cancer cells undermine the efficacy of available therapies, but more effective treatments for advanced breast cancer can only be developed if the molecular mechanisms are better understood [3,4]. Recent evidence suggests that inflammation is often involved in the malignant progression of solid tumors, including breast cancer, and chronic inflammation is a major risk factor for breast cancer development, treatment resistance and recurrence [5,6]

Although the link between inflammation and cancer initiation is well established by its ability to generate a pro-proliferative and pro-angiogenic environment, its role in metastatic diseases, has been poorly explored. Prominent mediators of cancer inflammation include the oncogenic NF κ B and several cytokines like IL-6, IL-8 and IL-1 β , which are activated and overexpressed in breast cancer [7,8]. In addition, biologically active lipids like eicosanoids are implicated in inflammation and cancer too. The cyclooxygenase isoform-2 (COX-2) is frequently overexpressed in solid tumors and activated by different cytokines and growth factors to support inflammatory processes and migration [9,10]. It generates the metabolite prostaglandin E₂ (PGE₂), which promotes cancer cell growth, stimulates angiogenesis, migration and invasion, confers resistance to apoptosis and suppresses immunity [11,12]. Also in breast cancer, elevated expression of COX-2 and overproduction of downstream PGE₂ is associated with a metastatic phenotype and poor prognosis [13,14]. The biological effect of PGE₂ is mediated by binding to EP receptors of which four subtypes have been characterized [15,16]. Particularly, EP₂ and EP₄ have been found to be closely associated with an invasive phenotype in various cancer types [17,18]. In breast cancer cells COX-2 induced PGE₂ synthesis was shown to increase expression of the pro-invasive CCR7 chemokine via EP₂ and EP₄ activation, and in breast cancer tissues overexpression of COX-2 correlates with lymph node metastasis [14].

Recent findings corroborate that malignant progression and aggressiveness of breast cancer is also driven by processes involving the bioactive sphingolipid metabolite sphingosine 1-phosphate (S1P). S1P has a pro-inflammatory potential [19,20] and stimulates cancer-relevant responses like cell growth, migration, invasion and angiogenesis [21]. Although S1P shows also intracellular distribution, most of its biological effects result from binding to cognate G protein-coupled receptors (GPCRs) on the cell surface [22,23]. Five subtypes of S1P receptors (S1P₁₋₅), have been identified, each of it demonstrates tissue-specific expression and couples to different G proteins [24]. As shown in renal mesangial cells and smooth muscle cells, S1P binding to S1P₂ and S1P₃ can induce COX-

2 expression and PGE₂ synthesis [25,26], and from lung cancer cells there is evidence that it activates cPLA₂ to produce arachidonic acid via S1P₃ [27]. In line with these findings, S1P was also reported to induce PGE₂ synthesis through activation of S1P₁ and S1P₃ in non-tumorigenic granulosa cells [28]. Furthermore, increased formation of S1P with subsequent activation of S1P₁ and the STAT3 pathway links chronic intestinal inflammation to colitis-associated carcinogenesis [29], and in breast cancer S1P was shown to induce migration and invasion via S1P₃-mediated upregulation of MMP-9 [30]. S1P is also able to transactivate tyrosine kinase receptors such as the EGF receptor and thereby increase EGFR signaling in breast cancer cells [31]. On the other hand, in non-malignant cell types anti-inflammatory effects of S1P mediated by S1P₁, S1P₂ and/or S1P₃ have been described [32,33], a discrepancy which needs to be clarified by further investigations.

In the present study, we used the breast cancer cell line MDA-MB-231 and two sublines established from single cell-derived progenies derived from lung and bone metastases in nude mice [34,35] to investigate S1P-induced pro-inflammatory effects associated with their increased metastatic potential. We report that in both metastatic sublines S1P₃ is overexpressed and its activation by extracellular S1P stimulates COX-2 expression and PGE₂ synthesis. PGE₂ binding to EP₂ and also EP₄ on the metastatic cells increases migration and Matrigel invasion in an auto- and paracrine manner thus underpinning their aggressive phenotype.

2. Materials and methods

2.1. Chemicals and reagents

The PGE₂-specific ELISA was obtained from Enzo Life Science (Lörrach, Germany); human recombinant IL-1 β was from PeproTech (Hamburg, Germany). Secondary HRP-coupled IgGs, hyperfilm MPR and enhanced chemiluminescence reagents were purchased from GE Health Care Systems (Glattbrugg, Switzerland). Antibody against COX-2 was from Cell Signaling (Schwalbach, Germany); antibody against microsomal prostaglandin E synthase-1 (mPGES-1) was from Cayman Chemicals Inc. (Ann Arbor, MI, US); antibody against β -actin (clone AC-15) was from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland); S1P, VPC23019 and W146 were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA); JTE013, TY52156, SC51322, PF04418948, L-798.106, were from Tocris Bioscience (Bristol, United Kingdom); AH23848, digitonin, celecoxib, CYM5541, PGE₂, 5'-aza-2'-deoxycytidine (aza-C), PCR primers and KICqStart[®]SYBR[®]Green qPCR ReadyMix[™] (SYBRgreen) were obtained from Sigma Aldrich Chemie GmbH (Buchs, Switzerland); fura-2/AM was from LuBio Science GmbH (Luzern, Switzerland); TurboFect[™] was from Fermentas Life Science/ThermoFisher Scientific (Zug, Switzerland); First Strand DNA Synthesis Kit, was from MBI Fermentas (St-Leon-Roth, Germany); TRIZOL[®] reagent (5-Prime), Corning[®] Matrigel[®] Basement Membrane Matrix and growth factor reduced (GFR) (Matrigel) were from VWR International AG (Dietikon, Switzerland); all cell culture media, supplements and Oligofectamine were purchased from Invitrogen AG (Basel, Switzerland);

2.2. Cell lines and cell culture conditions

The human breast cancer cell line MDA-MB-231 and its sublines 4175 and 1833, which were derived by single-cell cloning from lung and bone metastases of nude mice, respectively [34], are from Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). Cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 10 mM HEPES pH 7.4, 100 units / ml penicillin and 100 μ g/ml streptomycin. All cells were grown at 37⁰ C in a humidified atmosphere containing 5 % CO₂.

