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**Sphingosine 1-phosphate receptor expression profile and regulation of migration in human thyroid cancer cells**

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## ABSTRACT

Sphingosine 1-phosphate (S1P) receptor expression and the effects of S1P on migration were studied in one papillary (NPA), two follicular (ML-1, WRO) and two anaplastic (FRO, ARO) thyroid cancer cell lines, as well as in human thyroid cells in primary culture. Additionally, the effects of S1P on proliferation, adhesion and calcium signalling were addressed in ML-1 and FRO cells. All cell types expressed multiple S1P receptors. S1P evoked intracellular calcium signalling in primary cultures, ML-1 cells and FRO cells. Neither proliferation nor migration was affected in primary cultures, whereas S1P partly inhibited proliferation in ML-1 and FRO cells. Low nanomolar concentrations of S1P inhibited migration in FRO, WRO and ARO cells, but stimulated ML-1 cell migration. Consistently, S1P<sub>1</sub> and S1P<sub>3</sub>, which mediate migratory responses, were strongly expressed in ML-1 cells and S1P<sub>2</sub>, which inhibits migration, was the dominating receptor in the other cell lines. The migratory effect in ML-1 cells was mediated by G<sub>i</sub> and phosphatidylinositol 3-kinase. Both S1P and the S1P<sub>1</sub>-specific agonist SEW-2871 induced Akt phosphorylation at Ser-473. However, SEW-2871 failed to stimulate migration, whereas the S1P<sub>1</sub>/S1P<sub>3</sub> antagonist VPC 23019 inhibited S1P-induced migration. The results implicate that aberrant S1P receptor expression may enhance thyroid cancer cell migration and thus contribute to the metastatic behaviour of some thyroid tumours.

## INTRODUCTION

Sphingosine 1-phosphate (S1P) is a bioactive lipid that regulates central cellular processes, such as cell growth, survival, differentiation and motility [1-4]. S1P is present in plasma and serum at high nanomolar concentrations [5,6]. Platelets store S1P and release it into the blood [5,7] where its concentration is tightly controlled by ectoenzyme activity and distribution among plasma proteins [6,8]. Extracellular S1P may also be produced locally by other cell types, e.g. glioma cells [9], mouse melanoma cells, and fibroblasts [10]. The effects of extracellular S1P are mediated by G-protein coupled receptors, termed S1P<sub>1-5</sub> [11], which regulate multiple intracellular pathways [3,12,13]. Some of the effects of S1P are mediated by intracellular S1P acting on unknown targets [14].

S1P may either inhibit or stimulate cellular motility, depending on cell type and S1P concentration [4,12]. Differences in migratory responses correlate well with differences in S1P receptor expression, both *in vitro* and *in vivo* [4,15-18]. S1P receptors regulate migration by modulating the activity of the Rho family of GTPases [4,12]. S1P<sub>1</sub>- and S1P<sub>3</sub>-induced migration involves G<sub>i</sub>-mediated activation of phosphatidylinositol 3-kinase (PI3K), Akt, and Rac [12]. S1P<sub>2</sub> signals via G<sub>12/13</sub> to activate Rho and to inhibit Rac-dependent signalling, which results in an inhibitory effect of S1P on migration [12,16,19]. S1P<sub>4</sub> is mainly expressed in cells of the immune system [13], and stimulates migration of transfected CHO cells by activating cdc42 in a pertussis toxin-sensitive manner [20]. The effects of S1P<sub>5</sub> on migration are to our knowledge not clarified.

S1P is implicated in several of the key cellular events in cancer growth and metastasis [21]. On the other hand, S1P has been shown to inhibit metastasis of mouse melanoma cells *in vivo* [16]. S1P modulates central intracellular cascades, as well as regulates proliferation of rat thyroid cells, which express S1P<sub>2</sub>, S1P<sub>3</sub> and S1P<sub>5</sub> [22-25]. However, a possible role for S1P in human thyroid cells has not previously been investigated, nor has the importance of S1P in thyroid tumour growth and metastasis been addressed.

We have determined the relative expression level of S1P receptors in one human papillary (NPA), two human follicular (ML-1, WRO) and two human anaplastic (FRO, ARO) thyroid cancer cell lines, as well as in primary cultures of normal human thyroid cells. Furthermore, we have investigated the cellular responses to S1P stimulation (proliferation, adhesion, migration, and calcium signalling) in all of these cell types. The S1P receptor expression pattern differs significantly between the cell types investigated, which is reflected in the responses to S1P stimulation. Most notably, the follicular thyroid tumour cell line ML-1, which was originally isolated from an aggressive tumour [26], showed high expression of the migration-inducing S1P receptors S1P<sub>1</sub> and S1P<sub>3</sub>. In line with this, nanomolar concentrations of S1P induced migration of ML-1 cells, whereas migration was inhibited by S1P in the other cell lines. Furthermore, the S1P<sub>1/3</sub> antagonist VPC 23019 inhibited migration towards S1P in ML-1 cells. In primary cultures of thyroid cells, S1P evoked a modest calcium response, but did not affect migration or proliferation. Our results implicate that aberrant S1P receptor expression may contribute to the aggressive phenotype of some thyroid tumours.

## MATERIALS AND METHODS

### Materials

Dulbecco's modified Eagle's medium (DMEM), bovine TSH, porcine insulin, fatty acid-free bovine serum albumin (BSA) and pertussis toxin were purchased from Sigma (St. Louis, MO). Coon's modified Ham's F-12 medium, foetal calf serum, penicillin/streptomycin, trypsin and L-glutamine were from Gibco (Grand Island, NY). Type 2 collagenase was from Worthington Biochemicals (Lakewood, NJ). Non-essential amino acids and RPMI were obtained from Cambrex Bio Science (Verviers, Belgium). Cell culture plastic ware as well as human type I and type IV collagen and fibronectin were purchased from Becton Dickinson Biosciences (Bedford, MA). [methyl-<sup>3</sup>H]-thymidine (82 Ci/mmol) was from Amersham Biosciences, Amersham, UK) and CellTiter 96® AQueous One Solution cell proliferation assay from Promega Corp (Madison, WI). Transwell™ inserts for migration assays and 96-well plates for adhesion experiments were from Corning Inc (Corning, NY). Sphingosine 1-phosphate and SEW-2871 were from Biomol (Plymouth, PA). VPC 23019 was obtained from Avanti Polar Lipids (Alabaster, AL), SB20350, Wortmannin and Y-27632 from Calbiochem (San Diego, CA), and *C. botulinum* C3 exotoxin from List Biological Laboratories Inc (Campbell, CA). The ProJect transfection reagent, and the BCA protein assay reagent kit were purchased from Pierce Biotechnology (Rockford, IL). The FACE™ AKT ELISA kit was from Active Motif (Carlsbad, CA). The Akt and phospho-Thr-308-specific Akt antibodies used in Western blotting, as well as the positive and negative protein controls for phosphorylated Akt, were purchased from Cell Signaling Technology (Danvers, MA).

The HRP-conjugated goat anti-rabbit antibody, and the Aurum™ total RNA isolation kit were obtained from BioRad laboratories (Hercules, CA). Nitrocellulose membranes were from Schleicher & Schuell (Dassel, Germany). The RiboGreen® RNA quantification reagent, and Fura 2-AM were purchased from Molecular Probes (Eugene, OR). SuperScript III reverse transcriptase was from Invitrogen (Carlsbad, CA), and oligo(dT) primers from Promega Corp (Madison, WI). The LightCycler FastStart DNA Master SYBR Green kit was obtained from Roche Diagnostics (Mannheim, Germany). PCR primers were synthesized by TAGC Copenhagen (Denmark). All chemicals for RNA isolation and RT-PCR were of molecular biology grade, and all other chemicals of reagent grade.

#### Cell culture

ML-1 thyroid follicular cancer cells, kindly provided by Dr Johann Schönberger (University of Regensburg, Germany), were cultured essentially as previously described [26] in DMEM medium supplemented with 2 mM L-glutamine, 10% foetal calf serum (FCS), and 100 U/ml penicillin-streptomycin. NPA papillary and WRO follicular thyroid cancer cells were a kind gift from Dr Sylvia Asa (University Health Network and Toronto Medical Laboratories, Canada). FRO and ARO anaplastic thyroid cancer cell lines were generously provided by Dr James Fagin (University of Cincinnati, Ohio, USA). ARO, FRO, WRO and NPA cells were cultivated in RPMI medium supplemented with 0,1 mM non-essential amino acids, 2 mM L-glutamine, 10% foetal calf serum and 50 U/ml penicillin-streptomycin. Human thyroid follicular cells were prepared from surgical specimens as previously described [27]. In brief, thyroid tissue (normal and multinodular

goitre) was digested using 0.2% collagenase. Follicles were plated in Coon's F12 modification supplemented with 0.3 U/l TSH, 1 mg/l insulin,  $10^5$  U/l penicillin, 100 mg/l streptomycin and 1% FCS. After 72 h serum was omitted and the medium was changed every 3 days. All cell cultures were maintained in 37°C and 5% CO<sub>2</sub> in a humidified cell culture chamber.

#### Relative quantitative RT-PCR

RNA was isolated with the Aurum total RNA isolation kit according to the manufacturer's instructions. RNA was quantitated with RiboGreen reagent and RNA quality and integrity was checked by absorbance spectrometry and agarose gel electrophoresis. Reverse transcription reactions were performed with SuperScript III reverse transcriptase and oligo(dT) primers from 0.5 µg total RNA as described by the manufacturer.

