Interactions between sphingosine-1-phosphate and vascular endothelial growth factor signalling in ML-1 follicular thyroid carcinoma cells

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Abstract

Sphingosine-1-phosphate (S1P) induces migration of human ML-1 thyroid follicular cancer cells and inhibits migration of human FRO anaplastic thyroid cancer cells. As tumour cells often secrete vascular endothelial growth factor (VEGF), we investigated a possible interaction between S1P and VEGF signalling in the regulation of thyroid tumour cell migration. We found that both ML-1 and FRO cells secreted VEGF-A (3.6 and 0.1 ng/10^6 cells/day respectively) and VEGF-C (3.0 and 0.14 ng/10^6 cells/day respectively). S1P stimulated VEGF-A secretion in both cell lines, and blocking S1P receptors 1, 2 and 3 attenuated the S1P-evoked secretion of VEGF-A. Neither TSH nor insulin affected the amount of secreted VEGF-A or -C in ML-1 cells, while simultaneous stimulation with insulin and S1P increased VEGF-C secretion in FRO cells. Both cell lines expressed VEGF receptor 2 (VEGFR-2) mRNA and proteins. Serum-evoked migration of both ML-1 and FRO cells was attenuated when VEGFR-2 was inhibited. Moreover, inhibiting VEGFR-2 in ML-1 cells resulted in a rapid downregulation of S1P1 mRNA expression and S1P1 protein levels, suppression of S1P-induced migration and a decrease in S1P-induced Akt phosphorylation. A VEGF-neutralizing antibody also reduced S1P-induced migration. In ML-1 cells, S1P phosphorylated VEGFR-2. In addition, VEGFR-2 inhibition resulted in the upregulation of S1P3 mRNA within 24 h, but a significant increase in S1P3 protein levels was not observed. VEGFR-2 inhibition, but not a VEGF-neutralizing antibody, reduced ML-1 cell proliferation independently of S1P stimulation. The results indicate a complex interaction between S1P and VEGFR-2 in ML-1 cells, particularly in regulating migratory responses.

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Introduction

Sphingosine-1-phosphate (S1P) is a bioactive sphingomyelin derivative that is present in plasma and serum at high nanomolar concentrations (Yatomi et al. 1997, Murata et al. 2000). Extracellular S1P is produced mainly by platelets and released into the bloodstream (Yatomi et al. 1997, 2001), but S1P may also be produced locally by other cell types, e.g. fibroblasts or tumour cells (Yamamura et al. 1997, Edsall et al. 2001). Most cell types express specific G-protein-coupled S1P receptors (S1P1–5; Chun et al. 2002) that modulate multiple intracellular pathways and thereby regulate central cellular processes, such as survival, proliferation and cytoskeletal organization (Takuwa 2002, Anliker & Chun 2004, Ishii et al. 2004, Radeff-Huang et al. 2004, Chalfant & Spiegel 2005). Some of the effects of S1P are mediated by intracellular S1P acting on unknown targets (Spiegel & Milstien 2003).
S1P affects several of the key events in tumorigenesis, such as cell growth, migration and angiogenesis (Ogretmen & Hannun 2004, Visentin et al. 2006), and is therefore considered as a tumour-promoting agent. The vascular endothelial growth factor (VEGF) family and its tyrosine kinase receptors VEGFR-1 (Flt-1), VEGFR-2 (KDR/Fk-1) and VEGFR-3 are also of importance in cancer growth and metastasis, mainly because of their angiogenic and lymphangiogenic effects (Carmeliet 2005, Tammela et al. 2005, Olsson et al. 2006). Interactions between S1P and VEGF have been studied mainly in endothelial cells, where several levels of crosstalk have been described. First, S1P may phosphorylate and transactivate VEGF receptor 2 (VEGFR-2) in a VEGF-A-independent manner (Endo et al. 2002, Tanimoto et al. 2002). Secondly, VEGF regulates S1P1 and S1P3 expression (Igarashi et al. 2003, Hughes et al. 2005, Fieber et al. 2006). Thirdly, S1P and VEGF cooperate in regulating some signalling cascades: on the one hand, VEGF enhances S1P-induced Akt activation (Igarashi et al. 2003), and on the other hand, VEGF-induced expression of Akt3 kinase is S1P3 dependent (Fieber et al. 2006). Fourthly, VEGF may affect intracellular S1P concentrations by activating sphingosine kinase (Shu et al. 2002). In some types of endothelial cells, VEGF and S1P signalling interact to regulate migration (Endo et al. 2002, Hughes et al. 2005; but see Liu et al. 2001, Morales-Ruiz et al. 2001 for an alternative interpretation). Moreover, S1P is needed for VEGF-induced blood vessel formation, indicating that the interaction between S1P and VEGF in endothelial cells is important for tumour angiogenesis (Visentin et al. 2006). Interestingly, VEGFRs are expressed in some types of tumour cells (Vieira et al. 2005), opening the possibility that crosstalk between VEGF and S1P signalling may be of importance for tumour progression by regulating not only angiogenesis but also the survival, proliferation and migration of the tumour cells.

Recently, the expression of VEGF and its receptors was detected in thyroid carcinoma cells and tissue (Turner et al. 2003, Vieira et al. 2005, Kim et al. 2007), creating a potential autocrine loop that possibly regulates cancer cells. The effects of S1P on thyroid cancer development have to our knowledge not been studied. The S1P receptor expression of several thyroid tumour cell lines differs substantially from that seen in normal thyroid cells, as does their migratory response to S1P (Balthasar et al. 2006), suggesting a role for S1P as a potent regulator thyroid cancer cell migration and proliferation.