For S1P₂ and S1P₃ downregulation, cells were transfected with Oligofectamine and 100 nM of a Smartpool siRNA of hS1P₂ (Dharmacon, ON-TARGETplus SMARTpool S1PR2 (L-003952) or hS1P₃ (Dharmacon, ON-TARGETplus SMARTpool S1PR3 (L-005208)) as recommended by the manufacturer in 24-well-plates. Control siRNA transfection was performed with non-targeting siRNA (On-TARGETplus Control D-001810). 48 h after transfection cells were incubated under serum free conditions for 24 h and then stimulated with S1P or vehicle for 2 h.

2.3. S1P₃ overexpression in MDA-MB-231 cells

The human S1P₃ pcDNA3.1 construct was kindly provided by Dr. D. Guerini (Novartis, Basel). Transfection was performed using TurboFect™ following the manufacturer's instructions. Briefly, parental MDA-MB-231 cells were subcultured into a 6-well plate for 24 hours to achieve ~70% confluency on the day of transfection. 2 µg of DNA and 6 µl of transfection reagent were mixed in 200 µl serum free DMEM and placed drop-wise to each well for 48 hours. Stable transfectants were obtained under selection conditions in the presence of 0,5 mg/ml G418 for 10 -15 days.

2.4. Quantitative real-time PCR

Total RNA (1 µg) isolated with TRIZOL® reagent (5-Prime) was used for reverse transcriptase PCR using the First Strand DNA Synthesis Kit and a random hexamer primer for amplification. The real-time PCR reaction was performed using SYBRgreen and a BioRad iQ5 Cyclor Detection System (BioRad Laboratories AG, Glattbrugg, Switzerland). The following primers were used: human S1P₁: forward CCTCTAGCGTTCGTCTGG; reverse TTTCCCGTGTAGTTGTAATGC; human S1P₂: forward TCAAGACGGTCACCATCG; reverse ACAGGCATAGTCCAGAAGG; human S1P₃: forward CTTACGACGCCAACAAGAG; reverse CAGGATGGTAGAGCAGTCAG; human S1P₄: forward TGTTCTGCACTACAACCACTC; reverse AAGTTCTCCAGCACCACCAG; human S1P₅: forward TCTTCCTGCTGCTGTTGCTC; reverse GCGTGTAGATGATGGGGTTCAG; human COX-1: forward CTAAAGATTGCCCCACACC; reverse GTTTCCCATCCTTAAAGAGCC; human COX-2: forward TGACAGTCCACCAACTTACAATGC; reverse AATCATCAGGCACAGGAGGAAGG; human EP₁: forward CACGTGGTGCTTCATCGGCCTGGGTC; reverse CACCACCATGATACCGACAAG; EP₂: forward TCCTGTTCTGAGACTAATGCGTTC; reverse GTTTACRGGCATCTGACTGTGTAG; EP₃: forward GGGCCTGATGGAAGGTGTTT; reverse TCTATCATGAGAACTGCACCAAGTC; EP₄: forward TCTGACCTCGGTGTCCAAAAATCG; reverse TGGGTACTGCAGCCGCGAGCTA. The fold induction values were obtained according to the $\Delta\Delta C_T$ method after normalization to the housekeeping gene 18S RNA.

2.5. Cell homogenization and Western blotting

Cells were washed with phosphate-buffered saline (PBS), scraped into ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X100, 2 mM EDTA, 2 mM EGTA, 40 mM β -glycerophosphate, 50 mM sodium fluoride, 10 µg ml⁻¹ leupeptin, 10 µg ml⁻¹ aprotinin, 1 µM pepstatin A, 1 mM phenylmethyl sulphonyl fluoride) and homogenized by passing 10 times through a 26-G needle fitted to a 1 ml syringe. Samples were centrifuged

for 10 min at 13'000 x g. The supernatant was taken for protein determination and 30 µg protein was separated by SDS-PAGE, transferred to a nitrocellulose membrane and subjected to Western blotting as described [36] using the antibodies indicated in the figure legends.

2.7. Measurement of $[Ca^{2+}]_i$ in cell suspension

Intracellular Ca^{2+} was measured essentially as described [37]. Briefly, after starvation for 24 hours, cells were washed and suspended in HBSS (118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM D-glucose and 15 mM Hepes (pH 7.4), and loaded for 45 min with 1 µM of the Ca^{2+} indicator dye fura-2/AM. Then, the cells were washed twice in HBSS and fura-2 fluorescence was monitored in a spectrofluorometer (Hitachi) using excitation wavelengths of 340/380 nm and an emission wavelength of 510 nm. At a stable baseline (250 sec), S1P was added to a final concentration of 1 µM and the change of fluorescence was recorded. At the end of each scan (550 sec), 10 µM of digitonin was added to determine the maximum Ca^{2+} and 10 mM of the chelating agent EDTA was added (750 sec) to achieve the minimum concentration of Ca^{2+} in the cell. Data were calculated using the FL WinLab program (PerkinElmer AG, Schwerzenbach, Switzerland).

2.8. Measurement of secreted prostaglandin E_2

PGE_2 in cell culture supernatants was quantified using a competition immunoassay (PGE_2 -ELISA/EIA kit from Enzo Life Sciences, Lörrach, Germany). 100 µl cell supernatants were processed according to the manufacturer's instructions. The absorbance at 405 nm was measured by spectrophotometry and the PGE_2 concentration was determined with a calibration curve using PGE_2 as standard.