Quantitative RT-PCR was performed on a LightCycler instrument (Roche) with LightCycler DNA Master Kit reagents, and the following cycling conditions: preincubation 95°C 10 min, amplification 45-50 cycles (95°C 15 sec, annealing T°C 5 sec, 72°C 9 sec). The primers used (0.5 µM), and the primer-specific amplification conditions are listed in table 1. Relative mRNA levels were calculated with the LightCycler software. Control analyses, in which reverse transcriptase was omitted from the cDNA synthesis reaction, were performed for each of the RNA preparations to ensure that the preparations were not contaminated with genomic DNA. A melting curve analysis was performed for each run to ensure correct amplification. The data was related



to the amount of total RNA [28]. For comparison, the data was also analyzed with porphobilinogen deaminase (PBGD) as a reference gene.

#### Measurement of intracellular free calcium concentrations $[Ca^{2+}]_i$

The cell lines were harvested with calcium-free Hank's buffered saline solution ( $Ca^{2+}$ -free HBSS; NaCl, 118 mM; KCl, 4.6 mM; glucose, 10 mM; Hepes, 20 mM; pH 7.2) containing 0.02% EDTA and 0.1% trypsin. After washing the cells three times with HBSS (containing 1 mM  $CaCl_2$ ) by pelleting, the cells were incubated with 1  $\mu$ M Fura 2-AM for 30 min at 37°C. After washing twice, cells were kept in darkness for a minimum of 10 min and washed again prior to the experiment. The cell suspension was added to a quartz cuvette, kept at 37°C, and stirred throughout the experiment. A 125  $\mu$ M stock solution of S1P was made in HBSS containing 4 mg/ml fatty acid-free BSA. The cells were stimulated with the indicated concentrations of S1P and Fura 2 emission was measured with a Hitachi 2000 fluorescence spectrophotometer. The excitation wavelengths were 340 and 380 nm, and emission was measured at 510 nm. The signal was calibrated by addition of 0.05% Triton X-10 to lyse the cells and obtain maximal fluorescence. Tris-base was used to elevate pH above 8.3 and extracellular  $Ca^{2+}$  was chelated with 10 mM EGTA to obtain minimal fluorescence. Intracellular free calcium concentrations ( $[Ca^{2+}]_i$ ) was calculated as described in [29], using a computer program designed for the fluorimeter and a  $K_d$ -value of 224 nM for Fura 2.

The primary thyroid cells, cultured on poly-(D)-lysine-coated coverslips, were washed and incubated with 2  $\mu$ M Fura 2-AM as described above, and  $Ca^{2+}$  signals were measured as described before [30]. Cells were placed on the stage of an upright

microscope (Olympus ~~W~~61), and visualized through a 60x water-immersion objective. Images were captured using an intensified GenIV camera (Universal Imaging, Marlow, ~~U~~), and analysed with MetaFluor software (Universal Imaging Corporation, West Chester, PA). After measuring a baseline for 60 seconds, the cells were perfused with SIP for 60 seconds after which the perfusion was turned off for 30 seconds. Then SIP was washed away with HBSS, and the measurement was continued for five more minutes.

#### Measurement of intracellular inositol phosphates

Inositol phosphates were measured essentially as described by Oldham [31]. Cells were plated onto 100 mm cell culture plates at a density of 750 000 cells/plate, and cultured as described above for four days. [<sup>3</sup>H]-*myo*-inositol (1 µCi/ml) was added, and the cells were allowed to grow for an additional 48 hours. Cells were detached with trypsin solution, and washed with HBSS, after which they were incubated with 100 nM sphingosine 1-phosphate for 30 sec at 37°C. The reaction was terminated by adding an equal volume of ice-cold 10% perchloric acid, and then centrifuged for 5 min at +4°C. The supernatant was transferred to fresh tubes, and neutralized with freon:tri-n-octylamine 1:1. The samples were centrifuged briefly, and the upper phase was passed through Amprep™ columns that had been activated with 1M KOH. After washing the columns with distilled water to remove neutral compounds, inositol phosphates were eluted with 5 ml 0.17 M KOH. 1 ml of the effluent was mixed with 4 ml scintillation fluid, and radioactivity was measured using a Wallac 1410 scintillation counter.

### Measurement of [<sup>3</sup>H]-thymidine incorporation and proliferation

The thyroid cancer cell lines were grown in medium containing 10% dextran-treated, charcoal-stripped FCS (stripped FCS) for two days. Primary cultures of thyroid cells were grown in serum-free medium with or without TSH 0.3 U and/or insulin (1 mg/l). The cells were stimulated with S1P for 24 h or 48 h, with 0.4-1 µCi/ml [<sup>3</sup>H]thymidine added for the last 4 hours of the incubation. 10% FCS was used as a positive control for the primary thyroid cultures. At the end of the experiment the cells were washed three times with cold PBS, and the assays were terminated by addition of ice-cold 5% trichloroacetic acid (TCA). After 10 min on ice TCA was aspirated, the TCA-insoluble precipitate was dissolved in 0.1 N NaOH, and the radioactivity was measured. The results were confirmed with the CellTiter proliferation assay, as described by the manufacturer, and by cell counting.

### Cell adhesion

Cell adhesion experiments were performed as described previously [32-34] with modifications. The cells were washed with PBS, and changed into serum-free medium containing 0.1% fatty-acid free BSA (SFM) the day before the experiment. Then the cells were preincubated with S1P for 1 or 24 h. 96-well plates were coated with 5 µg/cm<sup>2</sup> collagen IV, collagen I or fibronectin for 1 h, after which the wells were rinsed, and blocked with 3% BSA for 30 min. Cells in SFM, with or without S1P, were added to each well and placed in 37°C in the cell culture chamber. The cells were allowed to attach for 1 h. The attached cells were fixed with 70% ethanol for 20 min, and stained with 1 mg/ml crystal violet in 20% methanol for 10 min. After washing with water the

wells were allowed to dry. The crystal violet was dissolved in 10% acetic acid, and the absorbance measured at 590 nm.

### Cell migration

Migration experiments were performed on 6.5mm-diameter Transwell chambers with 8  $\mu$ M pore size. The membranes were coated with 5  $\mu$ g/cm<sup>2</sup> collagen I, and reconstituted with SFM for 1h at 37°C in the cell culture incubator prior to the experiment. FRO cells failed to migrate on a collagen I matrix, and therefore collagen I (5  $\mu$ g/cm<sup>2</sup>) was used. The cell lines were changed into SFM the day before the experiment. In some experiments the cells were preincubated with one of the following inhibitors: 100 ng/ml pertussis toxin (Ptx) overnight; 5  $\mu$ M SB203580 for 1 hour; 10  $\mu$ M LY293784 for 1 hour; 100 ng/ml Wortmannin for 1 hour; 1  $\mu$ M PI3K inhibitor for 1 hour; 100 ng/ml C3 exotoxin in the presence of Protein transfection reagent for 4 hours. 200  $\mu$ l cell suspension was added to the upper wells, and 800  $\mu$ l SFM with or without S1P and/or stripped FCS to the lower wells. In experiments with inhibitors, the inhibitor was present in both chambers during the experiment. With ML-1 cells, additional experiments where S1P was added to both the upper and the lower wells were performed to test whether the S1P-induced migration was due to chemotaxis or chemokinesis. The cells were allowed to migrate in the incubator for 4-8h towards S1P-stripped FCS or 20 h towards S1P alone, after which the unmigrated cells from the top side of the membrane were wiped off with a cotton swab. The migrated cells were fixed in 2% paraformaldehyde (PFA) in PBS for 10 min, and stained with 0.1% crystal violet in 20% methanol for 5min. The membranes were rinsed and allowed to dry. The cells were

counted with 40x magnification in eight microscopic fields in a straight line bisecting the membrane.

#### Akt phosphorylation assay

The ML-1 cells (10 000 cells/well) were plated on 96-well plates and allowed to grow for 24 hours, after which the medium was changed to SFM. The following day the cells were stimulated with S1P for 10 min. In some experiments, the cells were treated with Ptx (100 ng/ml, oh) or Wortmannin (100 ng/ml for 1 h) before and during stimulation. The cells were fixed with 4% PFA in PBS 20 min. The FACEAkt EISA was performed with an antibody recognizing Ser-473-phosphorylated or total Akt according to the instructions provided by the manufacturer, and the absorbance was read at 450 nm.

#### Western blotting

ML-1 cells were cultured in SFM for one day and stimulated with 100 nM S1P for 10, 30 or 60 minutes. In some experiments, the cells were stimulated with 1  $\mu$ M SEW-2871 for 10 minutes. At the end of the stimulation, the cells were briefly rinsed with ice-cold HBSS and extracted in 200  $\mu$ l ice-cold lysis buffer (Tris-base, 10 mM; NaCl, 150 mM; EDTA, 7 mM; NP-40, 0.5%; PMSF, 0.2 mM; pH 7.7) with a cell scraper. Protein concentration was determined with a BCA protein assay reagent kit following the manufacturer's instructions. The cell extracts were mixed with 0.5 volumes of 3xSDS-buffer (glycerol, 30%; SDS, 3%; Tris-HCl, 0.1875 M, pH 6.8; bromophenol blue, 0.015%;  $\beta$ -mercaptoethanol, 3%) and boiled for 2 min. 10-50  $\mu$ g of protein samples

and 15  $\mu$ l of commercial control extracts were separated by SDS-PAGE on 10% polyacrylamide gels. The proteins were transferred electrophoretically to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (NaCl 150 mM; Tris-base 20 mM; pH 7.5) with 0.1% Tween 20 for 1 h. The blots were then incubated with phospho-Thr-308-specific Akt antibody (1:1000) diluted in TBS containing 5% non-fat dry milk and 0.1% Tween 20 overnight at +4°C, washed, and incubated with peroxide-conjugated rabbit antibodies (1:3000) for 1h at room temperature. The proteins were incubated in ECL solution (luminol, 1.25 mM; p-coumaric acid, 0.68 mM; H<sub>2</sub>O<sub>2</sub>, 0.01%) for 1 min, and the membranes were exposed to Hyperfilm™ (Amersham Biosciences, Buckinghamshire, UK) for 1-5 min. The membranes were stripped with 0.1 M glycine (pH 2.5), rinsed with 1M Tris-HCl (pH 7.6), and reprobed with total Akt antibody (1:1000) as described above.