The present investigation aimed at determining whether there is an interaction between VEGF and S1P signalling in the regulation of migration or proliferation in cell lines derived from thyroid tumours. VEGFR expression, VEGF secretion and the combined effect of VEGF and S1P signalling on proliferation and migration were studied in the human thyroid carcinoma cell lines ML-1 and WRO (follicular carcinoma cells), FRO and ARO (anaplastic carcinoma cells) and NPA (papillary carcinoma cells). Of these cell lines, only ML-1 and FRO cells expressed VEGFRs, and were thus chosen for further studies. These cell lines differ in their S1P receptor expression pattern as well as their migratory responses to S1P: FRO cells express predominantly S1P2, while ML-1 cells also express S1P1, S1P3 and S1P5 (Balthasar et al. 2006). S1P2 is known to inhibit migration, whereas S1P1 and S1P3 induce migration (Takuwa 2002, Taha et al. 2004). In line with this, nanomolar concentrations of S1P inhibit FRO cell migration and induce ML-1 cell migration (Balthasar et al. 2006). We found that ML-1 cells secrete large amounts of both VEGF-A and -C compared with FRO cells, but S1P stimulates VEGF-A secretion in both cell lines. We show that VEGFR-2 signalling is involved in S1P-induced Akt phosphorylation and migration of ML-1 cells. Furthermore, VEGFR-2 regulates S1P receptor expression in ML-1 cells: inhibition of VEGFR-2 downregulates S1P1 and upregulates S1P3 mRNA. A significant downregulation of S1P1 protein levels was also observed. Our data also reveal a stimulatory effect of VEGF-2 on ML-1 cell proliferation, which is not dependent on stimulation with S1P. Taken together, our results show that there is an interaction between S1P and VEGFR-2 signalling, which regulates ML-1 cell migration. This crosstalk may be relevant for the migratory properties of some thyroid tumours cells. Furthermore, S1P may also enhance angiogenesis of thyroid tumours by stimulating the release of VEGF.

Materials and methods

Materials

Dulbecco’s Modified Eagle’s Medium (DMEM), bovine insulin, forskolin, 3-isobutyl-1-methylxanthine (IBMX), deguelin, fatty acid-free BSA and BSA were purchased from Sigma. Bovine thyrotrophin (TSH) was a kind gift from Dr A P Parlow (National Hormone and Pituitary Program, Torrance, CA, USA). Foetal calf serum (FCS), penicillin/streptomycin, trypsin and L-glutamine were from Invitrogen. Non-essential amino acids and RPMI medium were obtained from Cambrex Bio Science (Verviers, Belgium). Cell culture plasticware, as well as human type I and type IV collagen, was purchased from Becton Dickinson
Biosciences (Bedford, MA, USA). The CellTiter 96 AQueous One Solution cell proliferation assay was from Promega Corp. Transwell inserts for migration assays were from Corning Inc. (Corning, NY, USA). Lipid-stripped fetal calf serum (LS-FCS) was from HyClone (Logan, UT, USA). S1P and SEW-2871 were from Biomol (Plymouth, PA, USA). JTE-013 was obtained from Tocris Bioscience (Elliswille, MO, USA) and VPC23019 from Avanti Polar lipids (Alabaster, AL, USA). Human recombinant VEGF-165 was from BioVision (Mountain View, CA, USA), and VEGFR-2-inhibitor 1 and CBO-P11 were both obtained from Calbiochem (San Diego, CA, USA). VEGF-neutralizing antibody and mouse IgG2B isotype control were from R&D Systems (Minneapolis, MN, USA). Rabbit β-actin antibody, rabbit anti-VEGFR-2 and rabbit anti-phospho-VEGFR-2 (Tyr1175) were from Cell Signalling Technology (Danvers, MA, USA) and HRP-conjugated goat anti-rabbit from Bio-Rad Laboratories. Rabbit S1P1 polyclonal antibody was from Cayman Chemical (Ann Arbor, MI, USA). The rabbit anti-EDG3 (SIP3) antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The TSH receptor antibody was from Affinity BioReagents (Golden, CO, USA). [2,8-3H]cAMP (30.1 Ci/mmol) was from Perkin–Elmer Life and Analytical Sciences (Boston, MA, USA), MultiScreen MAFB was from Millipore Corp. (Bedford, MA, USA) and SuperMix was from Wallac (Turku, Finland). The FACE AKT ELISA kit was from Active Motif (Carlsbad, CA, USA), the VEGF-A ELISA kit from Pierce Biotechnology (Rockford, IL, USA) and the VEGF-C ELISA kit from Zymed laboratories Inc. (San Francisco, CA, USA). The Aurum total RNA isolation kit was obtained from Bio-Rad Laboratories. The ribogreen RNA quantification reagent was purchased from Molecular Probes (Eugene, OR, USA). SuperScript III reverse transcriptase was from Invitrogen and oligo(dT) primers from Promega Corp. DynaZyme EXT DNA polymerase and dNTPs were from Finnzymes (Espoo, Finland). Nitrocellulose membranes were from Schleicher & Schuell (Dassel, Germany). The LightCycler FastStart DNA Master SYBR Green kit was obtained from Roche Diagnostics. 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 described previously (Schönberger et al. 2000) in DMEM supplemented with 2 mM l-glutamine, 10% 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, OH, 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% FCS and 50 U/ml penicillin–streptomycin. The cell cultures were maintained in 37 °C and 5% CO₂ in a humidified cell culture chamber.

**RT-PCR for determination of VEGFR expression**

ML-1, FRO, NPA, WRO, ARO and human umbilical vein endothelial cell (HUVEC) total RNA was isolated and DNase-treated with the Aurum total RNA isolation kit according to the manufacturer’s instructions. RNA was quantified with Ribogreen reagent and RNA quality and integrity was checked by absorbance spectrometry and agarose gel electrophoresis. Reverse transcription (RT) was performed with SuperScript III reverse transcriptase from 0.5 μg RNA. The PCR primers for VEGFR-1, -2 and -3 were designed with EMBOSS software (Rice et al. 2000). The primers, product lengths and annealing temperatures were: VEGFR-1 (membrane-bound isoform), 5′-AGCATTAGCTGGCAGATATT-3′ (sense), 5′-GCCCTTTCTCTCTGCTC-3′ (antisense), 200 bp, 58 °C; VEGFR-2 (both splice variants), 5′-GGTATTGCGAGTGGAGGAA-3′ (forward), 5′-CATTTGCGCTTGGATT-3′ (reverse), 199 bp, 57 °C; VEGFR-3 (both splice variants), 5′-TGTCATGAAAATCCCTTCA-3′ (forward), 5′-GCCCTCTGTCACCTCCTTGAG-3′ (reverse), 198 bp, 59 °C. The primers for HPRT have been described previously (29). The PCR was performed in 50 μl reactions (5 μl cDNA; 1 μM primers; 200 nM each of dATP, dCTP, dGTP and dCTP; 0.5 U DynaZyme EXT polymerase) on a Mastercycler Gradient thermal cycler (Eppendorf, Hamburg, Germany) with the following program: 94 °C 5 min, 30 cycles of amplification (94 °C 30 s, annealing T 60 s, 72 °C 60 s), 72 °C 5 min. Control analyses, where 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. The PCR products were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide.
**VEGF quantification**