2.9. Migration and invasion assays

Cell migration and invasion were measured with an adapted Boyden chamber assay. For invasion measurement, transwell filters had been precoated with 100 µl of 6 mg/ml Matrigel. Quiescent cells at a density of $2,5 \times 10^5$ cells in 200 µl starvation medium (0.1 mg/ml^{-1} fatty acid-free bovine serum albumin) were seeded into the transwell filters (6,5 µm diameter, 8 µm pore size). 750 µl of serum free or complete growth medium (for migration and invasion assays, respectively) was placed in the lower compartment and cells were incubated at 37°C for the indicated time periods to allow migration/invasion. Thereafter, transwell filters were removed and cells which had migrated/invaded through the filter into the lower chamber were counted in four different random fields for one sample using a Nikon TMS-F microscope (Nikon AG, Egg/ZH, Switzerland).

2.10. Statistical analysis

Statistical analysis of data was performed using one-way ANOVA following Dunnett post-hoc test for multiple comparisons or unpaired t-test when only two groups were compared.

3. Results

3.1. S1P₃ is upregulated in metastatic breast cancer cells and its activation by S1P induces intracellular calcium mobilization

The role of S1P and its cognate cell surface receptors in breast cancer metastases is still unclear. Here, we used the breast cancer cell line MDA-MB-231 and the two sublines 4175 and 1833 derived from lung and bone metastases, respectively, in nude mice [34], to determine the S1P receptor subtype associated with the metastatic phenotype. First, parental and metastatic MDA-MB-231 cells were characterised for S1P receptor expression using quantitative PCR. As shown in Fig. 1, the parental MDA-MB-231 cells showed the highest mRNA levels for S1P₃ and S1P₅ among the different receptor subtypes, low expression of S1P₁ and S1P₂, whereas S1P₄ was undetectable. For comparison, in both metastatic sublines expression of S1P₃ was strongly enhanced and reached particularly high levels in 1833 cells. The S1P₁ was downregulated in both metastatic sublines whereas the other S1P receptors showed a similar expression pattern as in parental MDA-MB-231 cells (Fig. 1).

To verify that overexpression of S1P₃ in the metastatic MDA-MB-231 sublines is coupled to increased S1P₃ receptor signalling, we investigated whether Ca²⁺ mobilization as an early read-out of receptor activation [23,37] increased in the presence of extracellular S1P. To this end, cells were loaded with the Ca²⁺ indicator fura-2/AM and stimulated with S1P. As shown in Fig. 2A, the parental MDA-MB-231 cells responded to S1P with only a marginal increase in intracellular Ca²⁺ ([Ca²⁺]_i). In contrast, in both metastatic sublines, which had higher basal ([Ca²⁺]_i) levels, S1P induced a 6- to 8-fold increase of [Ca²⁺]_i compared to the parental cells (Fig. 2A, B). In all cell lines, pre-treatment with the S1P_{1/3} antagonist VPC23019 effectively blocked S1P-induced Ca²⁺ mobilization (Fig. 2C and S1). The S1P₁ antagonist W146 had no effect on any of the cell lines and the S1P₂-selective antagonist JTE013 suppressed [Ca²⁺]_i increase only in the parental cells (Fig. 2C, S1). A selective S1P₅ antagonist is not yet available and therefore, this receptor subtype could not be explicitly excluded. The significant increase in baseline Ca²⁺ levels measured upon pre-treatment of 4175 cells with VPC23019 (Fig. S1) might be due to antagonism of G_i-coupled S1P₁. The pattern of responsiveness to the various antagonists together with the receptor subtype expression profiles shown in Fig. 1 confirms that S1P₃ overexpressed on the

metastatic MDA-MB-231 cells is fully functional and represents the major receptor involved in S1P-induced Ca^{2+} mobilization.

3.2. COX-2 and mPGES-1 are upregulated in metastatic breast cancer cells

There is evidence that COX-2 and its product PGE_2 are implicated in breast cancer metastasis [13,14]. Here, we investigated whether COX-2 and PGE_2 are also upregulated in the two metastatic MDA-MB-231 sublines. As shown in Fig. 3 A, the untreated metastatic sublines already produce higher basal amounts of PGE_2 compared to the parental MDA-MB-231 cells. Treatment of cells with the inflammatory cytokine interleukin (IL)-1 β together with forskolin (Fk), a regimen known to synergistically upregulate PGE_2 [38], only very slightly increased PGE_2 in the parental cells but triggered a strong increase in both metastatic sublines (Fig. 3A). As shown in Fig. 3B and Fig. S2, basal COX-2 and COX-1 mRNA expression was higher in the metastatic cells compared to the parental cells. Most importantly, however, similar to PGE_2 synthesis IL-1 β /Fk strongly increased COX-2 mRNA expression (Fig. 3B) and protein expression (Fig. 3D) in the metastatic cells, whereas COX-1 expression even slightly decreased (Fig. S2). This strongly suggests that the high level of PGE_2 was produced by COX-2. Again, in the parental MDA-MB-231 cells the effect of IL-1 β /Fk on COX-2 and COX-1 expression was marginal (Fig. 3B, Fig. S2).

Another downstream enzyme which is involved in PGE_2 synthesis and inducible by inflammatory mediators is the microsomal prostaglandin E synthase-1 (mPGES-1). We found that in the metastatic MDA-MB-231 cells mPGES-1 is also upregulated on the mRNA and protein level, and is strongly inducible by IL-1 β /Fk (Fig. 3C, D). Again, in the parental MDA-MB-231 cells the effect of IL-1 β /Fk on mPGES-1 expression was low (Fig. 3C, D). Since IL-1 β /Fk is known as a strong inducer of COX-2 and mPGES-1 in many cell types, we wondered whether the here observed weak effect in the parental MDA-MB-231 cells was due to suppression by promoter silencing, as such epigenetic regulation was reported also for other genes during metastatic selection in this cell line [39]. In fact, treatment of the parental cells with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (aza-C) increased COX-2 mRNA and protein expression (Fig. S3A, B), suggesting that the COX-2 promoter is silenced in these cells by methylation.