## RESULTS

### S1P receptor expression in human thyroid cancer cell lines and primary cultures

To find out which S1P receptors are expressed in thyroid cancer cell lines and thyroid primary cultures, we performed relative quantification of mRNA for each receptor with RT-PCR. All cell types studied expressed several S1P receptors (Fig. 1). The results are presented relative to total RNA [28] with the detection limit for each gene defined as 1. For comparison, data was also normalized to the expression of the reference gene  $\beta$ -actin which gave similar results (not shown). S1P<sub>1</sub> and S1P<sub>5</sub> mRNA were expressed in all cells, and S1P<sub>2</sub> was the dominating receptor in FRO, ARO and WRO

cells (Fig. 1A). FRO cells expressed S1P<sub>2</sub> at clearly higher levels than the other cell lines. Primary cultures and ML-1 cells expressed large amounts of S1P<sub>3</sub> compared to the other cell lines (Fig. 1A). In addition, ML-1 cells showed a comparatively high expression of S1P<sub>1</sub> and S1P<sub>5</sub>. Thus, the expression pattern in ML-1 cells was strikingly different compared to the other cell lines. S1P<sub>4</sub> expression was absent or minimal in all cell types (Fig. 1B). Based on these results, and preliminary calcium measurements, proliferation and migration experiments, we chose the ML-1 and FRO cell lines for further studies.

#### Effects of S1P on intracellular calcium concentration

Extracellular S1P evokes release of intracellular calcium, and a subsequent calcium inflow in many cell types [35]. We show that high concentrations of S1P evoked a small increase in  $[Ca^{2+}]_i$  in primary thyrocytes ( $EC_{50}=654$  nM; Fig. 2A). ML-1 cells responded in a similar manner ( $EC_{50}=986$  nM; Fig. 2B), whereas FRO cells were one order of magnitude more sensitive to S1P stimulation ( $EC_{50}=51$  nM; Fig. 2C). Moreover, the maximal increase in  $[Ca^{2+}]_i$  was substantially larger in FRO cells, compared to ML-1 cells and primary cultures. The calcium response in ML-1 cells was not sensitive to pertussis toxin-treatment (data not shown), but 100 nM S1P stimulated inositol phosphate production (mean $\pm$ SEM 158 $\pm$ 23% of control levels;  $p<0.01$ ). The S1P<sub>1</sub>-agonist SEW-2871 did not evoke a calcium response in ML-1 cells. Furthermore, preincubation of the cells with the S1P<sub>1/3</sub>-specific antagonist VPC 23019 was without an effect on the S1P-evoked calcium response (data not shown).

### Effects of S1P on proliferation

Since S1P regulates the proliferation of many cell types, including cancer cells and rat thyroid cells, we next investigated whether S1P has an effect on the proliferation of normal or malignant thyroid cells. S1P had no effect on [ $^3\text{H}$ ]thymidine incorporation in thyroid primary cultures either in the presence or absence of insulin (1 mg/l) or TSH (0.3 IU) (Fig. 3A). However, the primary thyrocytes proliferated in response to 10% foetal calf serum, which was used as a control (Fig. 3A).

S1P inhibited [ $^3\text{H}$ ]thymidine incorporation in ML-1 cells (Fig. 3B). The inhibitory effect on proliferation was verified with cell counting (Fig. 3B). When FRO cells were stimulated with S1P, they reverted to a rounded morphology and were lost during the washing steps of the thymidine incorporation experiment. Therefore, the effect of S1P on FRO-cell proliferation was assessed with the CellTiter assay and cell counting, revealing a modest decrease in FRO viability (Fig. 3B).

### Effects of S1P on adhesion and migration

S1P may either stimulate or inhibit cellular adhesion and motility, depending on cell type and S1P concentration. We therefore tested the effect of S1P on the adhesion of thyroid cells. A 24-h preincubation with 100 nM or 3  $\mu\text{M}$  S1P did not significantly affect cellular adhesion to fibronectin or collagen IV in ML-1 or FRO cells (Fig. 4A). The adhesion properties of ML-1 cells were further tested in experiments using a short time of S1P incubation (1h) and in some experiments, a collagen I matrix. No significant effects of S1P on ML-1 cell adhesion were found in these experiments (data not shown). In contrast, 3  $\mu\text{M}$  S1P inhibited FRO cell adhesion to collagen I (Fig. 4B).



We next tested if S1P affects the migration of thyroid cells. The migration experiments were performed across a collagen I-coated Transwell-membrane, except for FRO cells, which failed to migrate on Matrigel, collagen I or fibronectin matrices. Therefore, collagen I was used as a matrix in experiments with FRO cells. The cells showed no or a very modest basal migration in the absence of a chemoattractant. S1P was without an effect on basal or serum-induced migration of primary cultures (not shown). In contrast, S1P inhibited the migration of all the cell lines that expressed predominantly S1P<sub>2</sub> (ARO, WRO and FRO; Fig. 5). The effect was strongest in FRO cells, where as little as 10 nM S1P completely blocked migration towards FCS (Fig. 6B).

In contrast to the other cell lines, nanomolar concentrations of S1P stimulated ML-1 cell migration (Fig. 6). The concentration-response curve was bell shaped, and maximal migration was achieved with 100 nM S1P (Fig. 6A,B). The migratory effects of 100 nM S1P and 10% FCS were additive (Fig. 6A,D). In contrast, 3  $\mu$ M S1P inhibited migration towards FCS (Fig. 6A,D). This could not be explained by the S1P-mediated inhibition of proliferation, since incubation times longer than 8 hours were needed to reveal the effect of S1P on cell number (data not shown).

#### S1P-induced ML-1 cell migration is mediated by S1P<sub>1/3</sub> and G<sub>i</sub>-PI3K

Previous results have shown that migration evoked through S1P<sub>1</sub> and S1P<sub>3</sub> is mediated by G<sub>i</sub> proteins [12]. Therefore we tested the effect of Ptx pre-treatment (100 ng/ml overnight) on S1P-induced migration. In Ptx-treated cells, the S1P-induced migration was totally abolished (Fig. 6A,C). Furthermore, Ptx reversed the effect of 100

nM S1P, revealing a strong inhibition on FCS-induced migration (Fig. 6A). D Ptx was without an effect on the inhibition of migration by 3  $\mu$ M S1P (Fig. 6D).

To investigate which of the S1P receptors are involved in S1P-induced ML-1 migration, we used the S1P<sub>1</sub> agonist SEW-28 [36,37] and the S1P<sub>1/3</sub>-antagonist VEC 23019. 1  $\mu$ M SEW-28 did not evoke migration, whereas a 1h pre-treatment with 1  $\mu$ M VEC 23019 potently inhibited S1P-evoked migration (Fig. 7A). In the presence of FCS, the S1P-induced migration was reduced but not abolished in VEC 23019-treated cells (Fig. 7B). On the other hand, 3  $\mu$ M S1P had no inhibitory effect on migration in VEC 23019-treated cells (Fig. 7B). Thus, our results suggest that the effects of S1P on ML-1 cell migration are mediated by S1P<sub>1</sub> and/or S1P<sub>3</sub>, but that activation of S1P<sub>1</sub> alone is not sufficient to induce migration.

Signalling through PI3K-Akt has been shown to mediate S1P-induced migration [4,12]. In addition, S1P-evoked Akt activation is p38MAPK-dependent in some model systems [39]. Treatment of ML-1 cells with the potent PI3K-inhibitor Wortmannin (100 ng/ml, 1h) inhibited S1P-induced migration, but did not affect S1P-induced inhibition of migration (Fig. 7C,D). However, inhibiting p38MAPK with SB305588 (5  $\mu$ M, 1h) did not change the effects of S1P on migration (results not shown).

Next, we studied the S1P-induced phosphorylation of Akt in ML-1 cells with phosphorylation site-specific antibodies. 100 nM S1P, but not 3  $\mu$ M S1P, increased Akt phosphorylation on Ser-473 (Fig. 8). Consistently with its effects on migration, the S1P<sub>1/3</sub> antagonist VEC 23019 inhibited S1P-induced Akt phosphorylation (Fig. 8A). However, although 1  $\mu$ M SEW-28 failed to stimulate ML-1 cell migration, it induced Akt Ser-473 phosphorylation more potently than S1P itself (Fig. 8A). In contrast, we were not

able to detect any phosphorylation of Akt Thr-308 with S1P or SW-28, or in cells cultured in the presence of 10% FCS (results not shown).

S1P was unable to increase Ser-473 phosphorylation of Akt in Ptx (100 ng/ml) - or Wortmannin (100 ng/ml)-pre-treated ML-1 cells (Fig. 3). These two inhibitors reduced the basal Ser-473 phosphorylation of Akt as well. Thus, our results strongly suggest that, in ML-1 cells, the Gi-PI3K pathway mediates both S1P-induced migration and S1P-induced Akt phosphorylation, but that Akt phosphorylation on Ser-473 alone is not sufficient for the migratory response.