A fixed number of ML-1 or FRO cells, cultured in medium with 1% LS-FCS, were stimulated with S1P (100 nM, 3 μM), TSH (0.3 mU/ml) and/or insulin (1 μg/ml) for 24 h. In some experiments, the cells were pre-treated with JTE-013 (10 μM), VPC23019 (1 μM) or a combination of both compounds, for 1 h prior to stimulation with S1P. In some experiments, the cells were stimulated with SEW-2871 (1 μM). The concentration of VEGF-A and -C in the culture medium was quantified with commercial ELISA kits (see Materials and methods section).

**Akt phosphorylation assay**

The ML-1 cells (10,000 cells/well) were plated on 96-well plates and allowed to grow for 24 h, after which the medium was changed to serum-free medium (SFM). The following day the cells were stimulated with S1P for 10 min with or without pre-treatment with VEGFR-2-inhibitor 1 (100 nM, 1 h) or CBO-P11 (10 μM, 1 h). In some experiments, VEGF-neutralizing antibody (1 μg/ml) was included in the reaction medium. The cells were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min. The FACE-Akt ELISA was performed with phospho-Akt (Ser473) antibody according to the instructions provided by the manufacturer, and the absorbance read at 450 nm. The use of the FACE-Akt ELISA kit gave results indistinguishable from those obtained using western blot and an antibody against Akt phosphorylated at Ser473 (Balthasar et al. 2006).

**Cell migration**

Migration experiments were performed on 6.5 mm diameter Transwell chambers with 8 μm pore size. The membranes were coated with 5 μg/cm² collagen IV (for ML-1 cells) or collagen I (for FRO cells) and reconstituted with SFM for 1 h at 37 °C in the cell culture incubator prior to the experiment. The cell medium was changed into SFM the day before the experiment. In some experiments, the cells were preincubated with vehicle, VEGFR-2-inhibitor 1 (100 nM) or the VEGF inhibitor CBO-P11 (10 μM) for 1 h, or with 100 nM deguelin overnight. A total of 100,000 cells in 200 μl medium was added to the upper wells and 800 μl SFM with or without S1P and/or LS-FCS to the lower wells. In some experiments, 50–500 ng/ml VEGF-A was used as a chemoattractant in the lower wells. In experiments with VEGFR-2-inhibitor 1, CBO-P11 or VEGF-neutralizing antibody or mouse IgG2b isotype control (1 μg/ml), these agents were added to both wells of the migration chamber. The cells were allowed to migrate in the incubator for 6 h (FRO cells) or 8 h (ML-1 cells) in experiments with LS-FCS in the lower well, or for 20 h (ML-1 cells) in serum-free experiments, after which the non-migrated cells from the top side of the membrane were wiped off with a cotton swab. The migrated cells were fixed in 2% PFA in PBS for 10 min and stained with 0.1% crystal violet in 20% methanol for 5 min. The membranes were rinsed and allowed to dry. The cells were counted with 40× magnification in eight microscopic fields in a straight line bisecting the membrane (~8% of the total membrane area). The range of migrated cells counted in the presence of LS-FCS only was 50–186/Transwell insert, compared with 128–329/Transwell insert in cells stimulated with both LS-FCS and S1P. In the absence of LS-FCS, the range of migrated cells counted in the presence of S1P was 82–152/Transwell insert.

**Relative quantitative RT-PCR of S1P receptors and VEGFR-2**

To determine the effect of VEGFR-2-inhibitor 1 on S1P receptor mRNA expression, total RNA from ML-1 cells preincubated with SFM for 24 h and treated with 100 nM VEGFR-2-inhibitor 1 for the indicated periods of time, was isolated, quantified and reverse transcribed as described above. For quantification of VEGFR-2 mRNA, total RNA from ML-1, FRO and HUVEC in normal growth medium was isolated. Quantitative RT-PCR was performed on a LightCycler instrument (Roche Diagnostics) with LightCycler DNA Master Kit reagents as described previously for S1P receptors (Balthasar et al. 2006). The VEGFR-2 PCR was performed as for S1P receptors, with 3 mM MgCl₂ and annealing at 62 °C. The primers and cycling conditions were optimized so that the PCR efficiency was close to maximal. Relative mRNA levels were calculated with the second derivative maximum method and LightCycler software. The data were normalized to the reference gene HPRT (Rey et al. 2000). Control analyses, where 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.

**Proliferation assay and incorporation of ³H-thymidine**

ML-1 cells cultured on a 96-well plate in a medium with 1% LS-FCS (5000 cells/well) were stimulated...
with VEGFR-2-inhibitor 1 (100 nM) or VEGF-neutralizing antibody (1 µg/ml) ± S1P and the cells were allowed to grow for 24 h. Proliferation was assessed using CellTiter cell proliferation assay according to the manufacturer’s instructions. In some experiments, the wells were coated with collagen IV (for ML-1 cells) or collagen I (for FRO cells). For the ³H-thymidine incorporation assay, ML-1 cells were seeded on 35 mm plates (75 000 cells/plate). The next day the medium was changed to SFM complemented with 0.2% fatty acid-free BSA, the cells were stimulated with VEGFR-2-inhibitor 1 (100 nM) or vehicle and allowed to grow for 24 h. Four hours prior to the end of the experiment 0.4 µCi/ml ³H-thymidine was added. The cells were washed with PBS, incubated for 10 min with 5% trichloric acetic acid, and thereafter incubated for 10 min with 0.1 M NaOH and the radioactivity assessed using liquid scintillation.