3.3. S1P induces COX-2 expression and PGE_2 synthesis in metastatic breast cancer cells via S1P₃

To investigate the possibility that the high COX-2 expression and PGE_2 synthesis in the metastatic MDA-MB-231 cells is induced by S1P via overexpressed S1P₃, we treated

parental and metastatic cells with exogenous S1P and measured COX-2 mRNA expression and PGE₂ synthesis at different time points. Fig. 4A shows that in parental MDA-MB-231 cells S1P induced only a marginal time-dependent increase of COX-2 mRNA expression, consistent with the finding that the COX-2 gene is silenced in these cells. On the other hand, in the two metastatic sublines S1P induced a strong time-dependent increase in COX-2 mRNA expression with peak values reached after 2 to 4 hours, which then returned to low basal levels. COX-2 protein expression behaved similarly, peaked at 2-4 hours and thereafter declined to control levels over the next hours (data not shown). As expected, quantification of PGE₂ in the cell culture supernatants revealed that S1P strongly accelerated PGE₂ synthesis in the metastatic cells compared to the parental cells (Fig. 4B). Notably, the secreted PGE₂ levels remained constantly high over 24 hours of S1P stimulation. The basal PGE₂ secretion over time was low but steadily increased over 24 h (Fig. S4)

To further investigate the mechanism underlying S1P-induced PGE₂ overproduction, the COX-2-selective inhibitor celecoxib was used. As shown in Fig. 5A, celecoxib dose-dependently blocked S1P-stimulated PGE₂ synthesis in the metastatic cells, confirming that particularly the COX-2 enzyme is responsible for PGE₂ overproduction induced by S1P. Furthermore, the use of various S1PR antagonists demonstrated that S1P-induced PGE₂ synthesis is effectively abolished by the S1P_{1/3} antagonist VPC23019 and the S1P₂ antagonist JTE013, but not by the S1P₁-selective antagonist W146, suggesting that S1P₂, despite its low expression, and S1P₃ are involved (Fig. 5B). The selective S1P₃ agonist CYM5541 and the selective S1P₂ agonist CYM5520 also enhanced PGE₂ synthesis (Fig. 5C), thus strongly suggesting a critical role of both receptors. In a different approach, we downregulated S1P₂ and S1P₃ in 4175 cells by transfection with specific siRNAs. This downregulated approx. 80% of S1P₂ and 85-90% of S1P₃ in lung and bone metastatic cells (Fig. S5) and both treatments also inhibited S1P-stimulated PGE₂ synthesis to a similar extent (Fig. 5D), thus confirming that both receptors are involved in PGE₂ synthesis.

In addition, to confirm that S1P₃ activates PGE₂ synthesis, we stably overexpressed S1P₃ in parental MDA-MB-231 cells (Fig. 6A). Consistent with the fact that the COX-2 promoter is silenced in these cells, overexpression of S1P₃ alone and upon stimulation with S1P did not increase PGE₂ production (Fig. 6B). However, when the S1P₃-overexpressing cells were stimulated with S1P in the presence of the demethylating agent aza-C, PGE₂ production was strongly increased compared to untransfected control cells (Fig. 6B).

3.4. S1P/S1P₃ increases migration of metastatic breast cancer cells in a COX-2 and PGE₂-dependent manner

PGE₂ is a major pro-migratory prostanoid and implicated in metastasis [12]. Here, we investigated whether the high expression level of S1P₃ contributes to the metastatic phenotype of the MDA-MB-231 sublines by mediating S1P-induced COX-2 and PGE₂ synthesis. To this end, the migratory potential of cells was determined using an adapted Boyden chamber assay. As expected, the metastatic sublines migrated significantly faster than the parental cells and also strongly responded to S1P (Fig. 7A). Similarly, PGE₂ added to the upper chamber further strongly enhanced the migration of the metastatic variants whereas the parental cells were only marginally responsive (Fig. 7A). As shown in Fig. 7B, the S1P_{1/3} antagonist VPC23019 and the recently developed S1P₃-selective antagonist TY52156 [40] both effectively offset S1P-induced migration. In contrast, the S1P₂ antagonist JTE-013 failed to reduce S1P-stimulated migration (Fig. 7C).

Next, we examined, whether COX-2 is directly involved in the pro-migratory effect of S1P. To this end, we treated cells with the selective COX-2 inhibitor celecoxib to abrogate PGE₂ synthesis. Fig. 7D shows that celecoxib indeed inhibited S1P-induced cell migration in both metastatic sublines. This suggests that the pro-migratory effect of S1P/S1P₃ signaling is mediated by increased PGE₂ synthesis and secretion, followed by activation of cognate EP receptors on the metastatic cells in an auto- and paracrine manner.

In a control experiment presented in Fig. 7E, we examined the pro-migratory effect of S1P on parental cells MDA-MB-231 cells overexpressing S1P₃ by transfection. In untransfected control cells S1P alone induced a measurable but marginal increase of migration as expected from the relative lack of COX-2 and PGE₂ expression in these cells. Surprisingly, however, we found that also aza-C had only a very marginal stimulatory effect and even in cells overexpressing S1P₃, which overproduce PGE₂ in the presence of S1P and aza-C (Fig. 7E), the migratory response did not markedly increase compared to the untransfected cells. This indicates that in parental MDA-MB-231 cells additional defects are involved which negatively affect migration.

3.5. PGE₂ stimulates migration and invasion of metastatic breast cancer cells by EP₂ and EP₄ activation

To investigate the role of the EP subtypes in PGE₂-stimulated cell migration, we measured mRNA expression of the four known receptor EP₁₋₄. EP₂ was highly upregulated in both metastatic sublines with the highest level measured in 1833 cells (Fig. 8A). Expression of

the other receptor subtypes was much lower and similar in the three cell lines, except for EP₄, which was slightly higher in 1833 cells (Fig. 8A).