#### The role of the Rho-ROCK pathway in S1P-induced migration of ML-1 cells

Previous investigations have indicated that the S1P<sub>2</sub> receptor suppresses S1P-induced migration by signalling through Rho and ROCK [19]. To investigate whether Rho and ROCK are involved in S1P-induced migration in ML-1 cells, the cells were treated with the Rho inhibitor C3 exotoxin (100 ng/ml, 4h) or the ROCKIII inhibitor Y27632 (10  $\mu$ M, 1h). Both inhibition of ROCKIII and Rho increased S1P-induced migration in the absence of serum, although the effect of C3 exotoxin was modest compared to that of Y27632 (Fig. 4A,B). Moreover, in Y27632-treated cells, 3  $\mu$ M S1P had a modest migratory effect (Fig. 4B). Likewise, C3 exotoxin increased S1P-induced migration towards FCS (Fig. 4C). In contrast, Y27632 did not abrogate the inhibitory effect of 3  $\mu$ M S1P on FCS-induced migration (Fig. 4D). Migration towards FCS was significantly increased in Y27632-treated cells, and could not be further enhanced by S1P (Fig. 4D). The results suggest that S1P and FCS regulate ML-1 cell migration by activating stimulatory and suppressor pathways simultaneously, and that the suppression

of migration involves Rho and ROCK signalling. However, high concentrations of S1P may suppress migration by activating some other pathway.

#### The stimulatory effect of S1P on ML-1 cells is due to both chemotaxis and chemokinesis

To test whether S1P-induced ML-1 cell migration is chemotactic or chemokinetic, experiments where 100 nM S1P was added to both the upper and the lower well of the migration chamber (chemokinesis) were compared to experiments where S1P was present only in the lower well (chemotaxis). S1P stimulated migration in both experimental settings, but migration was less prominent in the absence of a concentration gradient (Fig. 10A). When the experiment was repeated in the presence of 10% FCS in the lower well, 100 nM S1P was not able to significantly stimulate migration when added to both compartments of the Transwell chamber (Fig 10B). On the other hand, the inhibition of FCS-induced migration by 3  $\mu$ M S1P was similar in both experimental settings. These data indicate that S1P has both chemotactic and chemokinetic stimulatory effects on ML-1 cell migration, but that the inhibition of migration is independent of a concentration gradient.

## **DISCUSSION**

In the present investigation we have addressed the role of S1P in thyroid cell proliferation, adhesion, migration, and calcium signalling. We show that human thyroid cells, as well as human thyroid cancer cell lines, express several S1P receptors at the mRNA level. In primary cultures of human thyroid cells, S1P evokes intracellular

calcium signalling but neither stimulates proliferation nor affects motility. This is an important finding, indicating that under normal physiological conditions, S1P is not a major factor regulating growth and migration of thyroid cells. However, our results implicate that S1P may be of importance in thyroid cancer biology. We show that S1P can regulate the proliferation and adhesion of some types of thyroid cancer cells. Most importantly, S1P potentially modulates the migration of thyroid cancer cells.

S1P receptor activation regulates proliferation in many cell types [1]. Since S1P stimulates DNA synthesis in FRTL-5 rat thyroid cells [22], but inhibits DNA synthesis in PC Cl<sub>3</sub> rat thyroid cells [23], it was of interest to test the effect of S1P on human thyroid cell proliferation. However, our results show that S1P does not induce proliferation of human thyroid cells in primary culture. In contrast, S1P reduces cell number in some thyroid cancer cell lines, but we did not detect apoptosis (Athas and Tönquist, unpublished results). Furthermore, S1P modulates cellular adhesion to extracellular matrix proteins in some cell types [34,40]. We did not observe any significant effects of S1P on the adhesion to fibronectin, collagen I or collagen IV in ML-1 cells. In contrast, the adhesion to collagen I was attenuated in FRO cells preincubated with S1P.

There are many implications of S1P-receptor-mediated regulation of tumour cell invasiveness. S1P affects the migration of many tumour cell lines [10,15,16,40,41]. It is well established that S1P<sub>1</sub> and S1P<sub>3</sub> mediate stimulation of migration by coupling to G<sub>i</sub>, whereas S1P<sub>2</sub> mediates inhibition of migration via G<sub>12/13</sub> [4,12]. The S1P receptor expression pattern, and consequently the migratory response to S1P, may change upon cellular transformation [15]. Furthermore, overexpression of S1P<sub>1</sub> enhances *in vivo* metastasis of B6F10 mouse melanoma cells, whereas overexpression of S1P<sub>2</sub> inhibits it

[16]. In our hands, S1P does not affect the migration of normal human thyroid cells, although the cells express mRNA for several S1P receptors. However, at physiological concentrations S1P may either inhibit or activate migration of thyroid tumour cell lines. In three out of the five cell lines studied (i.e. WRO, FRO and ARO cells), the dominating S1P receptor expressed was S1P<sub>2</sub>. In line with this, nanomolar concentrations of S1P attenuated migration in all of these cell lines. The FRO cells were the most sensitive to S1P, and as little as 10 nM S1P completely inhibited serum-induced migration. Consistently, the FRO cells expressed the S1P<sub>2</sub> receptor abundantly, compared to the other cell lines.

The migratory response of ML-1 cells was strikingly different from the other cell lines: low nanomolar concentrations of S1P stimulated ML-1 cell migration, whereas micromolar concentrations inhibited it. The ML-1 cell line was originally derived from a follicular thyroid carcinoma that was locally invasive, and had metastasized to the lymph nodes [26]. Given that the active concentration of S1P in human plasma is in the low nanomolar range [6], this suggests that S1P may have contributed to the aggressive and metastasing behaviour of the original tumour. ML-1 cells strongly expressed S1P receptors known to stimulate migration (S1P<sub>1</sub> and S1P<sub>3</sub>), as well as the inhibitory receptor S1P<sub>2</sub>.

According to previous investigations, S1P-evoked migration is mediated by G<sub>i</sub>-dependent activation of PI3K and Akt kinase, resulting in the activation of Rac [4,12]. This seems to be the case also in ML-1 cells, based on the following observations. Inhibition of G<sub>i</sub> with Ptx completely abolished S1P-induced migration. Inhibition of PI3K with Wortmannin attenuated the migratory effect of S1P. Furthermore, S1P

phosphorylated Akt kinase on Ser-473, through a Ptx- and Wortmannin-sensitive mechanism. Akt activity is regulated by phosphorylation on Ser-473 and Thr-308, but the mechanism of these phosphorylation events is poorly understood and controversial [42]. In ML-1 cells, Thr-308 phosphorylation seems not to be needed for migration, since neither FCS nor S1P were able to induce it. The mechanism by which S1P phosphorylates Akt on Ser-473 in ML-1 cells remains to be investigated. Akt activation by S1P is p38 MAPK-dependent in many cell lines, including ovarian cancer cells [39], and p38 MAPK activity is involved in S1P-induced migration of endothelial and smooth muscle cells [43-45]. However, inhibition of p38 MAPK with SB 230580 did not affect S1P-induced ML-1 cell migration. This is similar to the situation in S1P<sub>1</sub>- or S1P<sub>3</sub>-transfected CHO cells where PI3K, but not p38 MAPK activity, is needed for S1P-induced migration [46].

We used the S1P<sub>1</sub>-specific agonist SEW-2871 to mimic signalling through S1P<sub>1</sub>. SEW-2871 has been reported to induce migration in S1P<sub>1</sub>-transfected CHO cells [36]. However, SEW-2871 did not stimulate migration of ML-1 cells, although it potently phosphorylated Akt. Antagonizing S1P<sub>1</sub> and S1P<sub>3</sub> with VPC 23019 inhibited S1P-induced migration substantially. These data suggest that S1P<sub>1</sub> and/or S1P<sub>3</sub> are involved in S1P-induced migration in ML-1 cells, but that S1P<sub>1</sub> alone is not able to induce migration. On the other hand, the S1P-evoked inhibition of migration towards FCS was abolished by VPC 23019, suggesting that S1P<sub>3</sub> may mediate different effects on migration at different agonist concentrations. This might be possible by switching G protein coupling from G<sub>i</sub> to G<sub>12/13</sub> [47]. Indeed, inhibiting G<sub>i</sub> with pertussis toxin reversed the effect of 100 nM S1P, resulting in a strong inhibition of ML-1 cell migration. The same effect of Ptx-

treatment has been reported in S1P<sub>3</sub>-transfected CHO cells [47]. Whether the inhibitory effect on migration is mediated by S1P<sub>3</sub>, S1P<sub>2</sub>, or both receptors in ML-1 cells remains to be tested.

S1P-receptors mediate inhibition of migration by G<sub>12/13</sub>-evoked stimulation of Rho and inactivation of Rac [12,16,19,46-48]. However, Rho activity is also important for carcinoma cell motility in general [49], as well as S1P-induced endothelial and smooth muscle cell migration [44,50]. The downstream effects of Rho are partly mediated by Rho kinases (ROCKI/II) whose effect on S1P-modulated migration is controversial. Inhibition of ROCK abolishes S1P-induced inhibition of migration in glioblastoma cells [19], but has no effect in S1P<sub>2</sub>-transfected CHO cells or B6 melanoma cells [47,48]. On the other hand, inhibiting ROCK blocks S1P-induced migration in endothelial cells [44]. In ML-1 cells, inhibition of Rho or the Rho kinases ROCKI/II further enhanced the stimulatory effect of 100 nM S1P. This suggests that S1P activates Rho/ROCK-dependent inhibitory signalling pathways simultaneously with the stimulatory G<sub>12/13</sub> pathway, and that the balance between these signals determines the final migratory response. The ROCKI/II inhibitor Y27632 clearly had a stronger effect on S1P-induced migration than the Rho inhibitor C3 exotoxin. In the presence of C3 exotoxin the ML-1 cells had a rounded morphology, indicating that the cells responded to C3 exotoxin. It is, however, possible that the inhibition of Rho was incomplete.