Western blotting

For membrane preparations, the cells were washed with ice-cold PBS and suspended into Buffer A (containing 20 mM Tris–HCl, 250 mM sucrose, 10 mM EDTA (pH 7.5), 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 100 µg/ml leupeptin). The samples were sonicated for 4×10 s bursts on ice. The samples were centrifuged for 1 h at 100 000 g at +4 °C, after which the cytosolic fraction was collected and the pellet was resuspended in Buffer A and centrifuged as above. The pellets were then resuspended in Buffer A containing 0.2% Triton X-100 and incubated for 45 min on ice. The samples were then centrifuged for 30 min at 100 000 g at +4 °C, and the supernatants were collected as the membrane preparation. To obtain whole cell lysates, the cells were briefly rinsed with ice-cold HBSS and extracted in 200 µl 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 3X Laemmli Sample Buffer (glycerol, 30%; SDS, 3%; Tris–HCl, 0.1875 M (pH 6.8); bromophenol blue, 0.015%; β-mercaptoethanol, 3%) and boiled for 2 min. The protein samples were separated by SDS-PAGE on a 6–10% polyacrylamide gel. The proteins were transferred electrophoretically to nitrocellulose membranes, and 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 0.1–0.3% Tween 20 for 1 h. The blots were then incubated with rabbit anti-VEGFR-2 antibody (1:2000) diluted in TBS containing 5% non-fat dry milk and 0.1% Tween 20 overnight at +4 °C. For detection of phosphorylated VEGFR-2, the blots were blocked 48 h at +4 °C with TBS containing 5% non-fat dry milk and then incubated with rabbit anti-phospho-VEGFR-2 (pTyr1175; 1:1000) diluted in 5% milk and 0.1% Tween 20 overnight at +4 °C. For the loading control, membranes were blotted with β-actin antibodies (1:1000). Membrane preparations of ML-1 cells treated with 100 nM VEGFR-2-inhibitor 1 for 24 h were incubated with either rabbit anti-S1P1 (1:150) or rabbit anti-EDG3 (1:800) in TBS containing 5% non-fat dry milk and either 0.1% or 0.3% Tween 20 respectively overnight at +4 °C. The blots were washed and incubated with peroxide-conjugated goat anti-rabbit antibodies (1:3000) for 1 h at room temperature. The membranes were incubated in ECL solution (luminol, 1.25 mM; p-coumaric acid, 0.68 mM; H₂O₂, 0.01%) for 1 min and exposed to Hyperfilm (Amersham Biosciences) for 1–15 min. The Image J analysis program (http://rsb.info.nih.gov/ij/) was used for the quantification of western blots.

cAMP assay

To measure TSH-stimulated cAMP production, the ML-1 and FRO cells were grown in a medium containing 1% LS-FBS for 24 h and preincubated for 30 min in SFM before the experiments. The cells stimulated with or without TSH (0.3 and 1 mU/ml) or forskolin (10 µM) were incubated for 60 min in the presence of 250 µM IBMX. The incubation was terminated with perchloric acid (final concentration 0.4 M), and the samples were neutralized with KOH. The cAMP content in the supernatants was determined using a protein binding method (Nordstedt & Fredholm 1990). The samples and standards (0–320 nM cAMP) were incubated with a protein extract from bovine adrenal cortex containing cAMP-binding protein and with [³H]cAMP for 2.5 h at +4 °C. The bound cAMP was separated from free cAMP by rapid filtration on Millipore MultiScreen MAFB filtration plates (Billerica, MA, USA), and the wells were washed three times with 300 µl of 50 mM Tris–HCl (pH 7.4). The plates were dried and 50 µl SuperMix scintillation cocktail (Wallac) was added to the wells. The plate was counted using a MicroBeta scintillation counter (Wallac).

Data presentation

All numerical data are presented as a mean ± s.e.m. for at least three independent measurements. The data were usually normalized to make a comparison of the different experiments feasible, as different batches of
serum and collagen gave some variations in the results. The data were analysed statistically with Student’s t-test or with one-way ANOVA and Dunnett’s and Holm’s post hoc tests, or Bonferroni’s post hoc test for selected samples, depending on the experimental setting. P < 0.05 was considered statistically significant.

Results

VEGFR expression and secretion of VEGF-A and -C

Recent investigations have indicated that thyroid carcinoma cells may express receptors for VEGF, and that VEGF-A and -C is produced in thyroid tumorigenic tissues (Turner et al. 2003, Vieira et al. 2005, Kim et al. 2007). To further investigate this possibility, RT-PCRs were performed to determine the expression of VEGFRs in ML-1, FRO, NPA, ARO and WRO thyroid cancer cell lines. None of the cell lines examined expressed VEGFR-3. VEGFR-1 was not detected in NPA, ARO and WRO cells. In ML-1 and FRO cells, a minuscule amount of VEGFR-1 mRNA was observed, far too low to quantify. However, ML-1 cells and FRO cells expressed VEGFR-2 mRNA (Fig. 1 A and B). A VEGFR-2-specific antibody identified ~210–230 kDa double band in both cell lines (Fig. 1C). A similar double band was seen in HUVECs (Fig. 1C). Our results are thus in line with the recent report by Vieira et al. (2005).

Next, the amount of VEGF-A and -C secreted by ML-1 and FRO cells was assessed. Both VEGF-A and -C (3.6 ± 0.75 ng/10⁶ cells and 3.3 ± 0.51 ng/10⁶ cells respectively) was detected in the medium from ML-1 cell culture after 24 h incubation. FRO cells secreted barely detectable amounts of VEGF-A (0.018 ± 0.0047 ng/10⁶ cells) and VEGF-C (0.14 ± 0.040 ng/10⁶ cells; Fig. 2A). FRO cells express predominantly S1P2, and ML-1 cells express several receptors for S1P (Balthasar et al. 2006, Rey et al. 2000). Furthermore, S1P and VEGF have been shown to cooperate in the regulation of cellular functions (Igarashi et al. 2003, Fieber et al. 2006). Thus, we wanted to investigate whether S1P could modulate VEGF secretion from the cells. Stimulating FRO and ML-1 cells with 100 nM S1P was without an effect on VEGF-A secretion, whereas stimulation with 3 μM S1P resulted in increased VEGF-A secretion in both cell lines (Fig. 2B and D). As the EC₅₀ values for the S1P receptors are in the low nanomolar range, it is unclear why only a high dose of S1P evoked secretion of VEGF-A. In contrast to an earlier report on the effect of TSH on VEGF secretion in thyroid cancer cells (Soh et al. 1996), TSH (0.3 mU/ml) had no effect on VEGF-A secretion in either cell line (data not shown). We were also unable to show an effect of 0.3 mU/ml TSH on cellular cAMP-levels in either cell line (data not shown), although receptors for TSH were detected with western blotting in both cell lines (data not shown). Furthermore, insulin (1 μg/ml) was without an effect on VEGF-A secretion in ML-1 cells (data not shown). A combination of insulin and S1P slightly increased VEGF-C secretion (Fig. 2C).