To investigate whether the highly expressed EP₂ is involved in the pro-migratory action of PGE₂ in metastatic 4175 cells, we used antagonists selective for all four receptor subtypes. Fig. 8B shows that antagonism of EP₂ (by PF04418948) and EP₄ (by AH23848) attenuated S1P-induced migration. In contrast, an EP₁ antagonist (SC51322) and an EP₃ antagonist (L-798,106) were inefficient. This indeed suggests a pro-migratory stimulation loop initiated by S1P binding to S1P₃, which results in PGE₂ overproduction and secretion followed by activation of EP₂ and EP₄. The low level of EP₂ expression on parental MDA-MB-231 cells might explain why S1P₃ overexpression and aza-C could only partially increase the migratory potential (Fig. 7D)

Finally, as another hallmark of metastasis we examined the invasive potential of the MDA-MB-231 cell lines resulting from EP₂ and EP₄ signalling. To this end, parental and metastatic cells were allowed to migrate through a membrane covered with Matrigel in a transwell system. Similar to migration, the metastatic cells showed stronger invasion compared to the parental cells and it further increased significantly upon stimulation with S1P or PGE₂ (Fig. 9A). Moreover, as shown in Fig. 9B for the metastatic 4175 subline, the selective S1P₃ antagonist TY52156, the COX-2 inhibitor celecoxib and the EP₂ and EP₄ antagonists (PF04418948 and AH23848, respectively) all significantly offset the stimulatory effect of S1P on invasion. Together with the data from the migration assay, this suggests a crucial role of EP₂ and EP₄ in the pro-metastatic activity of PGE₂ in breast cancer.

4. Discussion

Inflammatory breast cancer is a metastatic disease characterized by an aggressive and invasive phenotype [5,6]. To investigate the role of S1P and its cognate receptors in inflammation and metastasis, we used a breast cancer metastases model based on the MDA-MB-231 cell line and the two sublines 4175 and 1833 established from single cell-derived progenies with enhanced metastatic ability to lung and bone, respectively, by in vivo selection [34,35]. All three cell lines express a common poor-prognosis gene expression signature, demonstrating that the metastatic clones already pre-exist in the parental cell population, which is derived from a pleural effusion, but in addition show a gene expression profile characteristic for their organ-specific localization [34,35]. The selection of such pre-existing highly metastatic clones from a parental cell population offers the possibility to compare cells with a very similar genetic background and specifically identify differences associated with increased metastasis. Here, we investigated such

differences with focus on S1P receptor signaling as this pathway was shown to be implicated in inflammatory processes and to support pro-metastatic features like migration and invasion [20]. We did not further explore differences related to the site of metastasis of the MDA-MB-231 sublines, i.e. lung and bone. Interestingly, we found that the three cell lines revealed distinct profiles of S1P₃ receptor expression, which was much higher in both metastatic sublines compared to the parental cell population. We are unable to present data on the protein level as several commercially available S1P₃ antibodies revealed irrelevant unspecific bands by Western blotting (data not shown). This has recently been reported as a serious drawback if antibodies against GPCR proteins are used and may question published data on these targets [41]. S1P₃ overexpression has not yet been described by transcriptomic analyses of these cell lines so far, and little is known about its implication in breast cancer development and metastatic progression. The advantage of S1P₃ upregulation for cell survival and metastasis formation in the lung and bone is unclear. It is, however, plausible to assume that it represents only part of a complex pro-metastatic signature and that the biological consequences depend on the interplay with other phenotypic changes. Therefore, a simple one to one correlation between S1P₃ expression levels and the here measured biological effects can not be expected.

S1P₃ couples to G_i, G_q and G_{12/13} [23]. Upon S1P binding, one of the fast intracellular responses mediated by S1P₃/G_q is induction of PLC/IP₃ signaling and Ca²⁺ mobilization [30]. S1P₃ overexpressed on the metastatic sublines was fully functional and increased [Ca²⁺]_i upon stimulation with extracellular S1P. Accordingly, the basal level of [Ca²⁺]_i in these cells is higher compared to the parental cell line, and stimulation with S1P further induced [Ca²⁺]_i in the metastatic, but not in the parental cell lines. As expected, this response to S1P was mitigated particularly by the S1P_{1,3} antagonist VPC23019 [42] (Fig. S1), corroborating previous findings that S1P₃ and S1P₂ can both increase Ca²⁺ mobilization [43]. However, we are aware that the antagonists do not fully discriminate between all receptor subtypes and may even dose-dependently cross-react as partial agonists [42,44], these data thus only indicate a trend and must be considered in the context of the receptor expression profiles. Similarly, without S1P stimulation we also measured a higher baseline of intracellular Ca²⁺ in the presence of VPC23019 in the metastatic sublines, however, we hypothesize that this was due to suppression of the inhibitory activity of G_i-coupled S1P₃.

In the metastatic MDA-MB-231 sublines activation of S1P₃ also increased COX-2, but not COX-1 expression, and consequently synthesis of the biologically active prostanoid PGE₂, which could be measured in the culture supernatants. Enhanced COX-2 expression is frequently found in invasive breast cancer [13] and was identified as a hallmark and poor

prognosis gene expression signature of metastatic MDA-MB-231 cells [34,35]. In line with this finding we also measured constitutively high levels of COX-2 in the two metastatic sublines compared to the parental cells, and expression could be further increased by treatment with IL-1 β /Fk, a potent inducer of the enzyme [45]. In contrast, the parental cells expressed only very low levels of COX-2 and also did not respond to IL-1 β /Fk. Interestingly, further investigations using the demethylating agent aza-C [46] unveiled COX-2 promoter silencing in the parental cell population, which in the two sublines was apparently suspended during metastatic selection.