Inhibition of ROCK increased the migration towards serum *per se*, and S1P could not further enhance the migratory response. A possible explanation for this could be that in the presence of the ROCK inhibitor and serum, maximal migration is already achieved, and a further migration in response to S1P is not possible. Surprisingly, inhibition of



ROCKI/II did not reverse the inhibitory effect of 3  $\mu$ M S1P on ML-1 cell migration. This implicates that the inhibition of migration by high concentrations of S1P is mediated via a mechanism not dependent on ROCK.

S1P-induced activation of Rac requires calcium mobilization in endothelial cells [51] and calcium is involved in the regulation of motility [52]. All known S1P receptors can mediate increases in  $[Ca^{2+}]_i$  [3]. Furthermore, overexpression of S1P<sub>1</sub> mediates inhibition of calcium signals evoked by S1P or other substances [35]. In thyroid cells in primary culture, and in ML-1 cells, the S1P-evoked increase in  $[Ca^{2+}]_i$  was similar in sensitivity and magnitude to that observed in rat thyroid cell lines [22,25]. FRO cells were much more sensitive, both regarding the concentration of the ligand, and the amplitude of the response. Similar to what has been observed in rat thyroid cells [24,25], S1P stimulated inositol phosphate production in both ML-1 cells and FRO cells [53], indicating that the PLC-IP<sub>3</sub> pathway is involved. Neither pertussis toxin nor VPC 23019 affected S1P-evoked calcium signalling in ML-1 cells. Moreover, SEW-2871 failed to evoke an increase in  $[Ca^{2+}]_i$ , although it has been shown to evoke calcium responses in S1P<sub>1</sub>-transfected CHO cells [36]. These data, together with the high expression of S1P<sub>2</sub> in FRO cells, implicate that S1P<sub>2</sub> receptor expression is central in regulating calcium signalling in thyroid cancer cells. Whether or not the S1P-evoked calcium signal is involved in the regulation of ML-1 or FRO cell migration remains to be investigated.

Tumour growth and metastasis is dependent on adhesion, migration, invasion, angiogenesis, and proliferation. S1P has been shown in several investigations to affect all of these steps, and is therefore a candidate molecule for regulating metastatic behaviour. Our investigation is the first to show that S1P may be of importance in the metastatic

behaviour of aggressive thyroid tumours. As the five receptors mediating S1P responses have partly different signalling mechanisms, the receptor combination and level of expression is important for the effect of S1P. Our data indicate that S1P receptors, which mediate migratory responses may be up regulated in some thyroid tumours, and that S1P is important for the aggressive phenotype of these tumours. The co-expression of several S1P receptors differentially regulating migration in a tumour opens possibilities for therapeutic intervention.

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Table 1. PCR primers and cycling conditions for quantification of S1P-receptors in thyroid cancer cells.

mRNA	primer sequences	Product length	[MgCl <sub>2</sub> ], mM	Annealing T°C	Product melting T°C
S1P <sub>1</sub>	s GGCTGGAAGTGCATCAGTGCG as GAGCAGCGCCACATTCTCAGAGC	223	4	60	89-90
S1P <sub>2</sub>	s CCGAAACAGCAAGTTCCACT as CCAGGAGGCTGAAGACAGAG	197	2	61	90
S1P <sub>3</sub>	s AAGGCTCAGTGGTTCATCGT as GCTATTGTTGCTGCTGCTTG	201	2	61	92-93
S1P <sub>4</sub>	s CCTTCAGCCTGCTCTTCACT as AAGAGGATGTAGCGCTTGGA	223	4	64	94
S1P <sub>5</sub>	s AGGACTTCGCTTTTGCTCTG as TCTAGAATCCACGGGGTCTG	201	3	59	87
PBGD	s TCCAAGCGGAGCCATGTCTG as AGAATCTTGTCCCCTGTGGTGGA	204	3	62	89

The primers for S1P<sub>2-5</sub> were designed with the EMBOSS ePrimer3. The primer sequences for S1P<sub>1</sub> [54] and PBGD [55] are from the literature. s = sense strand, as = antisense strand.

## Figure legends

Fig. 1. S1P receptor expression in thyroid cancer cell lines and primary cultures.

A. Relative S1P receptor mRNA expression data presented as multiples of 1, 1 being the detection limit of the assay. ND = not detected. The bars give the mean  $\pm$  SEM, n = 4. B. The expression of S1P receptor mRNA visualized as PCR products separated on an agarose gel and stained with ethidium bromide. m = molecular marker; + = positive control cDNA (human brain for S1P<sub>1</sub>, S1P<sub>2</sub> and S1P<sub>5</sub>; human heart for S1P<sub>3</sub>; and HeLa cells for S1P<sub>4</sub>).

Fig. 2. S1P evokes intracellular calcium signals in thyroid cells.

Calcium concentration response curves and representative traces of changes in  $[Ca^{2+}]_i$  upon stimulation with 3  $\mu$ M S1P in primary cultures (A), ML-1 cells (B), and FRO cells (C). Note the different scales on the y-axes. The results are given as mean  $\pm$  SEM. In (A), the data is from at least 18 cells, in (B) and (C), n=3-8.

Fig. 3. The effect of S1P on thyroid cell proliferation.

A. S1P does not affect [<sup>3</sup>H]-thymidine incorporation in thyroid primary cultures. The experiments were repeated in the absence (left panel) and presence (right panel) of 0.3 U/l TSH and 1 mg/l insulin. In the absence of hormones, 10% FCS was used as a positive control. The data represents one batch of thyroid primary cells (mean  $\pm$  SEM, n=4) after a 48 h-stimulation with vehicle, S1P or FCS. Similar results were obtained with two other cell batches. B. S1P reduces ML-1 and FRO cell number, as shown by [<sup>3</sup>H]thymidine

incorporation in ML-1 cells (left panel, black bars), the CellTiter metabolic assay in FRO cells (right panel, white bars) and cell counting for both cell lines (grey bars). Cell counting was performed after a 48 h-stimulation, the other two assays after 24 h of stimulation with S1P. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ;  $n = 3$  (One-way ANOVA and Dunnett's *post hoc* test).

Fig. 4. S1P modulates the adhesion of FRO cells, but not ML-1 cells.

A. The adhesion of ML-1 (white bars) and FRO (black bars) cells to collagen IV after a 24 h preincubation with vehicle control (C) or the indicated concentrations of S1P. B. The adhesion of FRO cells to collagen I after a 1 h (hatched bars) or 24 h (black bars) preincubation with vehicle control (C) or S1P. The bars give the mean  $\pm$  SEM,  $n = 3-6$ . \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  (One-way ANOVA and Dunnett's *post hoc* test).

Fig. 5. S1P inhibits migration of thyroid cancer cells.

A. Migration of WRO (white bars) and ARO (black bars) cells towards 10% FCS in the presence of vehicle or 100 nM S1P. B. Migration of FRO cells towards 10% FCS and the indicated concentrations of S1P. C = vehicle control. The bars give the mean  $\pm$  SEM,  $n = 3-4$ . \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  (One-way ANOVA and Dunnett's *post hoc* test).

Fig. 6. S1P regulates ML-1 cell migration.

A. ML-1 cells allowed to migrate for 20 h in the absence (a-d) or 8 h in the presence (e-h) of 10% FCS, and stained with crystal violet. (a,e) control; (b,f) 100 nM S1P; (c,g) 3  $\mu$ M S1P; (d,h) 100 nM S1P+pertussis toxin (Ptx, 100 ng/ml, pre-treatment overnight). Bar =

100  $\mu$ M. B. ML-1 cell migration towards different concentrations of S1P. \*\*,  $p < 0.01$  (One-way ANOVA and Dunnett's *post hoc* test). C. ML-1 migration towards S1P in control cells and cells treated with Ptx. D. The effect of S1P on the migration of ML-1 cells towards 10% FCS without (white bars) or with (black bars) Ptx pre-treatment. Asterisks (\*) indicate a statistically significant effect compared to control cells and diamonds (◆) to Ptx-treated controls. The bars give the mean  $\pm$  SEM,  $n = 3-4$ . \*/◆,  $p < 0.05$ ; \*\*\*/◆◆◆,  $p < 0.001$  (One-way ANOVA and Holm's *post hoc* test).

Fig. 7. S1P-evoked ML-1 cell migration is inhibited by the S1P<sub>1/3</sub> antagonist VPC 23019 and the PI3K-inhibitor Wortmannin.

A. Migration towards the indicated concentrations of S1P in control cells (white bars) or cells pre-treated with 1  $\mu$ M VPC 23019 for 1 h (grey bars). The S1P<sub>1</sub>-specific agonist SEW-2871 (1  $\mu$ M, black bar) does not induce migration.  $n = 3$ . B. Migration towards S1P in control cells (white bars) or cells pre-treated with VPC 23019 (grey bars) in the presence of 10% FCS. The diamond (◆) indicates statistically significant difference compared to VPC 23019-treated control cells. The bars give the mean  $\pm$  SEM,  $n = 3$ . C. Migration towards S1P in control cells (white bars) or cells pre-treated with 100 ng/ml Wortmannin for 1 h (black bars). The bars give the mean  $\pm$  SEM,  $n = 4$ . D. Migration towards S1P in control cells (white bars) or cells pre-treated with Wortmannin (black bars) in the presence of 10% FCS. Asterisks (\*) indicate statistical significance as indicated by brackets. Diamonds (◆) indicate statistically significant difference compared to Wortmannin-treated control cells. The bars give the mean  $\pm$  SEM,  $n = 4$ . \*/◆,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*/◆◆◆,  $p < 0.001$  (One-way ANOVA and Holm's *post hoc* test).