To investigate which S1P receptors are involved in the S1P-evoked secretion of VEGF-A, we pre-treated the cells with either the S1P₁₃ antagonist VPC23019 (1 μM) or the S1P₂ antagonist JTE-013 (10 μM). Neither VPC23019 nor JTE-013 per se had an effect
on VEGF-A secretion in ML-1 or FRO cells (Fig. 2B and D). However, VPC23019 and JTE-013 blocked the secretion of VEGF-A evoked by 3 μM S1P in both ML-1 and FRO cells (Fig. 2B and D). Surprisingly, the S1P1 agonist SEW-2871 (1 μM) stimulated the secretion of VEGF-A in FRO cells, but not in ML-1 cells (Fig. 2B and D), although FRO cells do not express detectable amounts of S1P1 receptor.

VEGFR-2 signalling regulates ML-1 and FRO cell migration

In several cell types, VEGF-A and VEGFR-2 potently stimulate cell migration (Olsson et al. 2006). Therefore, we studied whether ML-1 or FRO cell migration is affected by VEGFR-2 signalling or VEGF-A. Stimulating the cells with 50–500 ng/ml VEGF-A alone for up to 24 h did not induce migration of either ML-1 or FRO cells (results not shown). Additionally, neither VEGF-neutralizing antibody (1 μg/ml) nor CBO-P11 (10 μM), which both interfere with VEGF-A/VEGFR interaction, affected FRO cell migration towards 10% LS-FCS (Fig. 3A). By contrast, treatment with 100 nM VEGFR-2-inhibitor 1, which inhibits receptor tyrosine kinase (RTK) activity, significantly attenuated FRO cell migration towards LS-FCS (Fig. 3A). This suggests that VEGFR-2 is involved in FRO cell migration in a VEGF-A-independent manner.

As ML-1 cells produce VEGF-A, autostimulation of VEGFR-2 is possible. Therefore, we tested the effect of VEGFR inhibitors and VEGF-neutralizing antibody on ML-1 cell migration. VEGF-neutralizing antibody and VEGFR-2-inhibitor 1 attenuated migration towards LS-FCS (Fig. 3B), suggesting that VEGF-A is of importance in the regulation of ML-1 cell migration. A control antibody was without any effects on the migration of the cells (data not shown).

Figure 2 ML-1 and FRO cells secrete VEGF-A and -C.

(A) VEGF-A and -C secretion by one million ML-1 cells (white bars) and FRO cells (black bars) during a 24 h incubation period. (B) VEGF-A secretion in ML-1 cells evoked by 3 μM S1P in cells treated with vehicle (white bars), JTE-013 (10 μM, preincubation for 1 h, hatched bars), VPC23019 (1 μM, preincubation for 1 h, black bars) or a combination of the two (striped bars). The lack of an effect of SEW-2871 (1 μM) is also shown. (C) VEGF-C secretion in FRO cells stimulated with vehicle (white bars) or 3 μM S1P (black bars) in the absence or presence of 1 μg/ml insulin. (D) VEGF-A secretion in FRO cells evoked by 3 μM S1P in cells treated with vehicle (white bars), JTE-013 (10 μM, preincubation for 1 h, hatched bars), VPC23019 (1 μM, preincubation for 1 h, black bars) or a combination of the two (striped bars). The effect of SEW-2871 (1 μM) is also shown. The incubation period in all experiments was 24 h. The data in all panels are presented as percentage release of control cells. Asterisks (*) indicate statistically significant differences of S1P treatment compared with respective control (vehicle or antagonist). Diamonds (◊) indicate statistically significant differences compared with S1P treatment alone. The data were analyzed with one-way ANOVA and Holm’s post hoc test. *P<0.05; **P<0.01, ***P<0.001. In each panel, the error bars give the mean±S.E.M. of the indicated independent experiments. (A) N=5–7, (B–D) N=3–4.
VEGF and VEGFR-2 are involved in ML-1 cell migration towards S1P

In thyroid ML-1 cancer cells, S1P is a potent stimulator of migration (Balthasar et al. 2006), and S1P may interact with VEGFRs to enhance migration of other cell types (Endo et al. 2002). Thus, we investigated whether VEGF signalling is involved in migration evoked by 100 nM S1P in ML-1 cells. This concentration of S1P induces maximal migration of ML-1 cells (Balthasar et al. 2006). Treatment of the cells with VEGFR-2-inhibitor 1 and CBO-P11 abolished the S1P-evoked migration in the presence of LS-FCS (Fig. 3B). S1P still evoked migration of cells in the presence of LS-FCS and a VEGF-neutralizing antibody, but the effect of S1P was significantly smaller than that in vehicle-treated cells (Fig. 3B). In the absence of LS-FCS, VEGFR-2-inhibitor 1, CBO-P11 and the VEGF-neutralizing antibody significantly attenuated S1P-evoked migration (Fig. 3C). A control antibody was without any effects on migration (data not shown). Thus, VEGFR-2 and its ligand VEGF-A cooperate with S1P in the regulation of ML-1 cell migration.