PGE₂ is implicated in various pathological processes such as inflammation and cancer, and its role in carcinogenesis and tumor growth is well established [12]. On the other hand, the PGE₂ analog 16,16-dimethyl-PGE₂ was shown to inhibit intestinal tumor formation and growth [47]. Here, we found that in both metastatic MDA-MB231 sublines the high levels of COX-2 correlated with PGE₂ synthesis, whereas PGE₂ was hardly detectable in the parental cell line (Fig. 3A). mPGES-1 is another inflammation-relevant enzyme which is implicated in the synthesis of inducible PGE₂ and its expression is also associated with tumor growth and metastasis [48]. In fact, compared to the parental MDA-MB-231 cell line we measured also higher levels of IL-1 β /Fk-inducible mPGES-1 in the metastatic sublines, suggesting that it also contributes to the higher basal level of PGE₂ synthesis in these cells.

The pro-inflammatory action of S1P may be due to its ability to stimulate COX-2 expression and subsequent synthesis of PGE₂ [25,26,28]. Indeed, we found that S1P induced COX-2 expression and PGE₂ synthesis particularly in the metastatic MDA-MB-231 cells, and the use of antagonists selectively binding to the different S1P receptor subtypes unveiled that this effect was due to activation of the overexpressed S1P₃. Further evidence for the role of S1P₃ in PGE₂ synthesis was provided by S1P₃ downregulation using siRNA and pharmacologically using the S1P₃ agonist CYM5541 [49], which showed a similar effect as S1P on the metastatic cells. These findings were corroborated by our data from parental MDA-MB-231 cells overexpressing S1P₃ upon transfection. However, as discussed above for COX-2 expression, despite very high S1P₃ levels, treatment with S1P alone was ineffective in the parental MDA-MB-231 cells and pretreatment with the DNA demethylating agent aza-C was required to stimulate PGE₂ synthesis. This suggests silencing of the COX-2 promoter as an epigenetic mechanism which was reported also for other genes involved in breast cancer metastasis [39]. These findings demonstrate that S1P by activation of S1P₃ has potential to induce the COX-2/PGE₂ pathway, thereby contributing to the pro-inflammatory environment in metastatic breast cancer. Interestingly, in S1P₃ transfected parental cells high PGE₂ synthesis did not increase migration, suggesting that

other pro-metastatic changes occurred during the process of dissemination to lung and bone, e.g., upregulation of EP₂.

The pro-migratory potential of S1P and PGE₂ has been described in various cell types [18,20,50,51]. In addition, in cancer, PGE₂ also stimulates the angiogenic switch and was shown to promote lymphatic invasion of breast cancer cells [14]. As expected from the several rounds of in vivo selection in nude mice [34], compared to the parental MDA-MB-231 cells the metastatic sublines, particularly 4175, exhibited a higher migratory potential and tissue invasion, which further increased in the presence of exogenous S1P or after the treatment with recombinant PGE₂. Using various S1P receptor subtype-selective antagonists we could confirm that this effect was mediated by S1P₃, which is overexpressed exclusively in the two metastatic MDA-MB-231 sublines. This finding is corroborated by recent data from lung cancer cells where knockdown of S1P₃ mitigated S1P-induced migration and invasion [52] and from a melanoma metastasis model where S1P₃ stimulated motility and invasiveness triggered by stroma-derived S1P [53]. In breast cancer patients high cytoplasmic S1P₃ expression in tumors was shown to correlate with shorter survival and nuclear S1P₃ expression to correlate with tumor size [54]. In vitro data from other cancer types also suggest different scenarios for this pro-metastatic S1P₃-dependent effect, including G_i-mediated activation of the Rho family GTPase Rac [51] or interaction of G_q with MMP-9 [30]. Using the COX-2 inhibitor celecoxib we clearly demonstrate that the COX-2/PGE₂ pathway is implicated in S1P/S1P₃-induced migration and invasion of the here used in vivo selected metastatic breast cancer cells.

Similar to S1P₃ we found that S1P₂ was also involved in PGE₂ synthesis and its antagonism disrupted S1P-induced PGE₂ synthesis. A role of S1P₂ in cyclooxygenase 2 expression was previously also reported by others. Thus, Li et al. [55] showed that in a Wilms tumor-derived cell line, S1P-stimulated COX-2 expression occurs in a S1P₂-dependent manner and that S1P₂ is greatly increased in Wilms tumor tissue. Also, in renal mesangial cells, COX-2 induction by S1P was mediated by S1P₂ [25], whereas Kim et al. [56] reported for amnion-derived WISH cells an exclusive regulation of COX-2 by S1P₁ and ₃, but not by S1P₂. Interestingly, although S1P₂ stimulation also increases PGE₂ synthesis in metastatic MDA-MB-231 cells, in contrast to S1P₃ it does not increase migration. This suggests that S1P₂ provides additional cellular signals which counteract the pro-migratory potential of S1P₃ and PGE₂. One such mechanism could be activation of the Rho-kinase 1 which was reported to be regulated by S1P₂ (reviewed in [57]). The outcome of increased migration of the metastatic MDA-MB231 sublines compared to the parental MDA-MB231 cells may thus be the result of S1P₃ overexpression which outweighs the anti-migratory function of S1P₂. Moreover, in the lung metastatic cell line 4175 we could not confirm that S1P₂

downregulates the breast carcinoma metastasis suppressor 1 (Brms1) (data not shown), a response which in other cancer cell types was reported to promote lung metastasis [58].

PGE₂ binds to four EP subtypes, i.e., EP₁₋₄ [15,16]. Particularly, EP₂ and EP₄ are often associated with an aggressive and invasive cancer phenotype [17,18] and EP₂ was shown to mediate the pro-angiogenic effect of PGE₂ in a breast cancer metastases model [59]. Both receptors signal through G_s and increase cAMP, which may explain how stimulation of COX-2/PGE₂ increases expression of the CCR7 chemokine. Furthermore, in breast cancer tissues overexpression of COX-2 correlates with lymph node metastasis [14]. We found that the four receptor subtypes were expressed in the parental and the metastatic MDA-MB-231 cell lines though at different levels. EP₂ showed the highest expression and was upregulated in the two metastatic sublines, whereas EP₃ was hardly detectable (Fig. 9A). Using receptor subtype-selective antagonists we identified EP₂ to be mainly responsible for the stimulatory effect of S1P on migration and invasion of the metastatic cells, whereas EP₄ revealed only a moderate effect. The very low level of EP₂ on the parental cells might at least partly explain the low pro-migratory effect of PGE₂, the synthesis of which was increased in the cells upon S1P₃ transfection.