Fig. 8. S1P induces Akt phosphorylation on Ser-473 in ML-1 cells.

A. Akt Ser-473 phosphorylation in control cells (white bars) or cells pre-treated with 1  $\mu$ M VPC 23019 for 1 h (black bars) after stimulation with S1P or SEW-2871 (1  $\mu$ M). B. Akt phosphorylation in control cells (C) or cells preincubated with Wortmannin (100 ng/ml, 1h) or pertussis toxin (Ptx, 100 ng/ml, 1 h) stimulated with vehicle (white bars) or 100 nM S1P (black bars). Asterisks (\*) indicate statistically significant differences versus vehicle control. Diamonds (♦) indicate statistically significant differences versus untreated cells stimulated with 100 nM S1P. The bars give the mean  $\pm$  SEM, n=3. \*, p<0.05; \*\*, p<0.01; \*\*\*/♦♦♦, p<0.001 (One-way ANOVA and Holm's *post hoc* test).

Fig. 9. S1P-evoked ML-1 cell migration is regulated by Rho and ROCK.

Experiments were performed in the absence (A,B) and presence (C,D) of 10% FCS. A,C: Cell migration in control cells (white bars) or cells preincubated with C3 exotoxin (100 ng/ml, 4h, grey bars). B,D: Cell migration in control cells (white bars) or cells preincubated with Y-27632 (10  $\mu$ M, 1h, black bars). Asterisks (\*) indicate statistically significant differences as indicated by brackets. Diamonds (♦) indicate statistically significant differences compared to control cells treated with Y-27632 or C3 exotoxin. The bars give the mean  $\pm$  SEM, n=3. \*, p<0.05; \*\*, p<0.01; \*\*\*/♦♦♦, p<0.001 (One-way ANOVA and Holm's *post hoc* test).

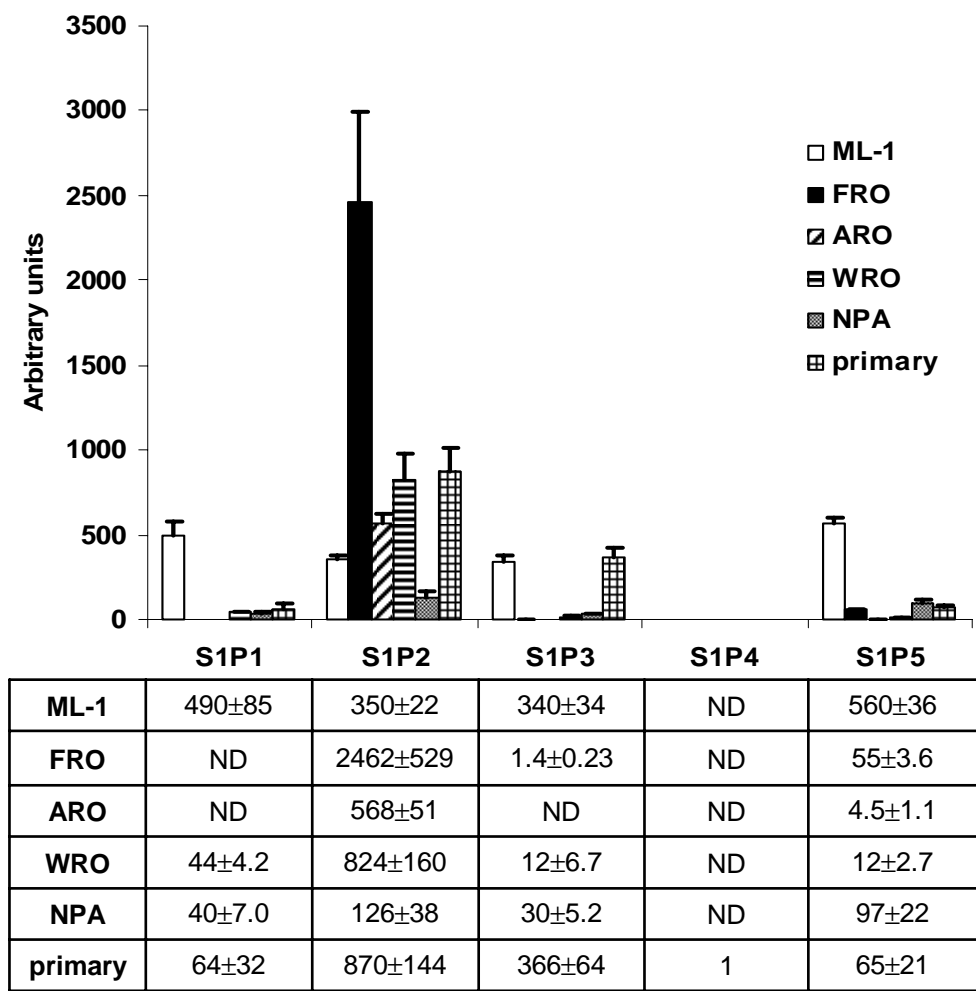
Fig. 10. S1P has both chemotactic and chemokinetic effects on ML-1 cell migration.

A. ML-1 cell migration evoked by 100 nM S1P in the absence (S1P in both wells of the migration chamber) or presence (S1P only in the lower well) of a S1P concentration gradient. B. ML-1 cell migration induced by 10% FCS and S1P in the absence (white bars) or presence (black bars) of a S1P concentration gradient. Asterisks (\*) indicate a statistically significant differences compared to control cells in the absence of a S1P concentration gradient. Diamonds (♦) indicate a statistically significant differences compared to control cells in the presence of a S1P concentration gradient. The bars give the mean  $\pm$  SEM, n=3. \*/♦, p<0.05 (One-way ANOVA and Dunnett's *post hoc* test).



Figure 1

A.



B.

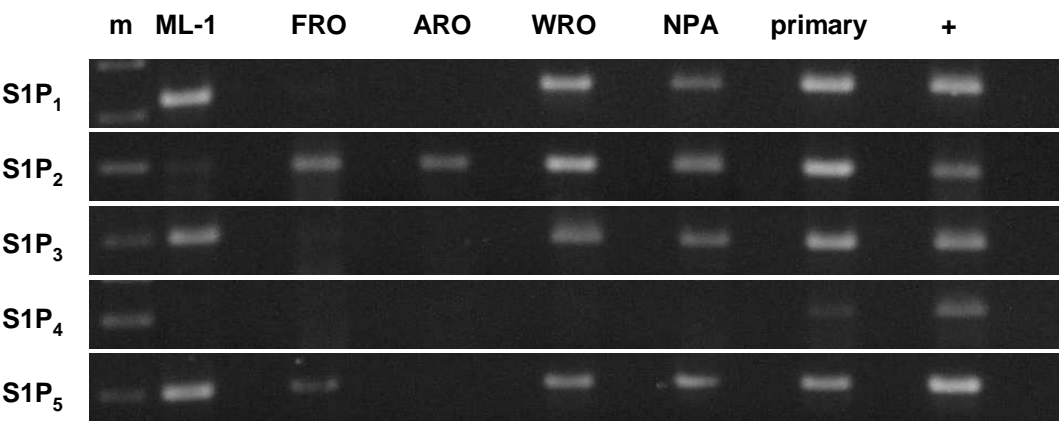
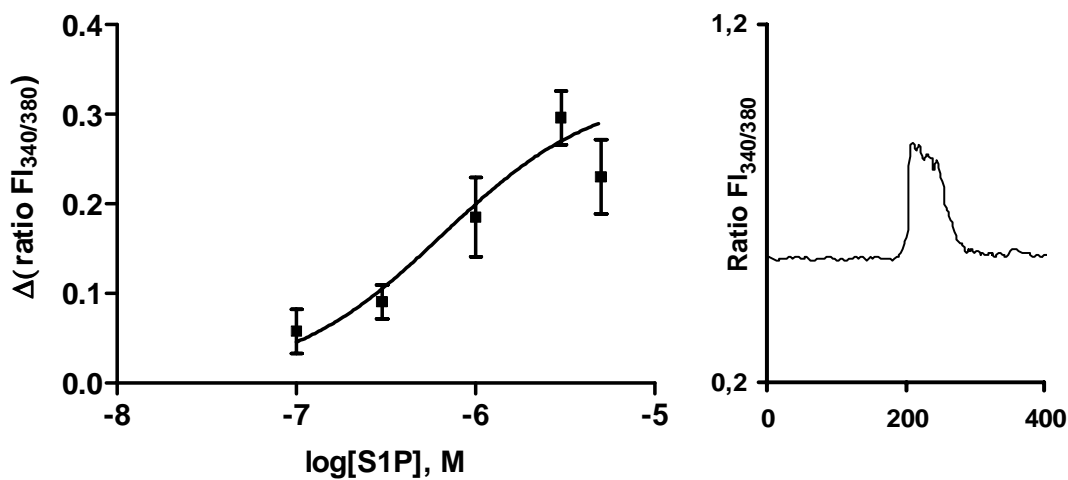
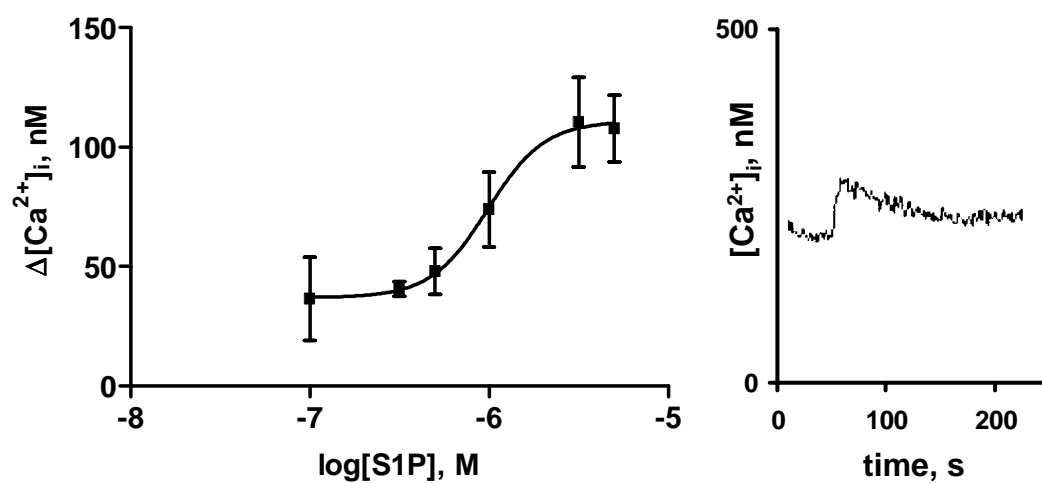