Effects of VEGFR inhibition on Akt phosphorylation in ML-1 cells

S1P-induced ML-1 cell migration is PI3K dependent (Balthasar et al. 2006). Furthermore, both S1P and the S1P1-specific agonist SEW-2871 stimulate Akt phosphorylation. S1P, but not SEW-2871, stimulates migration of ML-1 cells, implicating that S1P1 activation and Akt phosphorylation is not sufficient for a migratory response (Balthasar et al. 2006). As VEGF has been shown to enhance the S1P-evoked Akt phosphorylation (Igarashi et al. 2003), we investigated whether this also occurs in ML-1 cells. In cells pre-treated with VEGFR-2-inhibitor 1, the Akt phosphorylation evoked by either 100 nM S1P or VEGF signalling is involved in migration evoked by 100 nM S1P in ML-1 cells. This concentration of S1P induces maximal migration of ML-1 cells (Balthasar et al. 2006). Treatment of the cells with VEGFR-2-inhibitor 1 and CBO-P11 abolished the S1P-evoked migration in the presence of LS-FCS (Fig. 3B). S1P still evoked migration of cells in the presence of LS-FCS and a VEGF-neutralizing antibody, but the effect of S1P was significantly smaller than that in vehicle-treated cells (Fig. 3B). In the absence of LS-FCS, VEGFR-2-inhibitor 1, CBO-P11 and the VEGF-neutralizing antibody significantly attenuated S1P-evoked migration (Fig. 3C). A control antibody was without any effects on migration (data not shown). Thus, VEGFR-2 and its ligand VEGF-A cooperate with S1P in the regulation of ML-1 cell migration.

Figure 3 VEGF receptor signalling regulates S1P-induced migration in FRO and ML-1 thyroid cancer cells. (A) The effect of vehicle, VEGFR-2-inhibitor 1 (100 nM), CBO-P11 (10 μM) or VEGF-neutralizing antibody (VEGF ab; 1 μg/ml) on FRO cell migration towards 10% lipid-stripped FCS. The migration of vehicle-treated cells was considered as 100% migration.

(B) Migration of ML-1 cells treated with vehicle, VEGFR-2-inhibitor 1, CBO-P11 or VEGF-neutralizing antibody in the presence of lipid-stripped FCS with (black bars) or without (white bars) 100 nM S1P. The migration of vehicle-treated cells in the presence of S1P was considered as 100% migration. The brackets indicate statistically significant stimulation of migration by S1P. Asterisks (•) and diamonds (♦) indicate statistically significant differences in migration compared with control (vehicle) in the absence or presence of S1P respectively.

(C) Migration of ML-1 cells treated with vehicle, VEGFR-2-inhibitor 1, CBO-P11 or VEGF-neutralizing antibody in serum-free medium with (black bars) or without (white bars) 100 nM S1P. The migration of vehicle-treated cells in the presence of S1P was considered as 100% migration. The diamonds (♦) indicate statistically significant differences of treatment compared with control (vehicle) in the presence of S1P. The data were analyzed with one-way ANOVA and Holm’s post hoc test. *, P<0.05; **, P<0.01; ***, P<0.001. In each panel, the error bars give the mean±s.e.m. of three to five independent experiments.
1 μM SEW-2871 was abolished (Fig. 4A). The SEW-2871-induced Akt phosphorylation was likewise completely abolished by VEGF-neutralizing antibody (Fig. 4A). By contrast, VEGF-neutralizing antibody did not inhibit S1P-induced Akt phosphorylation. Neither VEGFR-2-inhibitor 1 nor the VEGF-neutralizing antibody per se had an effect on the basal phosphorylation of Akt (Fig. 4A). Taken together, these results suggest that S1P-induced Akt phosphorylation is dependent on VEGFR-2. In addition, the phosphorylation may be mediated by more than one S1P receptor interacting with VEGFR-2 in ML-1 cells, and the mechanisms of interaction between VEGFR-2 and respective S1P receptors may be different.

The above results and our previous publication (Balthasar et al. 2006) emphasize the importance of PI3K and Akt on S1P-induced migration of ML-1 cells. However, the importance of Akt per se has not been evaluated. In Fig. 4B, we show that preincubation of ML-1 cells with 100 nM of the Akt inhibitor deguelin overnight abolished migration of ML-1 cells. Deguelin was without an effect on the proliferation of ML-1 cells (results not shown). Thus, PI3K (Balthasar et al. 2006) and its downstream effector Akt are of crucial importance in the migration of ML-1 cells.

Effects of VEGFR inhibition on ML-1 and FRO cell proliferation

The inhibitory effects of VEGFR-2 inhibition on migration could be due to an effect on proliferation rather than on the migratory mechanism per se. To test this possibility, proliferation experiments with VEGFR-2-inhibitor 1, CBO-P11 and VEGF-neutralizing antibody were performed on ML-1 and FRO cells. VEGFR-2-inhibitor 1 (100 nM), but not CBO-P11 (1 μM) or VEGF-neutralizing antibody (1 ng/ml), attenuated ML-1 cell proliferation (Fig. 4C). Similar results were obtained using the [3H]thymidine incorporation assay or with cells grown on collagen (data not shown). The results suggest that VEGFR-2 has a VEGF-A-independent function in ML-1 cell proliferation, whereas the effect on S1P-induced migration is dependent on VEGF-A. In FRO cells, none of the treatments affected proliferation (Fig. 4C). Similar results were obtained when the experiment was repeated in the presence of 100 nM

Figure 4: Importance of VEGF receptor 2 and Akt on the migration and proliferation of ML-1 and FRO cells. (A) Effects of VEGF receptor signalling on Akt phosphorylation in ML-1 cells. The graph shows the effect of vehicle (white bars), 100 nM VEGFR-2-inhibitor 1 (black bars) or 1 μg/ml VEGF-neutralizing antibody (hatched bars) on relative Akt phosphorylation in control cells or cells treated for 10 min with 100 nM S1P or 1 μM SEW-2871. The phosphorylation of Akt was determined with a commercial ELISA kit as described in the Materials and methods section. (B) Akt is necessary for ML-1 cell migration. Migration was measured towards 10% lipid-stripped FCS in the presence or absence of S1P. The cells were incubated with (black bars) or without (white bars) 100 nM deguelin overnight prior to the migration assay. Deguelin was also added to the pre-treated cells during the migration assay. The migration of vehicle-treated cells in the absence of deguelin was considered as 100% migration. Diamonds (◆) indicate statistically significant differences in migration compared with control (vehicle) in the absence or presence of S1P respectively. (C) Effects of VEGF receptor signalling on the proliferation of thyroid cancer cells. ML-1 (white bars) and FRO (black bars) cell proliferation measured with the CellTiter assay in cells treated with vehicle, 100 nM VEGFR-2-inhibitor 1, 10 μM CBO-P11 or 1 μg/ml VEGF-neutralizing antibody. The data were analyzed with one-way ANOVA and Holm’s post hoc test. Asterisks indicate statistically significant differences of treatment compared with respective control. In each panel, the error bars give the mean ± S.E.M. of three to four independent experiments. **P<0.01; ***P<0.001; ****P<0.0001.
S1P, using the [3H]thymidine incorporation assay, or with cells grown on collagen (results not shown).