Altogether, our data demonstrate for the first time that S1P₃ overexpression is a potential marker of metastatic breast cancer and that its activation by S1P stimulates the pro-inflammatory COX-2/PGE₂ pathway, which uses the EP₂ and EP₄ receptor to promote cell migration and invasion in an auto- and paracrine manner. The finding that COX-2 expression is low in parental MDA-MB-231 cells line but high and inducible in the metastatic sublines, suggests an epigenetic silencing mechanism which becomes suspended during in vivo metastatic selection. We conclude that targeting S1P₃ holds promise for the treatment of inflammation-associated metastatic breast cancer.

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Legends to figures

Fig. 1. mRNA expression profile of S1P₁₋₅ receptors in parental and metastatic MDA-MB-231 cells.

Confluent cells were incubated in serum-free medium for 24 h. Thereafter, RNA was extracted and taken for quantitative PCR analysis of S1P₁₋₅ using the primers indicated in the Materials and Methods section. 18S RNA was used for normalization. Data are the mean $\pm$ SD (n=4). ***p< 0.0001 compared to S1P₁ expression in parental MDA-MB-231 cells.

Fig. 2. Effect of exogenous S1P and S1P receptor antagonists on intracellular calcium [Ca²⁺]_i mobilization in parental and metastatic MDA-MB-231 cells.

Cells were loaded with fura-2/AM prior to stimulation with 1 μ M S1P and [Ca²⁺]_i was measured using a spectrofluorometer as described in the Materials and Methods section. (A) Representative curves from parental MDA-MB-231 cells and the metastatic sublines. Results were calculated using the FL WinLab program. (B) Maximum S1P-induced peak level of Ca²⁺ expressed as % of the parental MDA-MB-231 cells. Data are the mean $\pm$ SD (n=3). ***p< 0.001 compared to parental MDA-MB-231 cells. (C) Dye-loaded cells were pretreated for 10 min with the S1P_{1/3} antagonist VPC23019 (V, 10 μ M), the S1P₁ antagonist W146 (W, 10 μ M) or the S1P₂ antagonist JTE013 (J, 10 μ M) prior to stimulation with 1 μ M S1P. [Ca²⁺]_i was measured and peak levels determined. Data are the mean $\pm$ SD (n=3). **p<0.01, ***p<0.001, compared to parental MDA-MB-231 cells; #p<0.05, ##p<0.01, ###p<0.001 compared to the corresponding S1P-treated cells.

Fig. 3. PGE₂ synthesis, and expression of COX-2 and mPGES-1 in parental and metastatic MDA-MB-231 cells.

Cells were incubated for 24 h in serum-free medium prior to stimulation for 24 h with either vehicle control (-) or a combination of IL-1 β (1 nM) plus forskolin (Fk, 5 μ M). (A) Supernatants were taken for PGE₂ quantification by ELISA as described in the Materials and Methods section. Data are expressed as pg of PGE₂ per ml supernatant and are the mean $\pm$ S.D. (n=3). (B and C) Cells were taken for RNA extraction and quantitative PCR analysis of COX-2 (B) and mPGES1 (C). Data are the mean $\pm$ S.D. (n=3). **p<0.01, ***p<0.001 compared to unstimulated parental MDA-MB-231 cells; ###p<0.001 compared to unstimulated 4175 cells; \$\$\$p < 0.001, compared to unstimulated 1833 cells. (D) Cells were taken for protein extraction, separation by SDS-PAGE (8 % gels for COX-2 and β -actin, 15 % gels for mPGES-1) and Western blotting using antibodies against COX-2 (upper panel),

mPGES-1 (middle panel) and β -actin (lower panel). Blots are representative of three independent experiments showing similar results.

Fig. 4. Time-dependent effect of S1P on COX-2 mRNA expression and PGE₂ synthesis in parental and metastatic MDA-MB-231 cells.

Cells were stimulated for 1-24 h with S1P (1 μ M). (A) Cells were taken for RNA extraction and subjected to quantitative PCR analysis of COX-2. 18S RNA expression levels were used for normalization. Data are the mean (n=2). (B) Cell supernatants were collected for PGE₂ determination by ELISA. Data are expressed as pg of PGE₂ per ml of supernatant and are the mean $\pm$ S.D. (n=3). **p<0.01, ***p<0.001 compared to the corresponding unstimulated control cells.

Fig. 5. Effect of celecoxib and S1PR modulators on S1P-induced PGE₂ synthesis in parental and metastatic MDA-MB-231 cells.

(A) Cells were stimulated for 24 h with either vehicle control (Co) or 1 μ M S1P in the absence (-) or presence of the indicated concentrations of celecoxib. (B) Cells were stimulated for 24 h with either vehicle (Co) or S1P (1 μ M) in the absence (-) or presence of the S1P₁₊₃ antagonist VPC23019 (V, 10 μ M), the S1P₂ antagonist JTE013 (J, 10 μ M), the S1P₁ antagonist W146 (W, 10 μ M). All antagonists were added 30 min prior to S1P stimulation. (C) 4175 cells were stimulated for 2 h with the S1P₃ agonist CYM5541 (0.5 and 5 μ M) or the S1P₂ agonist CYM5520 (2 and 10 μ M). (D and E) 4175 cells were transfected with either control siRNA (D and E, NT-siRNA), siRNA against S1P₂ (D, siS1P2) or siRNA against S1P₃ (E, siS1P3) as described in the Methods section. Thereafter, cells were stimulated for 2 h and 6 h with S1P (1 μ M). Supernatants were taken for PGE₂ quantification by ELISA. Data are the mean $\pm$ S.D. (n=3). **p<0.01, ***p<0.001 compared to the corresponding unstimulated vehicle-treated cells; #p<0.05, ##p<0.01, ###p<0.001 compared to the corresponding S1P-stimulated cells.