Figure 2

A



B



C

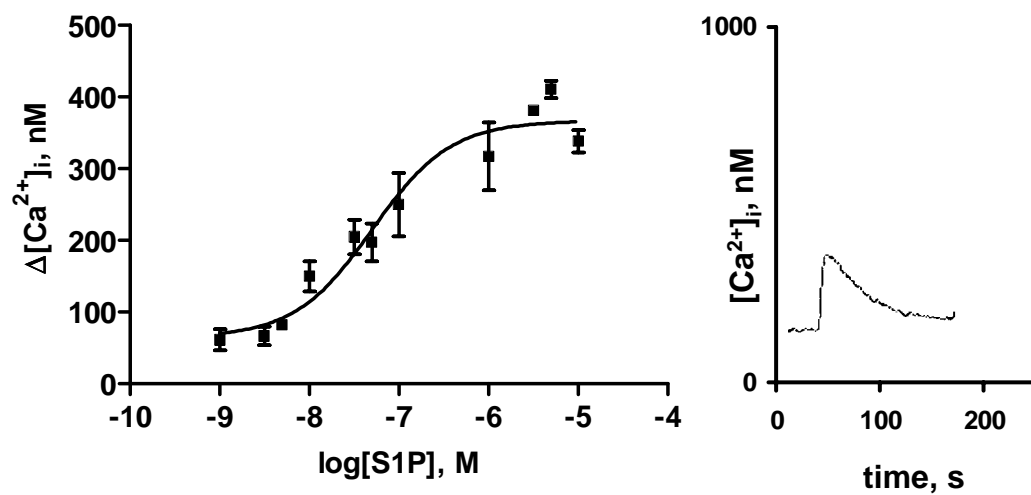


Figure 3

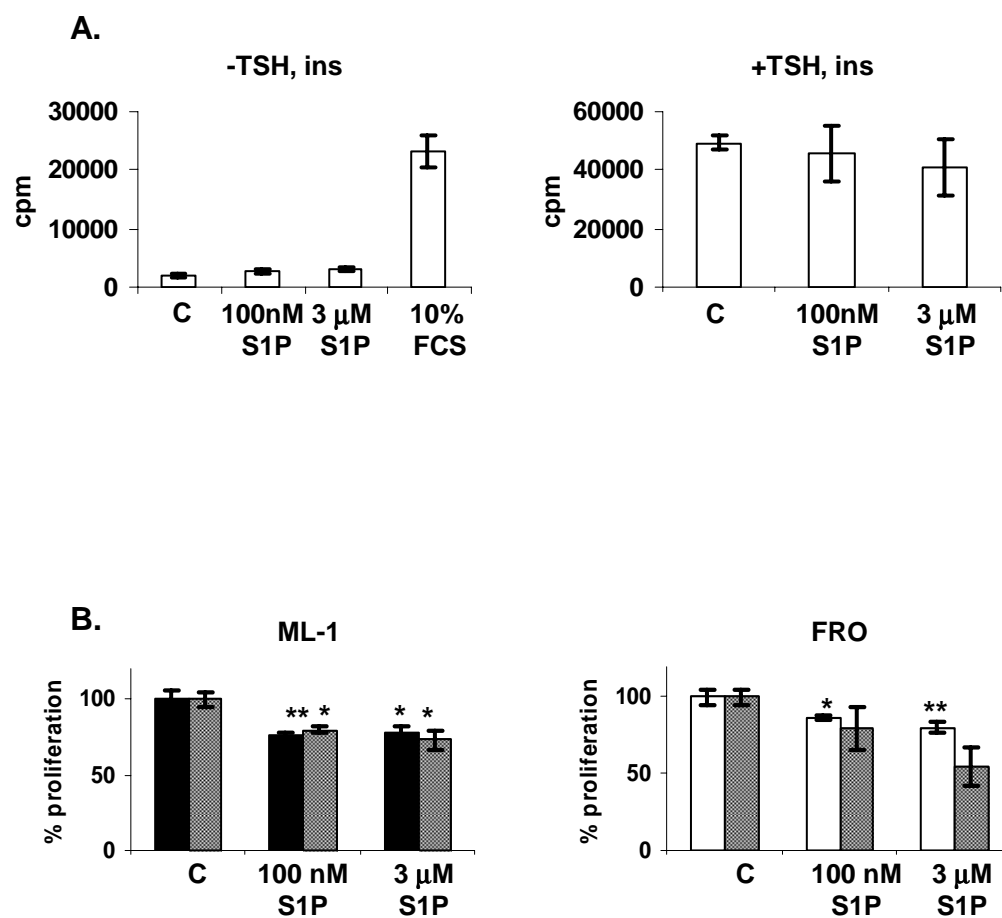


Figure 4

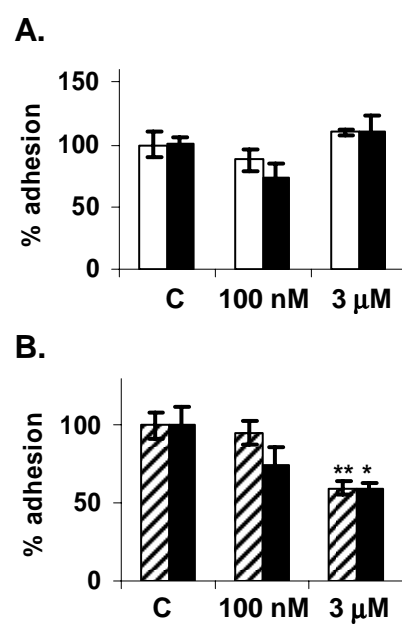
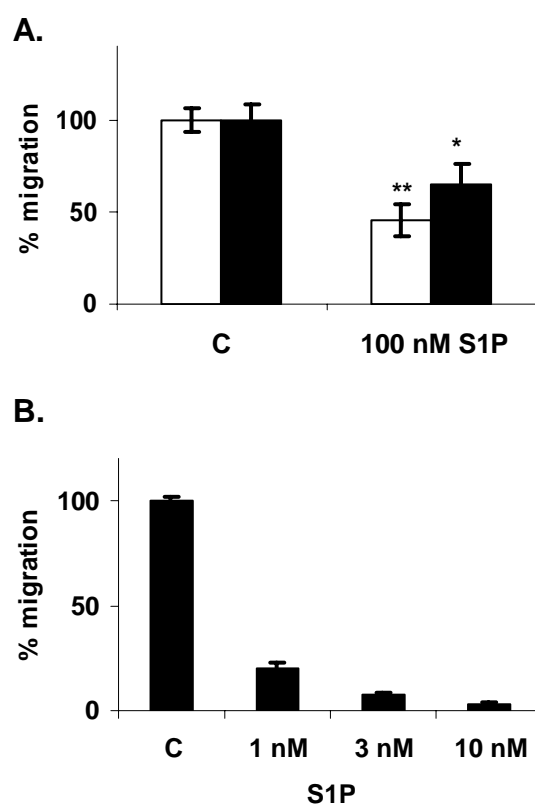