**Effects of S1P on VEGFR phosphorylation**

The above results suggest a complex interaction between S1P and VEGF signalling. As previous investigations have shown that S1P can phosphorylate VEGFR-2 (Tanimoto et al. 2002), we investigated whether stimulating the cells with S1P could phosphorylate VEGFR-2 in ML-1 or FRO cells. As can be seen in Fig. 5, both S1P and VEGF evoked a phosphorylation of VEGFR-2 in ML-1 cells within 10 min (Fig. 5). The effect of S1P was still evident after 30 min of stimulation (results not shown). In FRO cells, VEGF, but not S1P, evoked a phosphorylation of VEGFR-2 (results not shown). Thus, in ML-1 cells, S1P can transactivate VEGFR-2.

**Effects of VEGFR-2 inhibition on S1P1 and S1P3 mRNA expression and protein levels in ML-1 cells**

ML-1 cells express S1P1, S1P2, S1P3 and S1P5 mRNA (Balthasar et al. 2006) and protein (S Balthasar, N Bergelin and K Törnquist, unpublished observations). As VEGF has been shown to induce S1P1 receptor expression in endothelial cells (Igarashi et al. 2003), we investigated the effect of VEGFR-2 inhibition on S1P receptor mRNA expression in ML-1 cells. VEGFR-2-inhibitor 1 (100 nM) downregulated S1P1 mRNA expression within 2 h, and the expression level stabilized at 45% of control levels within 6 h (Fig. 6A). By contrast, S1P3 mRNA was 2.5-fold upregulated within 24 h (Fig. 6A), whereas S1P2 and S1P5 mRNA levels were not significantly altered (data not shown). Furthermore, the S1P1 protein level was significantly downregulated in membrane preparations after 24 h of incubation with VEGFR-2-inhibitor 1 (Fig. 6B). Shorter incubation periods (2, 6 and 12 h) did not reveal an effect of VEGFR-2-inhibitor 1 on the amount of S1P1 protein (data not shown). We were unable to observe a significant change in the S1P3 protein level (Fig. 6C). Thus, in ML-1 cells VEGFR-2 has a role in regulating the expression of at least the S1P1 receptor.

**Discussion**

In the present investigation, we show that two thyroid cancer cell lines, the ML-1 follicular cancer cell line and the FRO anaplastic thyroid cancer cell line,
express VEGFR-2. We were unable to show an expression of VEGFRs in the WRO, ARO or NPA thyroid cancer cell lines. In addition, ML-1 and FRO cells secrete VEGF-A and -C, creating a possible autocrine signalling loop for VEGF. We also show that S1P and VEGFR signalling interact to regulate human thyroid cancer cells. We base our conclusion on the following observations: first, S1P regulates VEGF-A secretion in both ML-1 and FRO cells; secondly, VEGF and its receptor VEGFR-2 are of crucial importance in S1P-induced migration of ML-1 cells; thirdly, S1P phosphorylates VEGF-2 in ML-1 cells; and, finally, VEGFR-2 regulates S1P1 and S1P3 receptor mRNA expression in these cells.

There are many examples of crosstalk between G-protein-coupled receptors (GPCRs) and RTKs (reviewed by Shah & Catt 2004, Waters et al. 2004). One group of GPCRs that interact with RTKs are the S1P receptors (e.g. Spiegel et al. 2002, Baudhuin et al. 2004, Shida et al. 2004). Interactions between S1P and VEGF signalling have been studied mainly in endothelial cells. These studies imply that crosstalk between S1P receptors and VEGFRs regulates angiogenesis and is thereby of major importance in tumour progression (Endo et al. 2002, Tanimoto et al. 2002, Igarashi et al. 2003, Fieber et al. 2006, Visentin et al. 2006). S1P has also been shown to stimulate the release of proangiogenic growth factors, including VEGF-A, from breast cancer cells (Visentin et al. 2006). The present study is, to our knowledge, the first investigation to show that S1P and VEGF signalling pathways may interact to regulate tumour cells directly.

VEGF-A expression is correlated with thyroid cancer growth, recurrence and metastasis and VEGF-C expression with lymph node metastasis (Turner et al. 2003), and receptors for VEGF have been found in thyroid cancer cells (Sato et al. 1995, Belletti et al. 1999, Susarla et al. 2005a, Vieira et al. 2005, Kim et al. 2007). Thyroid endothelial cells express VEGFRs (Susarla et al. 2005b). It is thus possible that the substantial constitutive secretion of VEGF-A and -C by ML-1 cells contributed to the recurrence, metastatic and invasive potential of the original tumour (Schönberger et al. 2000). The basal levels of VEGF-A and -C secretion were distinctly lower in FRO cells, but S1P was equally potent in stimulating VEGF-A secretion in both cell lines. This effect of S1P may be physiologically important for the tumorigenic potential of FRO cells (Pacífico et al. 2004) by stimulating angiogenesis and/or lymphangiogenesis. Our results do not support an effect of VEGF-A in the migration or proliferation of FRO cells. Interestingly, in both ML-1 and FRO cells, the S1P-evoked secretion of VEGF-A was blocked by the S1P1,3 antagonist VPC23019 and the S1P2 antagonist JTE-013. In FRO cells, but not in ML-1 cells, the S1P1 agonist SEW-2871 stimulated the secretion of VEGF-A. As FRO cells do not express detectable amounts of S1P1 receptor, the results suggest that SEW-2871 is not entirely specific for the S1P1 receptor.