Fig. 6. Effect of S1P₃ overexpression in parental MDA-MB-231 cells on PGE₂ synthesis.

(A) Parental MDA-MB-231 cells were either treated with vehicle control or transfected with a pcDNA3.1 vector containing the human S1P₃ cDNA (OE) for 48 h. Thereafter, cells were taken for RNA extraction and quantitative PCR analysis of S1P₃. Data are the mean $\pm$ S.D. (n=3). (B) Untransfected MDA-MB-231 cells and S1P₃ overexpressing cells (OE) were treated with vehicle control (Co) or 5 μ M aza-C (+) for 3 days (fresh aza-C was added every 24 h). Then, cells were starved for 4 h and subsequently stimulated for 2 h with S1P (1 μ M). Supernatants were taken for PGE₂ quantification by ELISA. Data are the mean $\pm$ S.D. (n=3). *p<0.05, ***p<0.001 compared to

the corresponding vehicle-treated untransfected control cells; ### $p < 0.001$ compared to the corresponding aza-C-treated cells.

Fig. 7. Effect of S1P and PGE₂ on the migration of parental S1P₃-overexpressing MDA-MB-231 cells and of metastatic MDA-MB-231 cells.

(A) 2×10^5 parental and metastatic cells were seeded onto transwell filters and treated for 20 h with either vehicle (Co), S1P (1 μ M) or PGE₂ (1 μ M) under serum-free conditions. (B) 2×10^5 parental and metastatic cells were seeded onto transwell filters and treated for 20 h with either vehicle (Co) or S1P (1 μ M) in the absence (-) or presence of the S1P₃ antagonists VPC23019 (V, 10 μ M) or TY52156 (TY, 1 μ M). (C) 4175 cells were seeded onto transwell filters and treated for 20 h with either vehicle (Co) or S1P (1 μ M) in the absence (-) or presence of the S1P₂ antagonist JTE013 (1 μ M and 10 μ M). (D) 2×10^5 parental and metastatic cells were seeded onto transwell filters and treated for 20 h with either vehicle (Co) or S1P (1 μ M) in the absence (-) or presence of celecoxib (CLX, 5 μ M). (E) 2×10^5 parental and S1P₃-overexpressing cells (OE) were seeded onto transwell filters and treated for 20 h with either vehicle (Co) or S1P (1 μ M) in the absence or presence of aza-C. Migrated cells were determined as described in the Materials and Methods section. Data are expressed as number of cells migrated to the lower chamber cells and are the mean $\pm$ SD (n=3). In A: ** $p < 0.01$, *** $p < 0.001$ compared to vehicle-treated parental MDA-MB-231 cells; ### $p < 0.001$ compared to vehicle-treated 4175 cells; § $p < 0.05$ compared to vehicle-treated 1833 cells. In B, C, D: ** $p < 0.01$, *** $p < 0.001$ compared to the corresponding vehicle-treated cells; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to the corresponding S1P-stimulated cells.

Fig. 8. mRNA expression of EP₁₋₄ in parental and metastatic MDA-MB-231 cells and effect of EP antagonists on S1P-induced migration.

(A) Cells were cultured for 24 h in serum-free medium before RNA was extracted for PCR analysis of the PGE₂ receptor subtypes EP₁₋₄. 18S RNA was used for normalisation. Data are the mean $\pm$ SD (n=6), *** $p < 0.0001$. (B) 2×10^5 4175 cells were seeded on transwell filters and treated for 20 h with vehicle control (Co) or S1P (1 μ M) in the absence (-) or presence of the EP₁ antagonist SC51322 (SC, 5 μ M), the EP₂ antagonist PF04418948 (PF, 5 μ M), the EP₃ antagonist L-798.106 (L, 5 μ M) or the EP₄ antagonist AH23848 (A, 5 μ M). Migrated cells were counted as described in the Materials and Methods section. Data are expressed as number of cells migrated to the lower chamber cells and are the mean $\pm$ SD (n=3). *** $p < 0.001$ compared to the corresponding vehicle-treated control cells; ## $p < 0.01$ compared to the corresponding S1P-stimulated cells.

Fig. 9. Effect of S1P and PGE₂ stimulation on invasion of parental and metastatic MDA-MB-231 cells. (A) 2×10^5 cells were seeded onto Matrigel-coated transwell filters and treated for 48 h with either vehicle control (Co), S1P (1 μ M) or PGE₂ (1 μ M). (B) 2×10^5 4175 cells were seeded onto Matrigel-coated transwell filters and treated for 48 h with vehicle control (Co) or S1P (1 μ M) in the absence (-) or presence of celecoxib (CLX, 5 μ M), the S1P_{1/3} antagonists VPC23019 (V, 10 μ M) and TY52156 (TY, 1 μ M), the EP₁ antagonist SC51322 (SC, 5 μ M), the EP₂ antagonist PF04418948 (PF, 5 μ M), the EP₃ antagonist L-798.106 (L, 5 μ M) or the EP₄ antagonist AH23848 (A, 5 μ M). Invaded cells were determined as described in the Materials and Methods section. Data are expressed as number of cells migrated through the Matrigel-coated membrane into the lower chamber and are the mean $\pm$ SD (n=3). In A: **p<0.01, ***p<0.001 compared to vehicle-treated parental MDA-MB-231 cells; #p<0.05 compared to vehicle-treated 4175 cells; §p<0.05 compared to vehicle-treated 1833 cells. In B: **p<0.01 compared to vehicle-treated 4175 cells; #p<0.05, ##p<0.01, compared to S1P-treated 4175 cells.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9