Figure 5



**Figure 6**

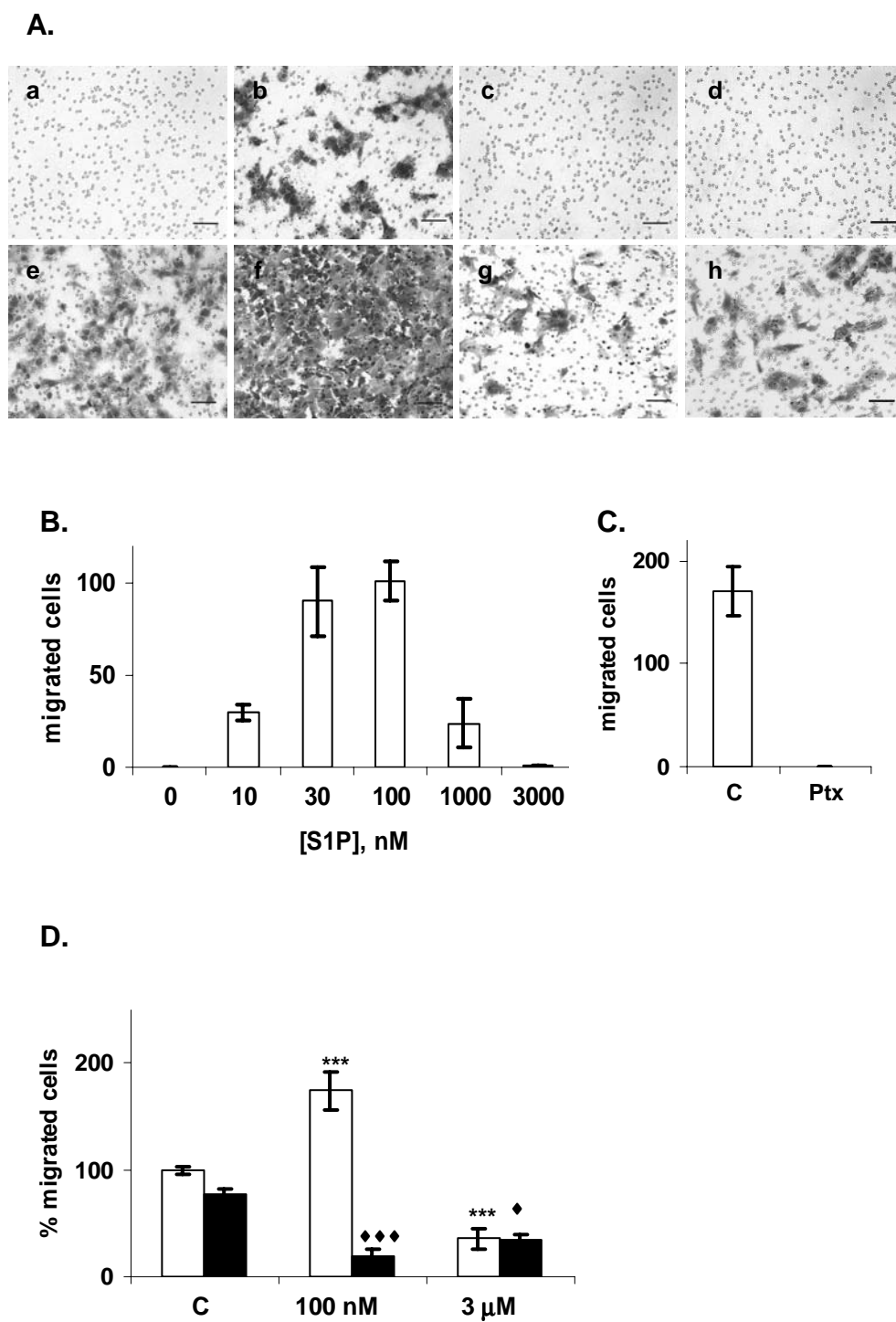
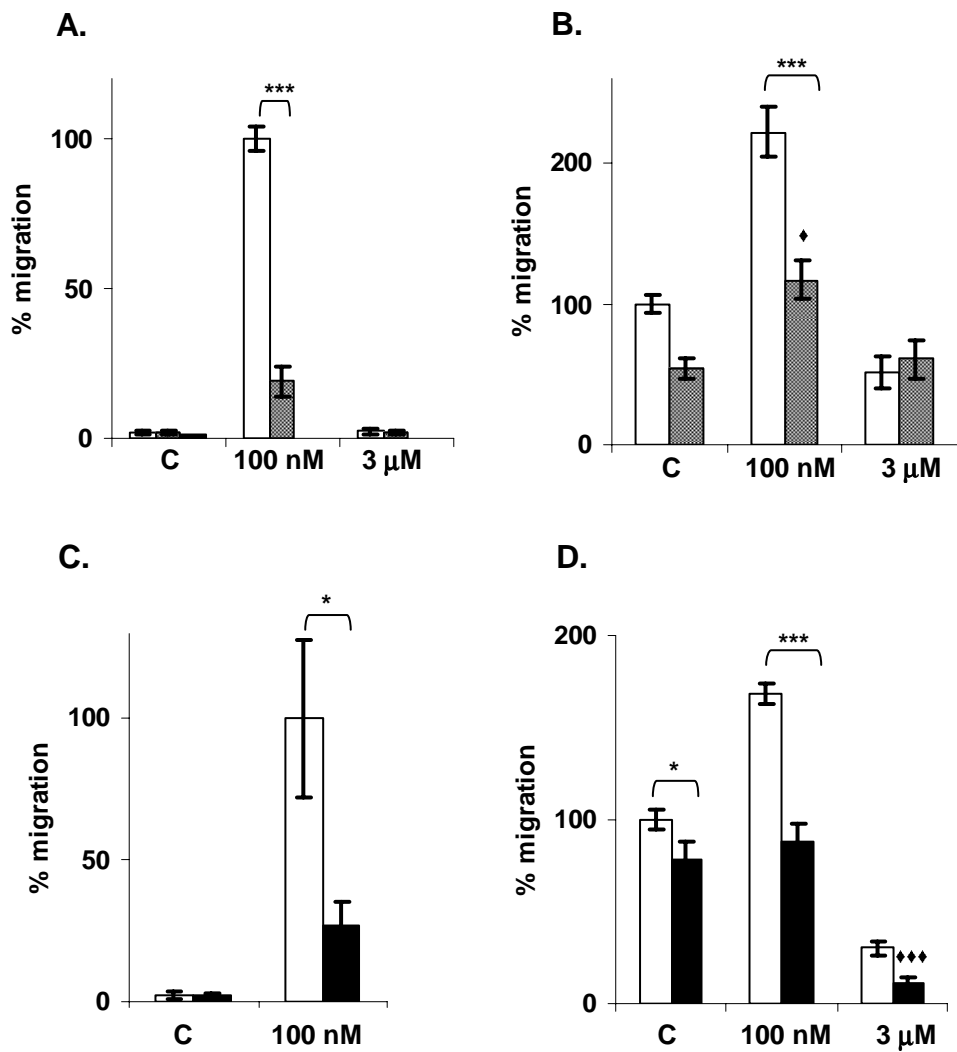
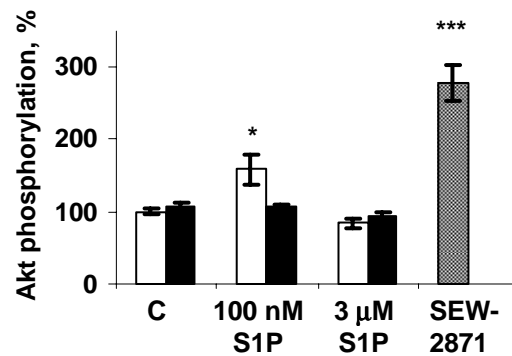


Figure 7



**Figure 8**

**A.**



**B.**

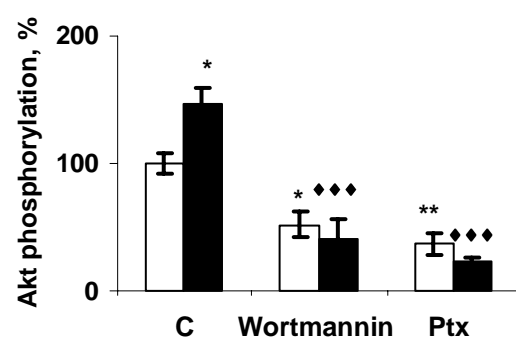




Figure 9

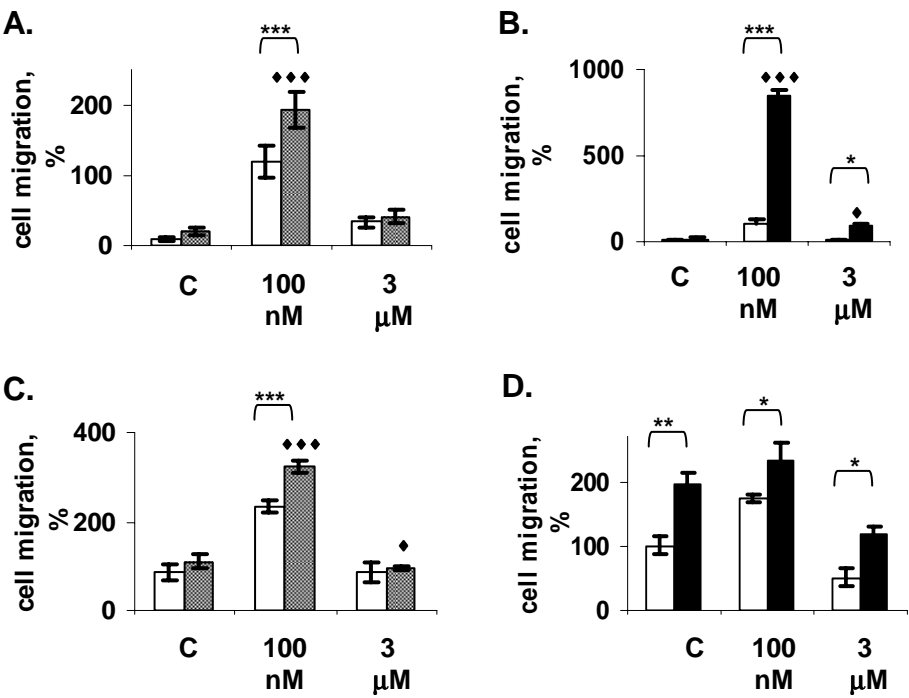


Figure 10