TSH stimulates VEGF-A secretion or expression in several thyroid cancer cell lines (Soh et al. 1996), human thyroid follicles (Sato et al. 1995) and rat thyrocytes (Viglietto et al. 1997). Insulin stimulates VEGF-A expression in human thyroid follicles (Sato et al. 1995) and IGF, via PI3K-Akt, stimulates VEGF-A secretion in SW579 thyroid cancer cells (Poulaki et al. 2003). Surprisingly, neither TSH (0.3 mU/ml) nor insulin (1 μg/ml) had major effects on VEGF-A or -C secretion in ML-1 and FRO cells, although both cell lines express TSH receptors (Moretti et al. 2000, Schönberger et al. 2000). Furthermore, we did not detect any effects of TSH on cellular cAMP, suggesting that our ML-1 cells are less sensitive to TSH, compared with the parent cell line. Moreover, S1P (10 nM to 3 μM) was not capable of phosphorylating Akt in FRO cells (results not shown), implicating that some other signalling pathway mediates S1P-induced VEGF-A secretion.

ML-1 cell migration and Akt kinase phosphorylation is potentely stimulated by 100 nM S1P via the Gt-PI3K pathway (Balthasar et al. 2006). The PI3K-Akt pathway is an important downstream effector of VEGFR-2 signalling in endothelial cells (Olsson et al. 2006). We found that inhibiting Akt with deguelin totally abolished migration in ML-1 cells, implicating that Akt is an important downstream effector in the Gt-PI3K pathway in these cells. As deguelin may not be a totally specific inhibitor of Akt (see Oh et al. 2007), we cannot exclude the possibility of unspecific effects of deguelin on migration. In addition, inhibiting VEGFR-2 blocked S1P-induced migration and Akt phosphorylation in ML-1 cells. Moreover, CBO-P11, which interferes with VEGF-A binding to its receptor, and a VEGF-neutralizing antibody inhibited migration towards S1P, implicating that autocrine stimulation of VEGFR-2 by VEGF-A secretion participates in ML-1 cell migration. However, secreted VEGF-A alone is not sufficient to induce migration, as no basal migration in the absence of a chemoattractant (i.e. LS-FCS or S1P) was observed. Moreover, exogenous VEGF-A did not evoke any migration of the cells. Interestingly, VEGFR-2-inhibitor 1 and VEGF antibody reduced migration towards LS-FCS. Presently, we do not know which component(s) in the LS-FCS acted upon VEGFR-2 and stimulated migration.
The S1P₁ agonist SEW-2871 is able to induce Akt phosphorylation, but not migration, in ML-1 cells (Balthasar et al. 2006), implicating that more than one S1P receptor may be involved in S1P-induced ML-1 cell migration. We used SEW-2871 to investigate whether S1P₁ interacts with VEGFR-2 in Akt phosphorylation. Both VEGFR-2-inhibitor 1 and VEGF-neutralizing antibody completely inhibited Akt phosphorylation induced by 1 μM SEW-2871. Thus, crosstalk between VEGFR-2 and S1P₁ requires activation of VEGFR-2 by a ligand. However, there is no requirement for VEGF-A binding to VEGFR-2 for S1P-induced Akt phosphorylation, as VEGFR-2-inhibitor 1, but not VEGF-neutralizing antibody reduced the phosphorylation. In conclusion, the results from migration experiments and Akt phosphorylation experiments suggest that there are both VEGF-A-dependent (S1P₁-mediated) and VEGF-A-independent components in VEGFR-2 interaction with S1P signalling.

As inhibiting VEGFR-2 attenuated ML-1 cell proliferation, one possibility is that it is proliferation (S1P independent), not migration towards S1P that is inhibited in VEGFR-2-inhibitor-1-treated cells. However, neither CBO-P11 nor VEGF-neutralizing antibody affected cell number although they inhibited migration, showing that VEGF signalling is indeed involved in the regulation of S1P-induced migration. It is an interesting point that VEGFR-2 seems to be able to stimulate ML-1 cell proliferation without the involvement of VEGF-A as a ligand. An interference of CBO-P11 with the binding of other ligands (VEGF-C and -D) to VEGFR-2 is to our knowledge not clarified. This leaves us with two possibilities: the effect of VEGFR-2 on ML-1 cell proliferation may be ligand independent or mediated by some other ligand than VEGF-A.

Inhibiting VEGFR-2 rapidly reduced S1P₁ receptor mRNA expression and protein levels in ML-1 cells. The effect is similar to that in BAECs and HUVECs, where stimulation with VEGF upregulated S1P₁ expression and enhanced cellular responses to S1P (Igarashi et al. 2003, Hughes et al. 2005). S1P₁ receptor downregulation may partly explain the inhibition of S1P₁-induced migration by VEGFR-2. However, S1P₁ stimulation alone is not sufficient for the migratory effect (Balthasar et al. 2006) and the role of the other receptors remains to be solved. In other cell systems, both S1P₁ and S1P₃ are involved in S1P₁-induced migration (Takuwa 2002, Taha et al. 2004). It is thus surprising that S1P₃ mRNA is upregulated by VEGFR-2 inhibition. Whereas S1P₁ downregulation is apparent within 2 h, S1P₃ upregulation is statistically significant within 24 h of stimulation with VEGFR-2-inhibitor 1. It is likely that the time course of the migration experiments in the absence of serum, 20 h, was too short to detect an effect of S1P₃ upregulation on migration.

Taken together, our study reveals a complex interaction between VEGF and S1P signalling involved in VEGF secretion, S1P receptor expression, Akt kinase phosphorylation, and the regulation of migration of ML-1 thyroid cancer cells. Moreover, the data suggest that VEGF regulates ML-1 cell migration by an autocrine mechanism. A schematic illustration of the interrelationships between VEGFR and S1P receptors is shown in Fig. 7. Together with the known synergistic effects of VEGF and S1P on angiogenesis, our results suggest that S1P and VEGFR-2 cross-communication may be important in the regulation of thyroid tumour migration, and thus metastasis.

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